Biosynthetic studies on the fungal metabolite tenelln

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by

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Declaration.

The work described in this thesis was carried out by the author, in the Department of Chemistry, University of Durham, between October 1989 and September 1992. It has not been submitted previously for a degree at this, or any other, university.

Memorandum.

" 'Curiouser and curiouser 'said Alice...."

Acknowledgements.

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Biosynthetic Studies on the Fungal Metabolite Tenellin.

Abstract.

This thesis is divided into seven chapters plus appendices. The first chapter introduces tenellin (1), its origins, structure and biogenetic relationships to other metabolites. The second chapter concentrates on polyketides and through three representative examples explains their relationship to the fatty acids and current theories and evidence for a processive mechanism during their biosynthesis. An investigation carried out into the biosynthesis of the polyketide sidechain of tenellin (1) from sodium [1-^{13}C, 2-^{2}H$_3$] and [2-^{13}C, 2-^{2}H$_3$] acetates and L-[*S-methyl*-^{13}C^{2}H$_3$]-methionine is described. The possible intermediacy of singly methylated diketides and doubly methylated triketides carrying stable isotopic labels during the biosynthesis of tenellin (1) is also tested experimentally and discussed.

In the third chapter possible intermediates to both the 2-pyridone moiety of tenellin (1) and the tropic acid (42) moiety of the tropane alkaloids are discussed and tested experimentally. 3-Amino-2-phenylpropionic acid (104) was synthesised and rejected as an intermediate to tenellin (1) and tropic acid (42). In the case of tenellin (1) a tetramic acid (139) was implicated as a possible intermediate by a biosynthetic feeding experiment. An alternative mechanism to account for 2-pyridone formation from a tetramic acid is put forward.

In the fourth chapter the determination of the absolute configuration of tenellin (1) is described. The configuration of the chiral methyl bearing centre of tenellin (1) is compared to that of similar fungal polyketide derived molecules and the mode of reduction by various fungal PKS enoyl reductase systems is assessed.

In the fifth chapter the syntheses and characterisations of the compounds labelled with ^{14}C, ^{13}C and ^{2}H that were used in the biosynthetic studies on tenellin (1) and tropic acid (42) are described. Chapter six describes in detail the experimental procedures used in the syntheses of these compounds. Chapter seven consists of a description of the biological materials and methods used during this study.

The appendices consist of abbreviations used in the text, and a list of lectures, seminars and colloquia arranged within the Department of Chemistry and attended by the author.

Contents.

Chapter 1.

Introduction.

1.1 Pigments of the genus *Beauveria* and related compounds.

1.2 The 2-pyridones and related metabolites.

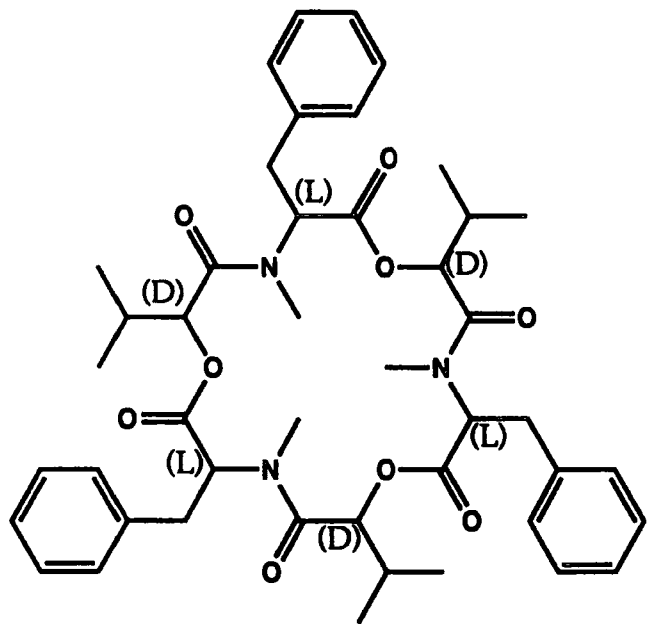
1.3 The polyketides.

1.4 The Biosynthesis of tenellin (1).

1.6 References.

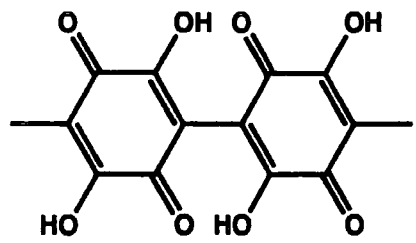
1.1 Pigments of the genus *Beauveria* and related compounds.

The genus *Beauveria* is a group of entomogenous fungi first described by Vuillemin in 1912[1]. The genus is responsible for red muscardine disease in insects. As the name suggests species of this genus are usually coloured, colours including reds, yellows and greens, and although colouration has been used as a criterion for distinguishing species, von Arx has recently outlined species classification along morphological lines[2]. The two most common species, *B. bassiana* (Bals.) Vuill. and *B. tenella* (Delacroix) Siem., have been isolated from diseased insects[3] and also as contaminants of submerged cultures of *Agaricus campestris* (field mushroom) and *Amanita muscaria* (fly agaric) respectively[4]. *B. bassiana* has been shown to be virulent towards *Melanoplus sanguinipes* (migratory grasshopper) by virtue of the excretion of an extracellular protease which has been shown to hydrolyse insect cuticle[5]. The excretion of the hexadepsipeptide beauvericin (3)[6,7,8] has been shown to be responsible for *B. bassiana* toxicity towards *Artemia salina* (brine shrimp), *Calliphora erythrocephala* (blowfly) and *Aedes aegypti* (mosquito)[9].



Beauvericin (3), the toxic principal of *B. bassiana*.

Vining *et al.* have studied the effect of nutrition upon the production of pigments by two species of *Beauveria* (*B. tenella and B. bassiana*)[3]. They established that red colouration was due to the diquinonoid metabolite oosporein (4)[10,11] and that under certain circumstances, namely when the culture media contained a high carbon/nitrogen ratio, yellow pigments could be produced and isolated[12]. These compounds were divided into two groups, tenellins and bassianins. Tenellin (1) produced mainly by *B. bassiana* and the closely related bassianin (2) produced by *B. tenella* were purified and their structures elucidated by a combination of physical, chemical and biosynthetic experiments[13].



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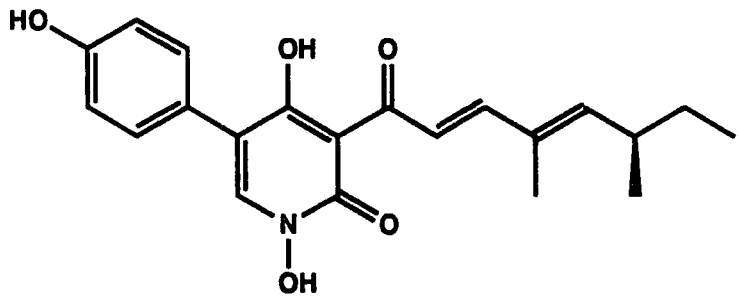
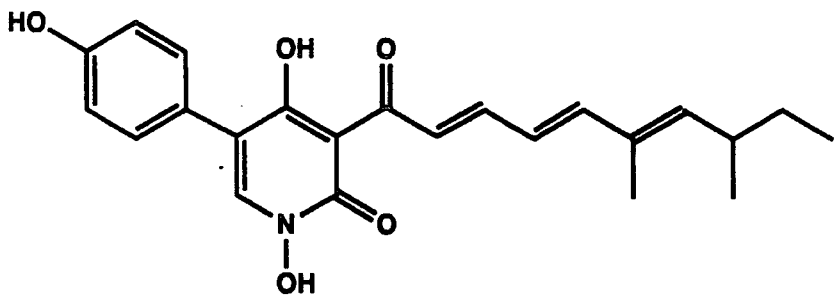


(1).



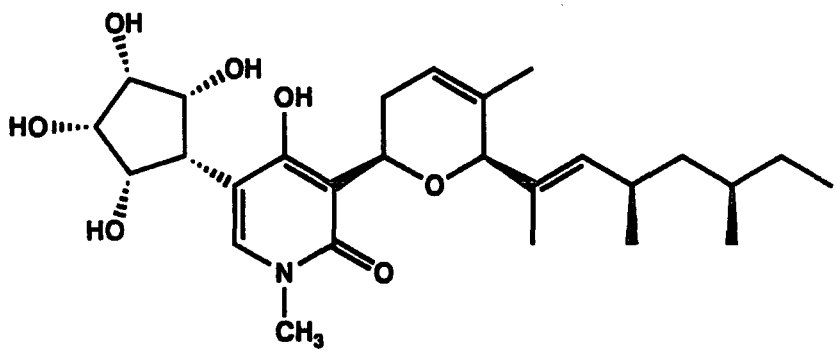
(2).

The principal pigments of *Beauveria bassiana*, oosporein (4), tenellin (1) and bassianin (2).

1.2 The 2-Pyridones and related metabolites.

Tenellin (1) and bassianin (2) differ only by an extra *trans* ethylene group in the side chain of bassianin (2). They are characterised by the presence of a 2-pyridone moiety substituted at the 3 position by a branched conjugated acyl side chain and at the 5 position by a para-hydroxy benzyl group[14].

These two metabolites belong to a small family of 2-pyridone containing fungal secondary metabolites which includes funiculosin (5) (*Penicillium funiculosum*)[15], ilicicolin-H (6) (*Cylindrocladium ilicicocola*)[16], leporin-A (7) (*Aspergillus leporis*)[17], viridicatin (8a)[18], viridicatol (8b) (*Penicillium viridicatum*)[19] and harzianopyridone (9) (*Trichoderma harzianum*)[20] and the structurally related atpenins (10a,b,c) (*Penicillium sp. FO125*)[21].



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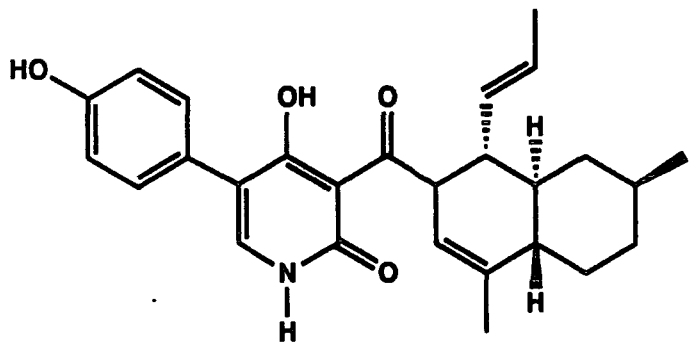
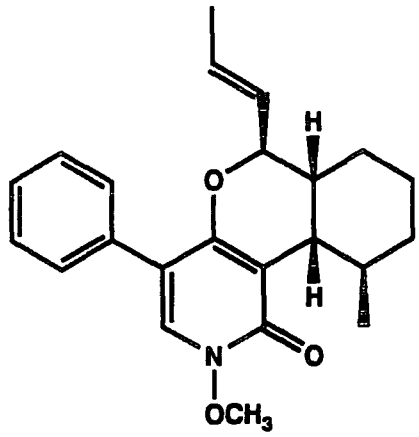


(6)

The fungal 2-pyridones funiculosin (5) and ilicicolin-H (6).



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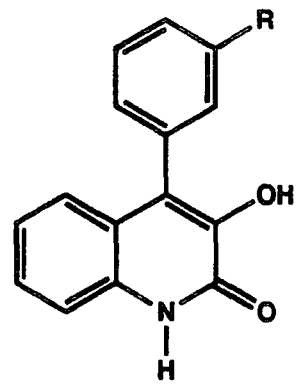
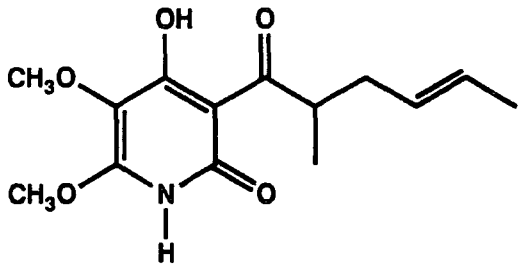


(8a) R=H.

(8b) R=OH.



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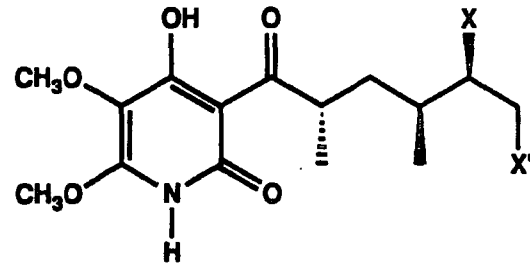
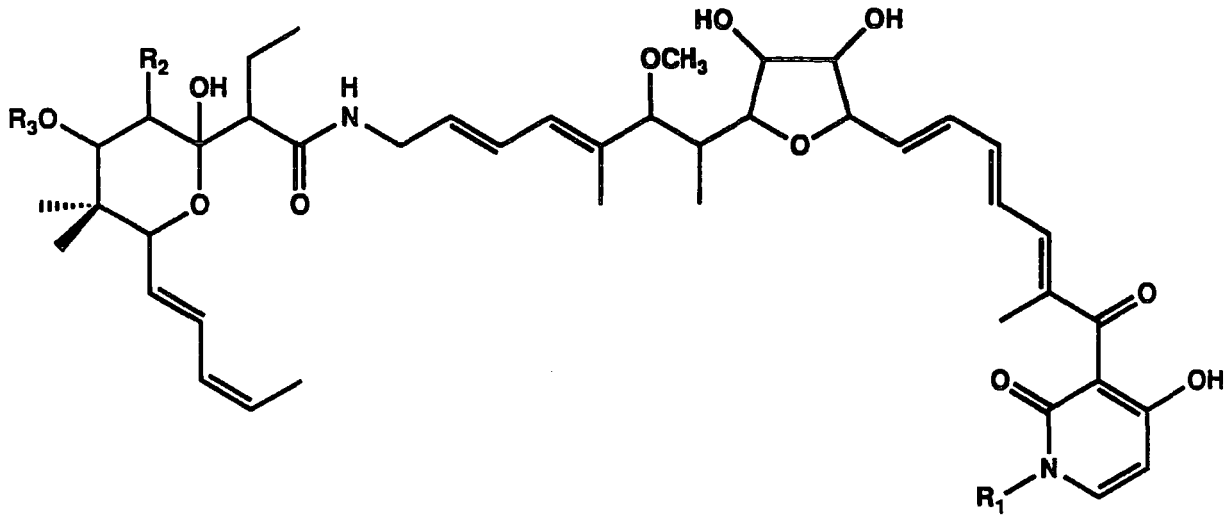


X	X'	
Cl	H	atpenin-A4 (10a)
Cl	Cl	atpenin-A5 (10b)
H	H	atpenin-B (10c)

The fungal 2-pyridones, leporin-A (7), viridicatin (8a), viridicatol (8b), harzianopyridone (9) and atpenins A4 (10a), A5 (10b) and B (10c).

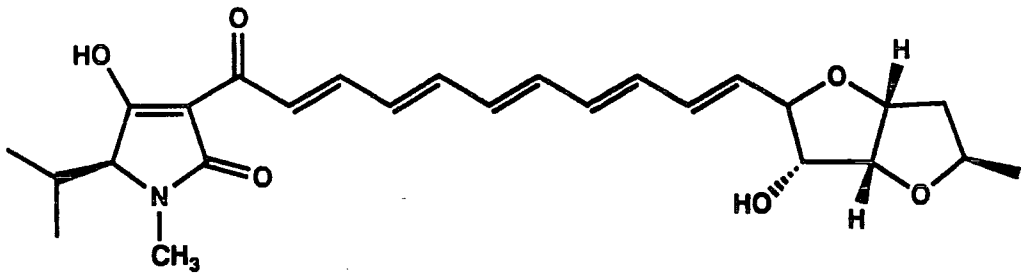
The interesting 2-pyridone unit also occurs in the bacterial efrotomycin family of antibiotics[22]. The parent glycone efrotomycin (11a) (*Streptomyces sp.*) co-occurs with the aglycones aurodox (11b) (also known as goldinomycin or antibiotic X5108) (*Streptomyces goldinensis*), kirromycin (11c) (*Streptomyces sp.*) and heneicomycin (11d) (*Streptomyces sp.*).



R_1	R_2	R_3	
CH_3	OH	Disaccharide	efrotomycin (11a)
CH_3	OH	H	aurodox (11b)
H	OH	H	kirromycin (11c)
CH_3	H	H	heneicomycin (11d)

The bacterial 2-pyridone efrotomycin family.

These metabolites belong to a larger family of metabolites elaborated by both fungal and bacterial systems which is made up of groups of molecules biosynthesised from a combination of amino-acid derived, and polyketide derived, moieties. The group also contains, the acyl tetramic acids, including erythroskyrine (12) (*Penicillium islandicum* Sopp.)[23,24], fuligorubin-A (13) (*Fuligo septica* (L.) Wiggers)[25,26], tenuazonic acid (14) (*Alternaria tenuis* auct.)[27], lydicamycin (17) (*Streptomyces lydicus*)[28], ikarugamycin (15) (*Streptomyces phaeochromogenes var. ikaruganensis*)[29,30,31] and the structurally interesting pseurotin-A (16) (*Pseudeurotium ovalis* Stolk)[32] amongst others; the cytochalasins (18)[33] synthesised by the imperfect fungus *Zygosporium masonii* (Hughes) in which phenylalanine and tryptophan provide the amino acid residues; the ochratoxins[34] (19a,b,c) elaborated by members of the genera *Aspergillus* and *Penicillium* in which L-phenylalanine forms an amide link to an acetate and methionine derived bicyclic residue; and the more distantly related *bis*-amino acids in which modified amino acids are condensed together to form the six membered rings of mycelianamide (20) (*Penicillium griseofulvum*)[35] and cryptoechinulin-A (22) (*Aspergillus spp.*)[36] and the seven membered rings of cyclopenin and cyclopenol (21a,b) (*Penicillium cyclopium*)[37].



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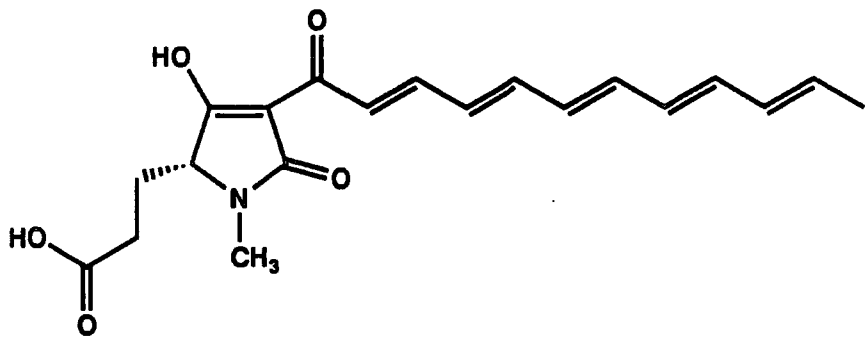

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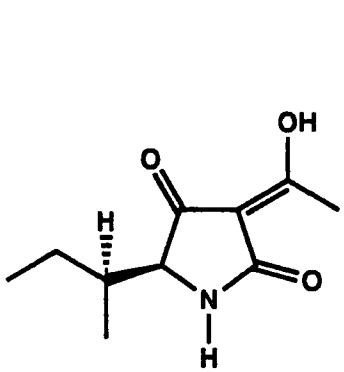

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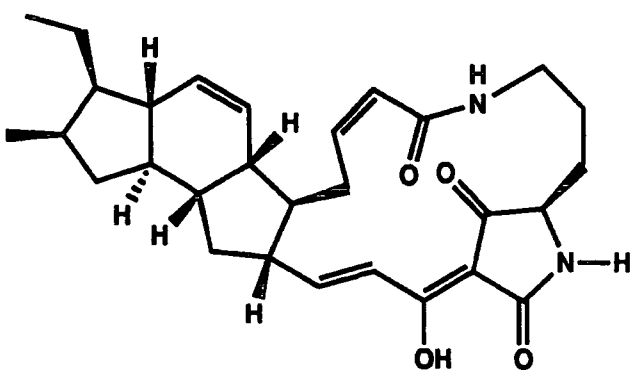

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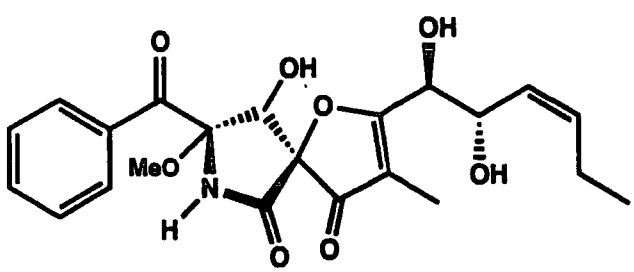
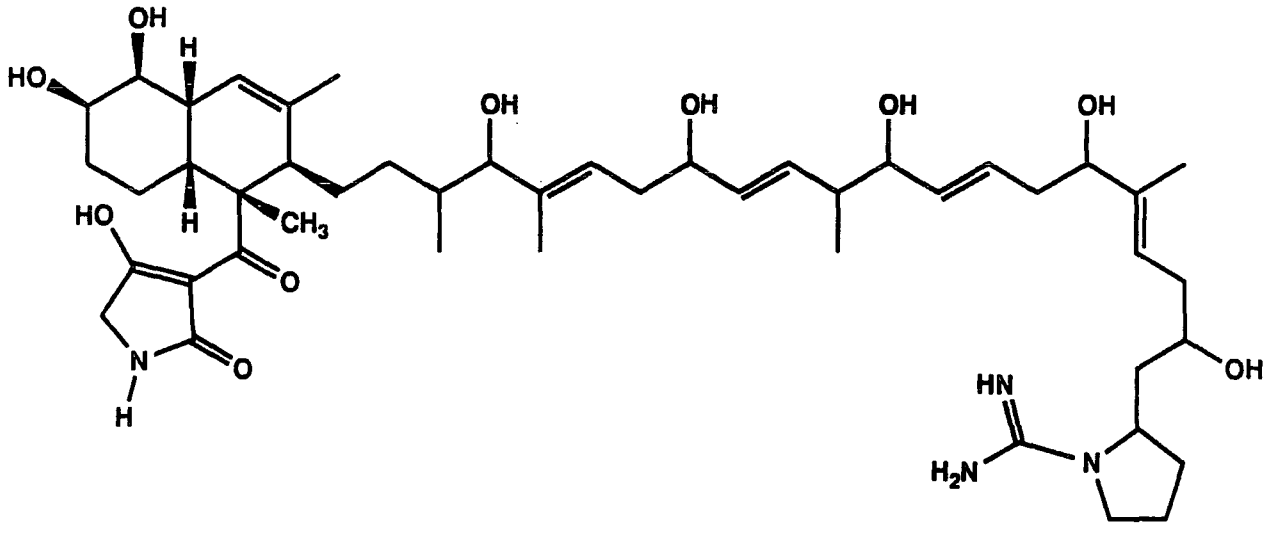
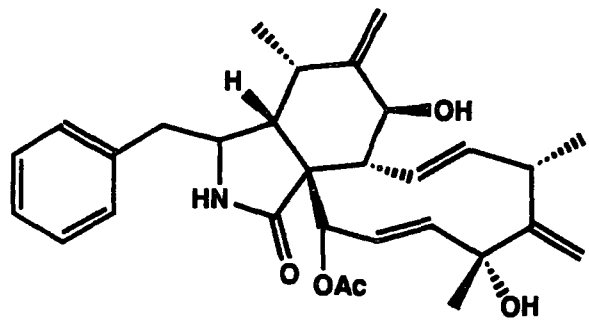


(16)

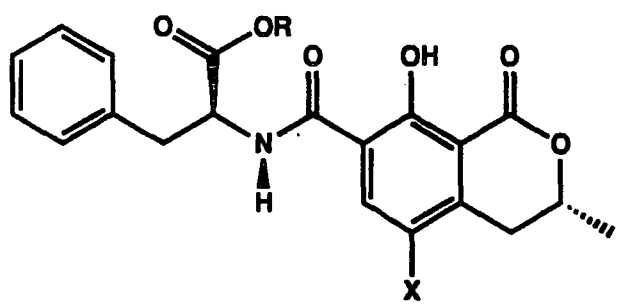
The acyl tetramic acids erythroskyrine (12), fuligorubin (13), tenuazonic acid (14), ikarugamycin (15) and pseurotin-A (16).



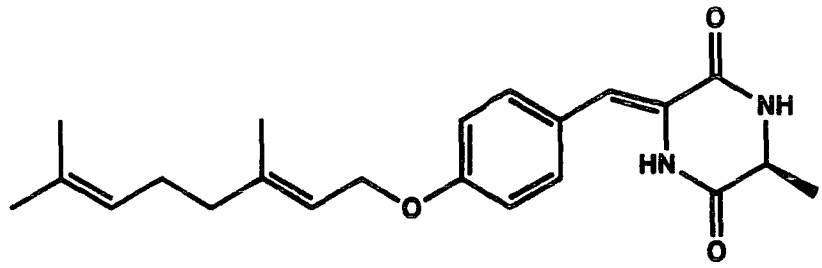
The acyl tetramic acid lydicamycin (17).



cytochalasin D (18a)



X	R	
Cl	H	ochratoxin-A (19a)
H	H	ochratoxin-B (19b)
Cl	Et	ochratoxin-C (19c)



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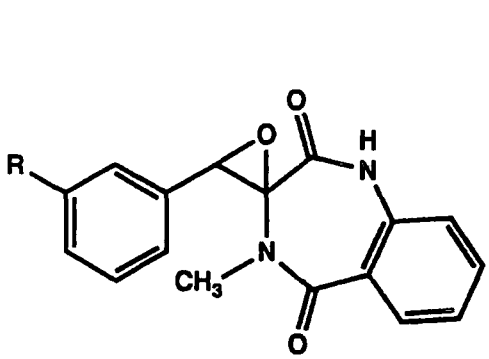
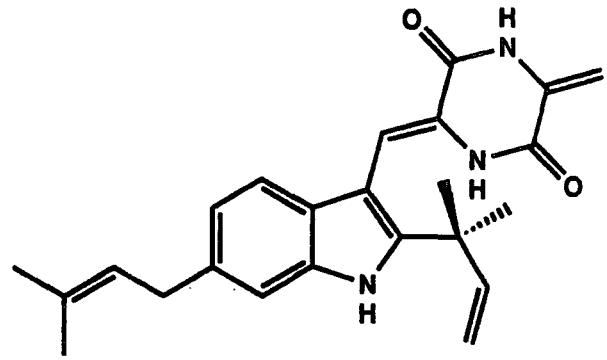




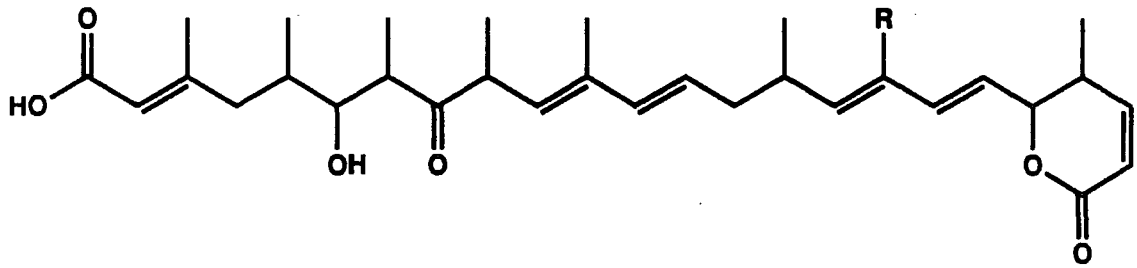
(22).

R	
H	cyclopenin (21a)
OH	cyclopenol (21b)

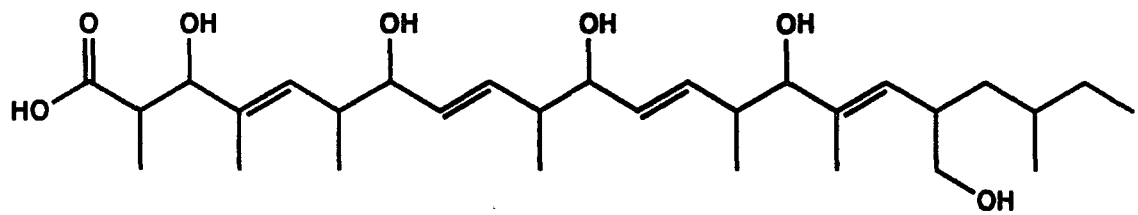
Mycelianamide (20), cryptoechinulin (22), cyclopenin (21a) and cyclopenol (21b).

1.3 The polyketides.

The polyketides make up a large and diverse group of secondary metabolites elaborated across a range of prokaryotic and eukaryotic organisms as varied as the bacteria, fungi and slime moulds, algae and marine sponges and corals. Examples include leptomycins-A and B (23a,b) (*Streptomyces 1287*)[38] and cubensic acid (24) (*Xylaria cubensis*)[39,40] the macrolide antibiotic erythromycin-A (26a) (*Saccharopolyspora erythraea*),[41] 6-methylsalicylic acid (25) (*Penicillium patulum*) an aromatic polyketide[42], the phytotoxin (-)-betaenone-B (27) (*Phoma betae* Fr.)[43,44], the polyether monensin-A (28) (*Streptomyces cinnamonensis*)[45] and the marine metabolite tedanolide (29) (*Tedania ignis*)[46]. Even a cursory glance at these molecules reveals the vast diversity of structures grouped under the innocuous title of the polyketides.



R	
CH_3	leptomycin-A (23a)
CH_2CH_3	leptomycin-B (23b)



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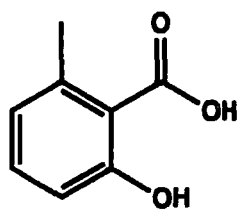
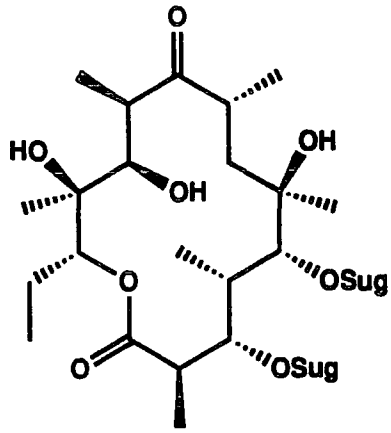
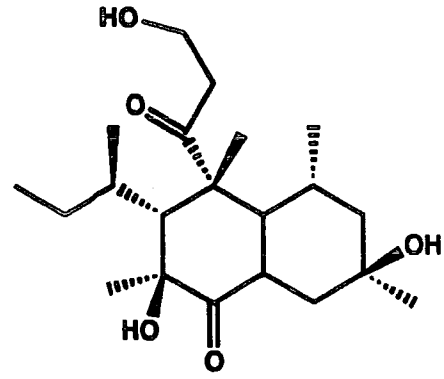


(25)

Leptomycin-A and B (23a,b), cubensic acid (24) and 6-methylsalicylic acid (25).



(26a)



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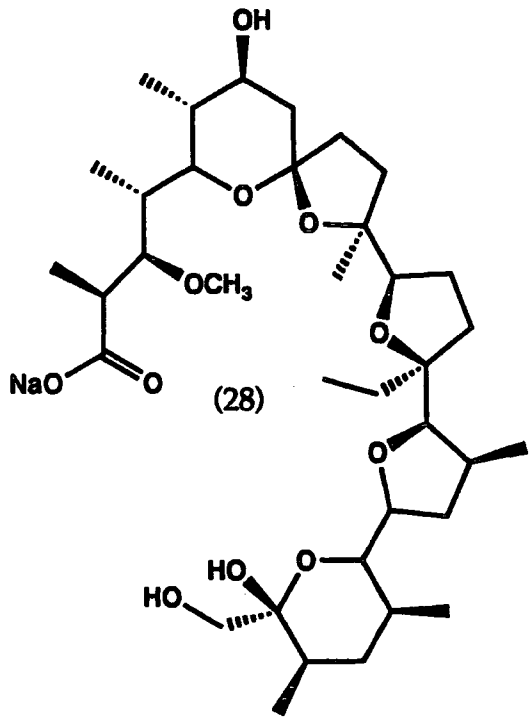

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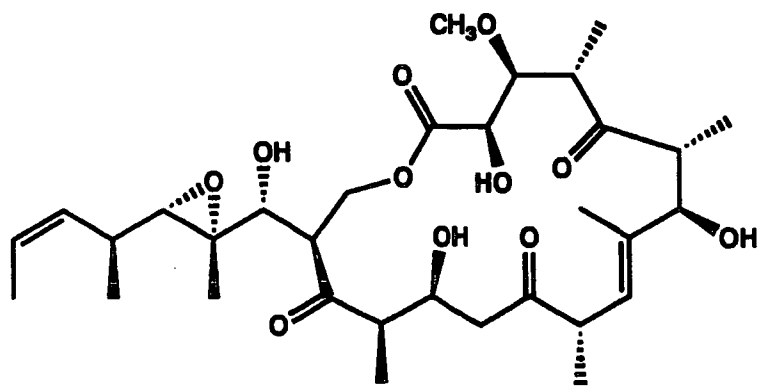


(29)

Erythromycin-A (26a), (-) betaenone-B (27), monensin-A (28) and tedanolide (29).

The enormous structural differences displayed by these metabolites, however, belies the simplicity of their biogenesis. Feeding experiments with labelled carbon units of four or (usually) fewer carbon atoms have highlighted the underlying similarities between structures. For example the macrolide dehydrocurvularin (30)[47] elaborated by the fungal plant pathogen *Alternaria cinerariae* is biosynthesised from eight acetate (77) units, condensed head to tail.

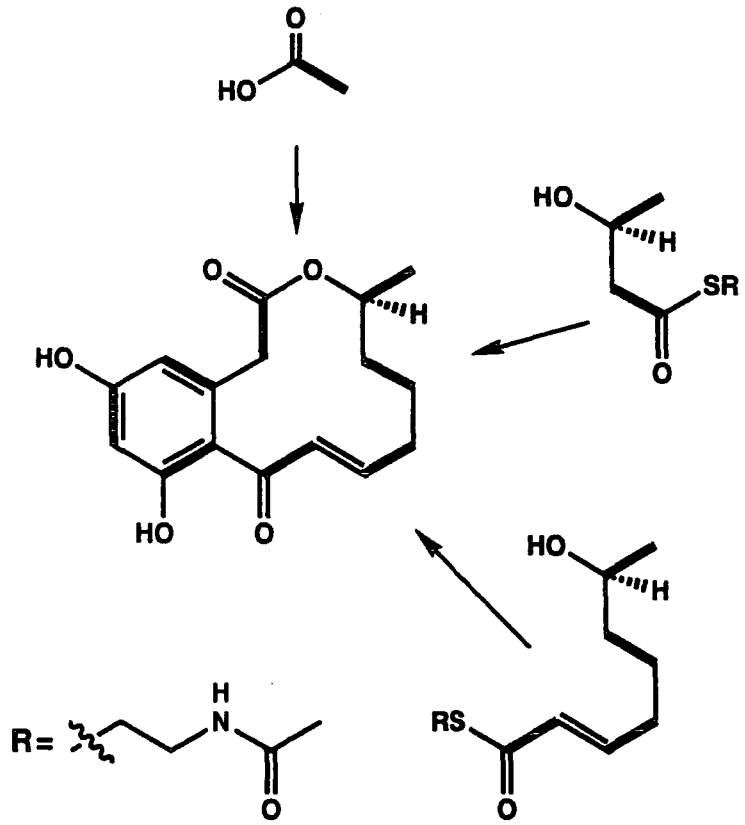


Fig 1.31. Dehydrocurvularin (30) is biosynthesised from eight acetate units in a stepwise manner via the diketide and tetrakedide shown.

More sophisticated feeding experiments using doubly labelled di- and tetra-ketides (that is, structures corresponding to two and four acetate units respectively) activated to N-acetylcysteamine thiolesters have shown that dehydrocurvularin (30) is built up in a processive manner using acetate units, reminiscent of classical fatty acid biosynthesis (see chapter 2, section 2.34).

In fungi chain methylations can occur to increase complexity. For the biosynthesis of cubensic acid (24), eight methyl groups are provided by L-methionine (90)[40], in addition to eleven acetates arranged head to tail.

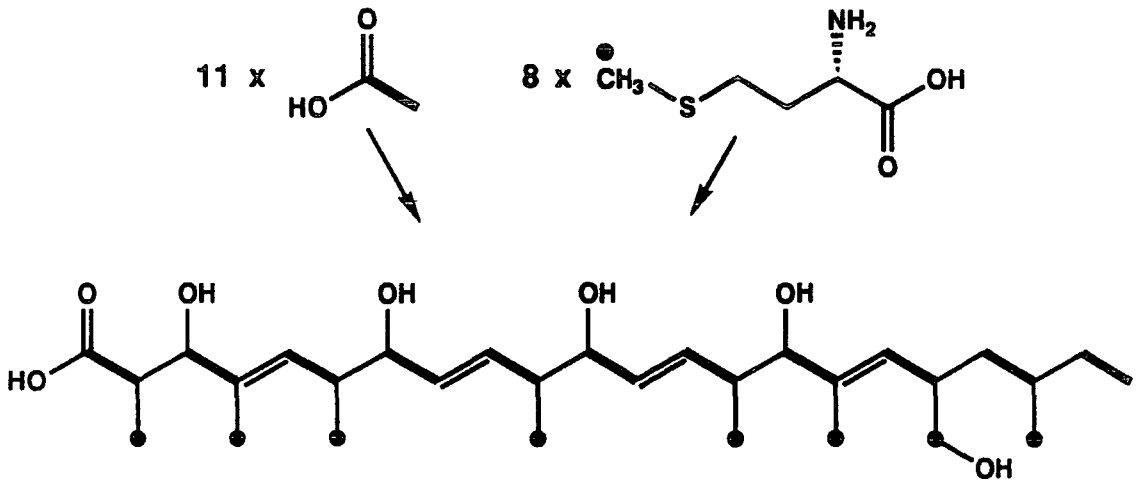


Fig. 1.32. The biosynthesis of cubensic acid (24) from eleven units of acetate and eight L-methionine (90) donated methyl groups.

In bacterial systems the incorporation of propionate (78) and butyrate (79) units as well as acetate (77) increases complexity. The erythromycin aglycone 6-deoxyerythronolide-B (26c)[48] produced by a mutant of *Saccharopolyspora erythraea* arises from the condensation of seven propionate (78) units, whilst the polyether monensin-A (28), elaborated by *Streptomyces cinnamonensis,* is built of five acetate (77), seven propionate (78) and one butyrate (79) units[49].

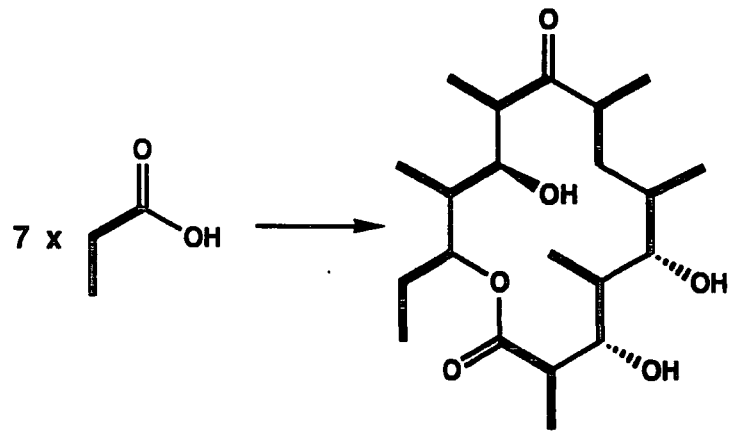


Fig. 1.33. 6-Deoxyerythronolide-B (6-dEB) (26c) is biosynthesised from seven units of propionate (78).

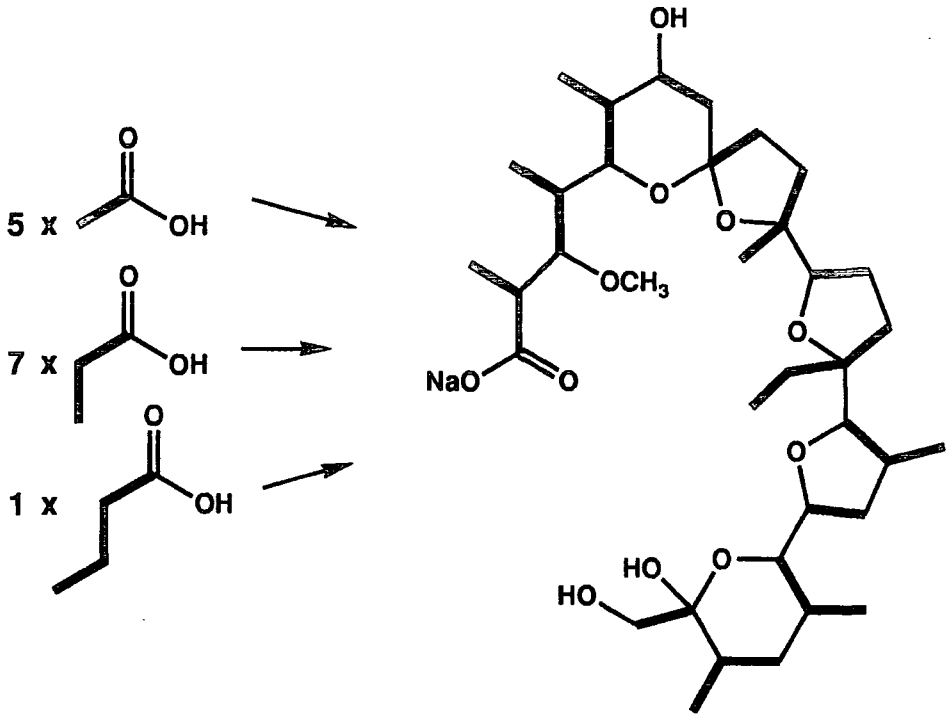
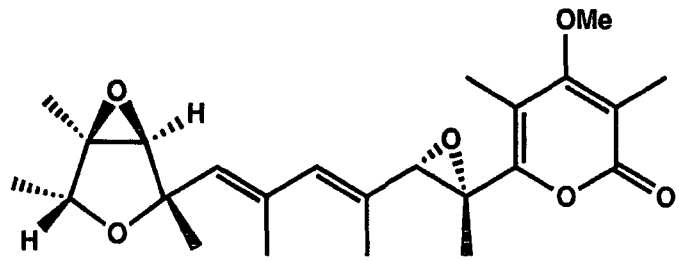


Fig. 1.34. Monensin-A (28) is biosynthesised from five units of acetate, seven units of propionate and one butyrate unit.

Post assembly modification of polyketides occurs in a number of ways to further increase structural diversity. Double bonds can be epoxidised as in the case of verrucosidin (31) (*Penicillium verrucosum. var cyclopium*),[50] a fungal octaketide bearing seven methionine donated methyl groups.



verrucosidin (31).

Epoxidation of polyketide double bonds also introduces scope for further elaboration to form tetrahydropyran and tetrahydrofuran rings and more complex bicycles. Thus it has been proposed that the hydroxylated furan ring occuring in citreoviridin (33)[51] (Fig. 1.35), a potent neurotoxin, produced by the fungus *Penicillium citroviride* derives from a di-epoxide of an acetate and methionine derived tetra-methylated nonaketide. Such a metabolite, citreomontanin (32), is already known as a product of *Penicillium pedemontanum*.[52,53]. More complex ring systems can be formed

by increasing the number of epoxidised double bonds, citreoviridinol (34) also produced by *Penicillium citroviride* is envisaged to derive from further epoxidation and cyclisation of citreoviridin (33)[54].

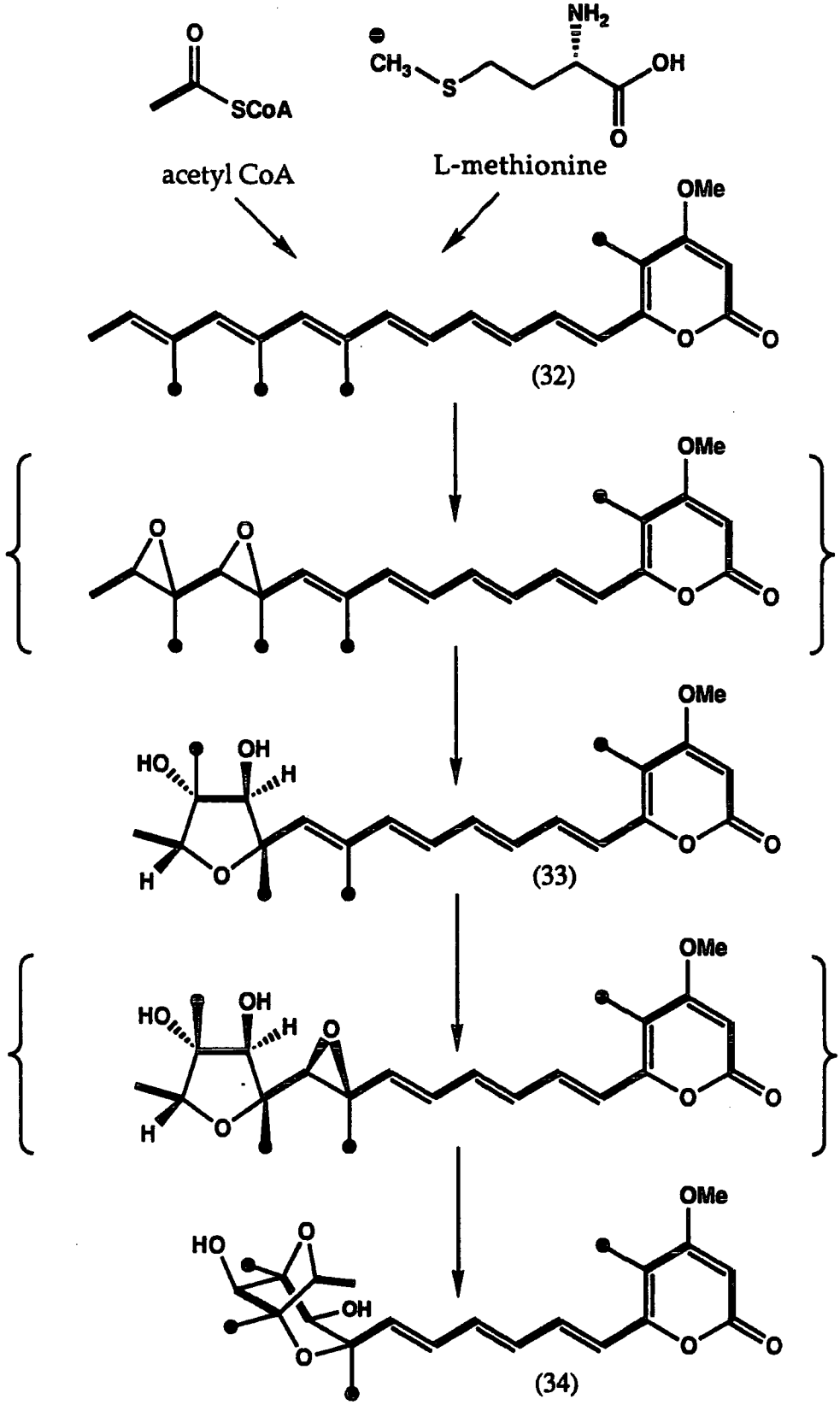


Fig. 1.35. Postulated biosynthesis of citreoviridin (33) and citreoviridinol (34).

Lactonisations between the terminal carboxyl group and a hydroxyl functionality further down the polyketide backbone can also occur, to form metabolites such as tylactone (37) produced by *Streptomyces fradiae*[55], one of the clinically important macrolide family, as well as the pyrone moieties typical of the polyene pyrone family (31-36).

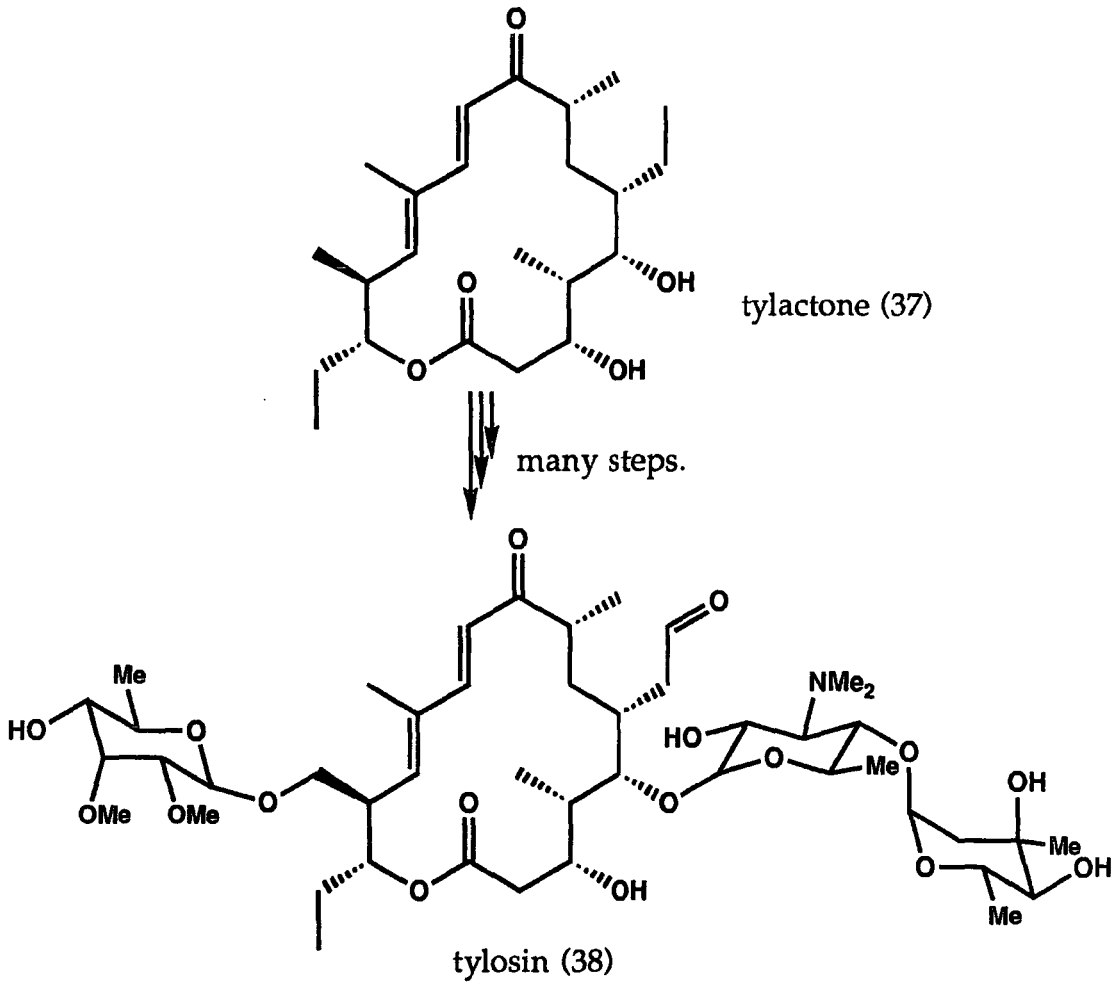
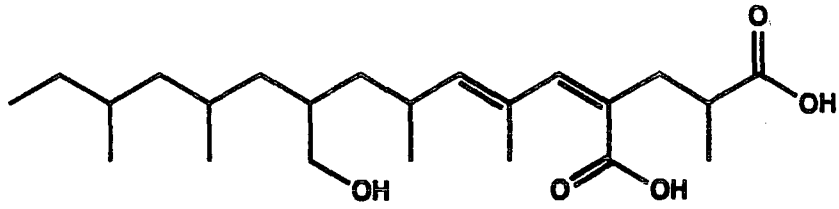
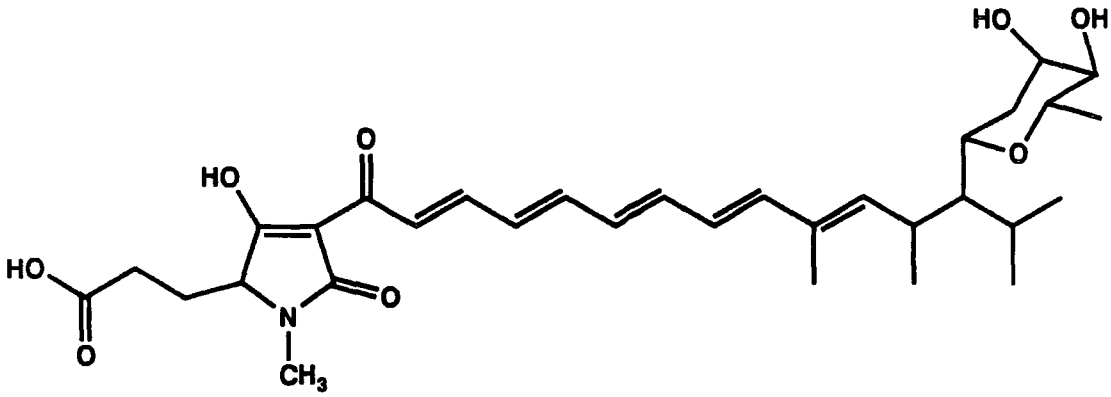


Fig. 1.36. Post assembly modification of the polyketide tylactone (37) leads to tylosin (38).

Oxidations are also a common modification step, for example leptomycins-A and B (23a,b) from *Streptomyces 1287* are oxidised at the terminal carbon of the starter acetate unit whilst the *Penicillium spp.* metabolite radiclonic acid (39)[56,57] contains two oxidised methyl groups, one partially oxidised to an alcohol and the other fully to a carboxylic acid. Derivatisations of alcohol groups also occur, especially with sugars, the acyl tetramic acid α–lipomycin (40)[58,59] elaborated by *Streptomyces aureofaciens* being a good example bearing a digitoxide residue. The oxidation and alcohol derivitisation steps leading from tylactone (37) to tylosin (38) are particularly well understood[60].



radiclonic acid (39)



α–lipomycin (40)

A further dimension of structural complexity can be introduced by carbon skeletal rearrangements and carbon-carbon bond cleavages. These changes often split the intact acetate units incorporated during polyketide biosynthesis *de novo* and thus tend to obscure the biogenetic origin of a particular metabolite derived by such means. Asteltoxin (35)[61] elaborated by the fungi *Aspergillus stellatus* and *Emericella variecolor* is a case in point[62]. The carbon skeleton of asteltoxin (35) is related to that of citreomontanin (32)[53], being modified only by the unusual incorporation of a propionate (78) rather than an acetate (77) starter unit. Feeding experiments with ^{13}C labelled acetates revealed the enrichment pattern shown in Fig. 1.37 and led to the conclusion that the carbon skeleton of asteltoxin (35) is derived from a linear tri-epoxide (Scheme 1.38) which has undergone a pinacol[63] type rearrangement[64]. The labelled feeding experiments also highlighted dual routes to the starter unit, this unit being derived from either intact propionate or from a combination of acetate and methionine. Asteltoxin (35) belongs to the aurovertin family of metabolites in which dual routes to propionyl starter units are a characteristic feature (see chapter 2, section 2.4)[65].

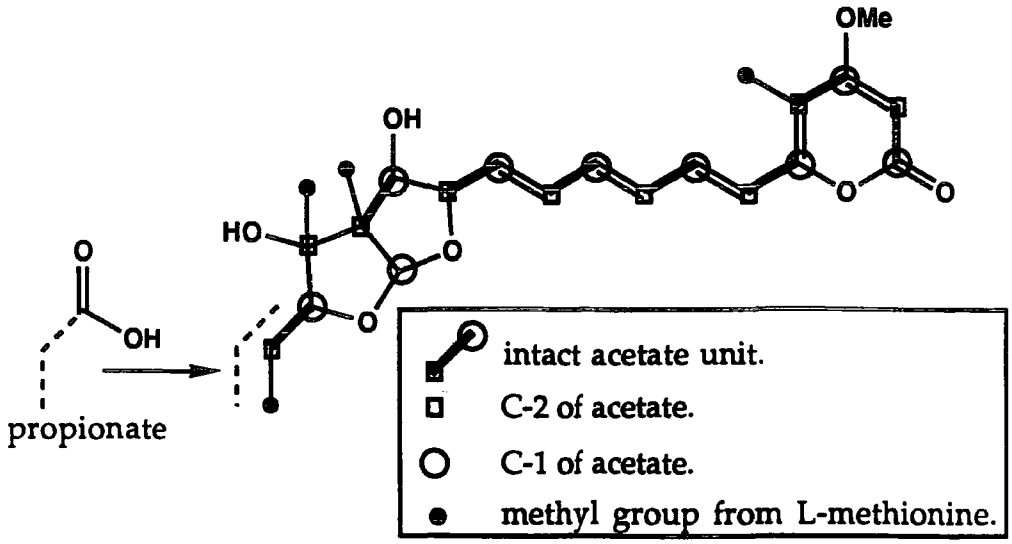
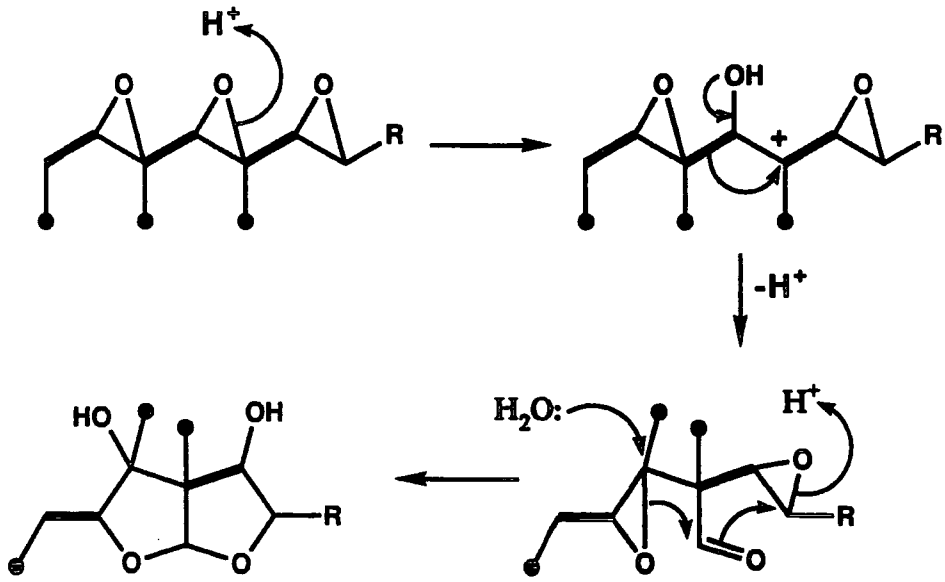


Fig. 1.37. The distribution of labels from acetate, propionate and L-methionine incorporated into asteltoxin (35) during biosynthesis.



Scheme 1.38. Postulated mechanism of chain rearrangement in the biosynthesis of the bicyclic moiety of asteltoxin (35).

1.4 The Biosynthesis of tenellin (1).

Vining et al. have elucidated the biosynthetic origin of all the skeletal atoms of tenellin (1) in a definative series of feeding experiments utilising acetates, L-methionine and phenylalanine isotopically enriched with the stable isotopes ^{13}C and ^{15}N[66].

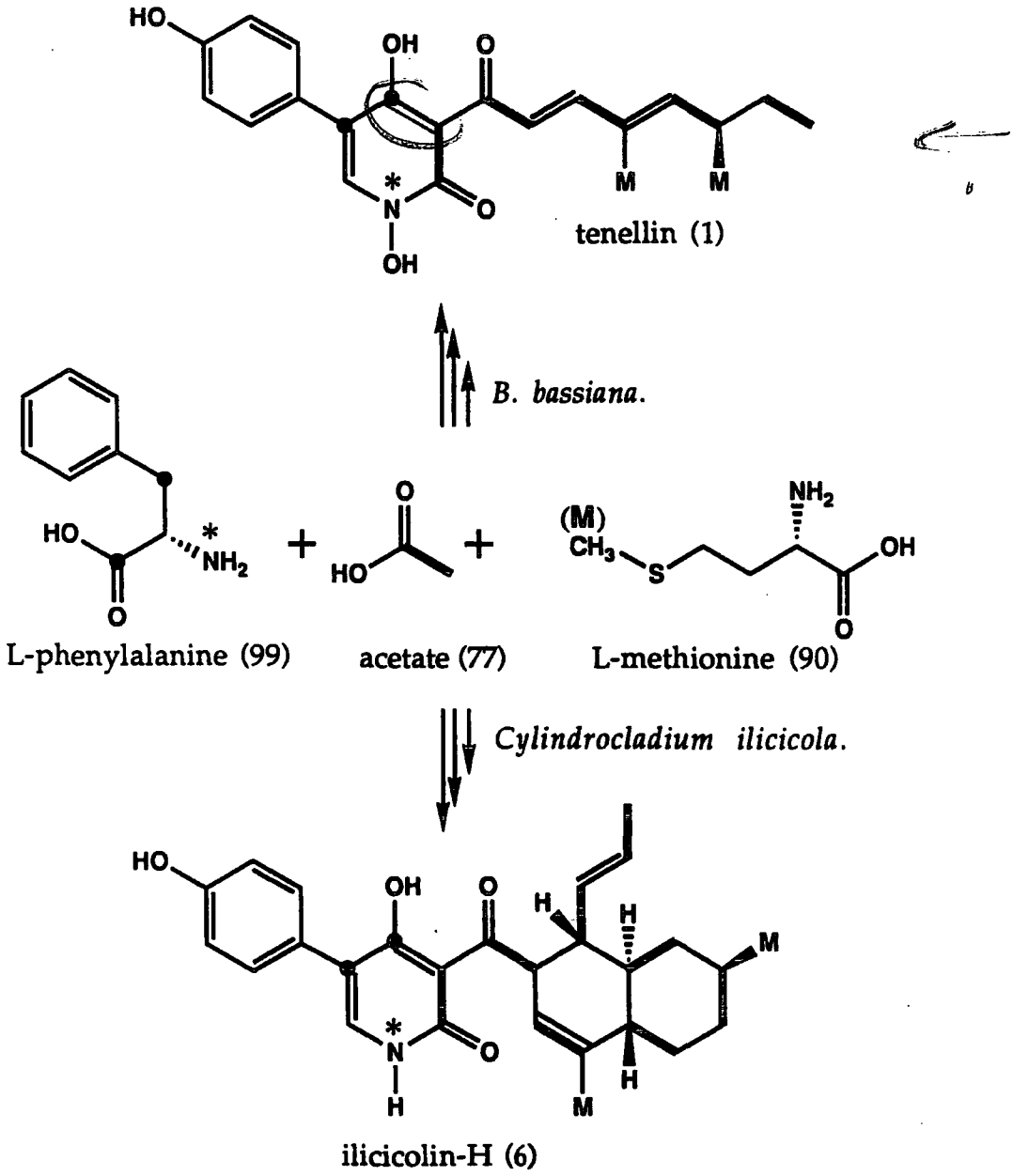
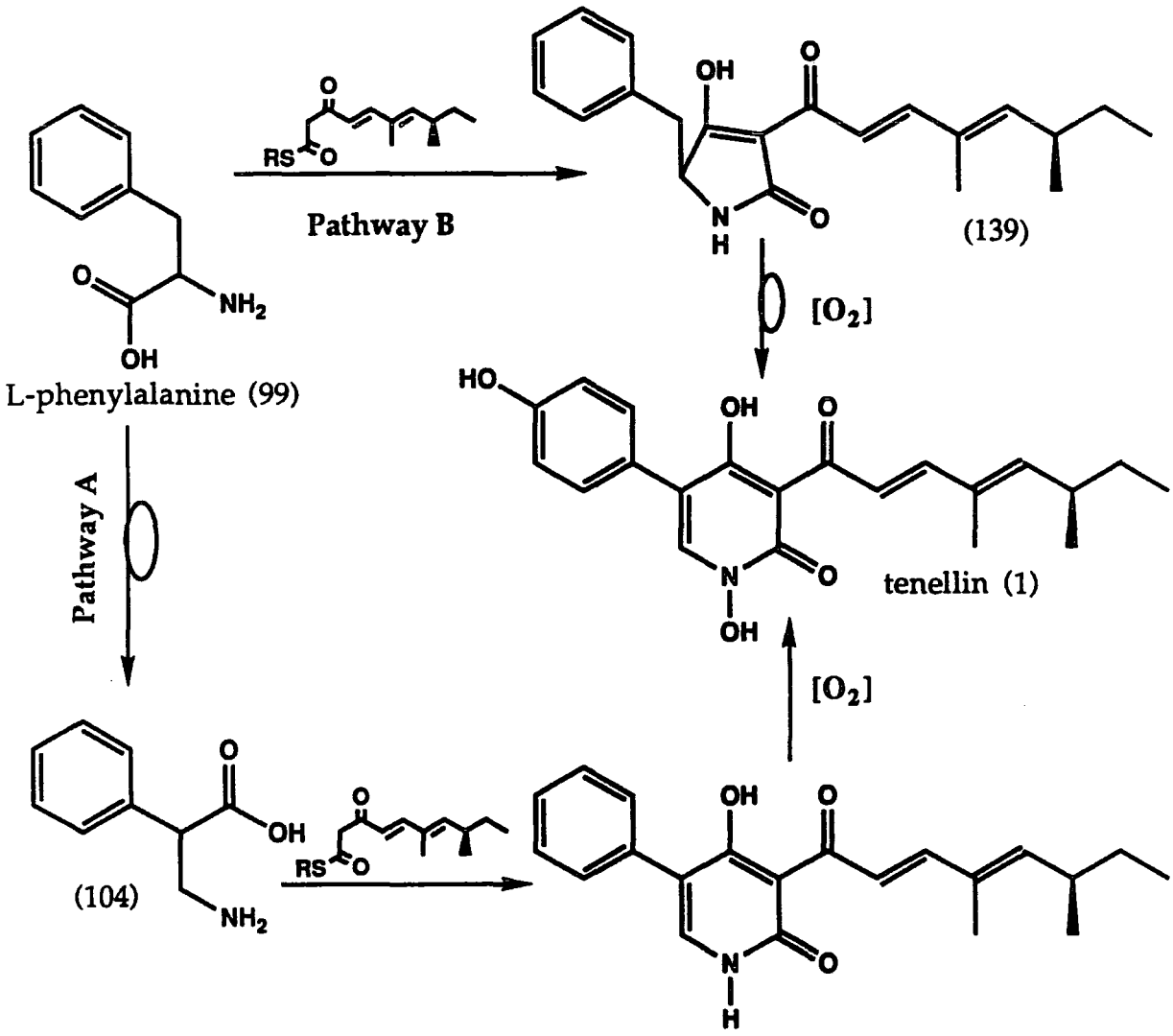


Fig. 1.401. Tenellin (1) and ilicicolin-H (6) are derived from L-phenylalanine (99), acetate (77) and L-methionine (90).

They demonstrated[66] that tenellin (1) arose by the coupling of an amino phenylpropanoid moiety derived intact from L-phenylalanine, to the terminal acetate residue of a typical polyketide chain made up from five intact acetate units coupled in a head-to-tail manner, with two methionine derived pendant methyl groups. The biosynthesis of ilicicolin-H (6), a structurally related metabolite, has also been investigated and the pyridone ring shown to be similarly derived[67].

Vining and his co-workers were unsurprised by the incorporation of acetate and methionine derived methyl groups into the sidechain which was wholly in accordance with the known course of polyketide biosynthesis in fungi. However the observation of the apparant rearrangement of phenylalanine during the biosynthesis of the pyridone ring of tenellin spurred their interest in the origin of this novel group. They further demonstrated in an elegant doubly labelled feeding experiment that the labels of DL-[1,3-$^{13}C_2$]-phenylalanine came together and showed ^{13}C-^{13}C coupling in the ^{13}C nmr spectrum of tenellin (1) obtained from a broth of *B. bassiana* supplemented with this material[68]. This experiment conclusively demonstrated the intramolecular nature of the rearrangement but left open the timing and mechanism of the process.

Two possibilities (at least) may be entertained (Scheme 1.402) to account for these observations concerning the rearrangement of phenylalanine during the biosynthesis of tenellin (1). In pathway **A** it may be envisaged that the amino acid is subect to direct rearrangement by a mutase enzyme before condensation with the polyketide group, forming the six membered pyridone ring directly, with subsequent oxidations furnishing tenellin (1). Alternatively, in pathway **B**, *suggested by Vining et al.*, condensation between L-phenylalanine (99) and the terminal residue of the polyketide chain leads to the five membered ring of an acyl tetramic acid. The six-membered pyridone ring of tenellin (1) could then be formed by a ring expansion of the tetramic acid. Both hypotheses are consistent with the observations of Vining *et al.* and both have biogenetic precedence.



Scheme 1.402. The biosynthesis of the 2-pyridone moiety of tenellin (1) could occur by either of pathways **A** or **B**.

Biosynthetic experiments with numerous members of the family of acyl tetramic acids lend significant support to pathway **B**. Members of the group are produced by both fungi and bacteria. The fungal metabolite erythroskyrine[24] (12) from *Penicillium islandicum* has been shown to arise from the condensation of L-valine (91) with the terminal residue of a decaketide chain[69], whilst the bacterial malonomicin (41) produced by *Streptomyces rimosus var. paramomcyinus* arises from the uncommon α–amino acid 2,3 diaminopropionic acid (DAP) coupled to an acetate derived side chain[70]. In the case of malonomicin (41), the amino acid serine (92) was also efficiently incorporated into the five membered ring, suggesting a direct pathway between serine and DAP.

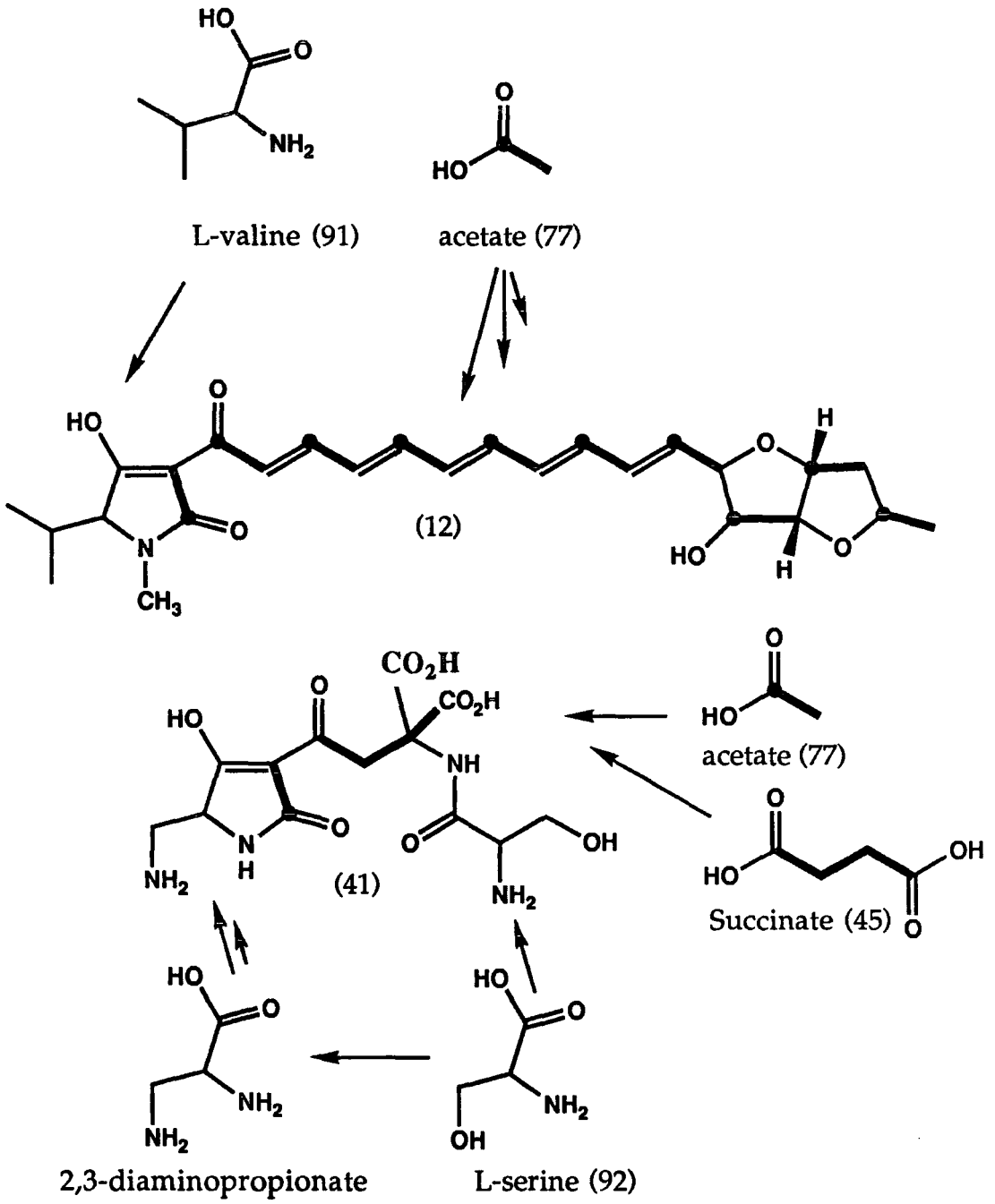


Fig. 1.403. Biosynthesis of erythroskyrine (12) and malonomicin (41).

Vining *et al.* suggested that the ring expansion required to form the six-membered pyridone ring of tenellin (1) from the five membered acyl tetramic acid analogue could procede *via* a quinonoid intermediate[66], with ring expansion promoting re-aromatisation of the pendant phenyl ring (Fig. 1.404). This mechanism is feasible in the cases of tenellin (1), bassianin (2), ilicicolin-H (6) and funiculosin (5) (which may derive ultimately from phenylalanine or tyrosine), but cannot be invoked to explain the biosynthesis of the structurally divergent 2-pyridones such as leporin-A (7) which lacks a para-hydroxyl moiety on the phenyl ring and the non-benzyl

substituted harzianopyridone (9) and atpenins (10a,b,c). A unifying mechanism would be expected for all of these metabolites bearing in mind the close relationship between their producing organisms. These possible mechanisms are discussed in more detail in chapter 3.

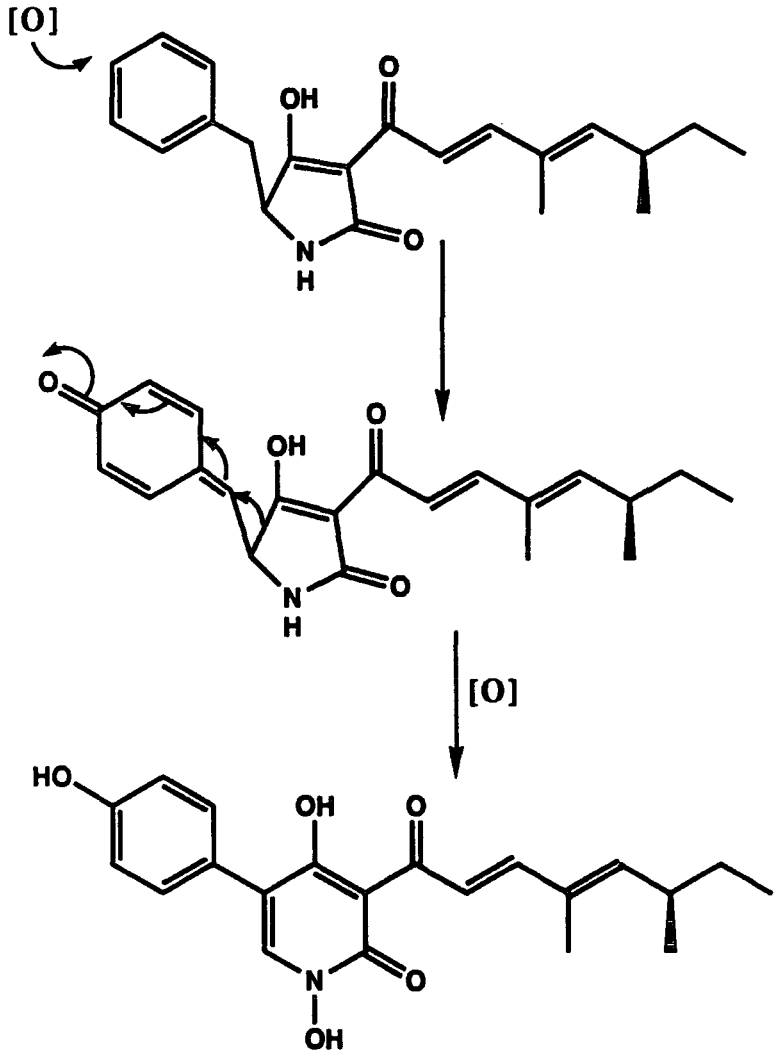
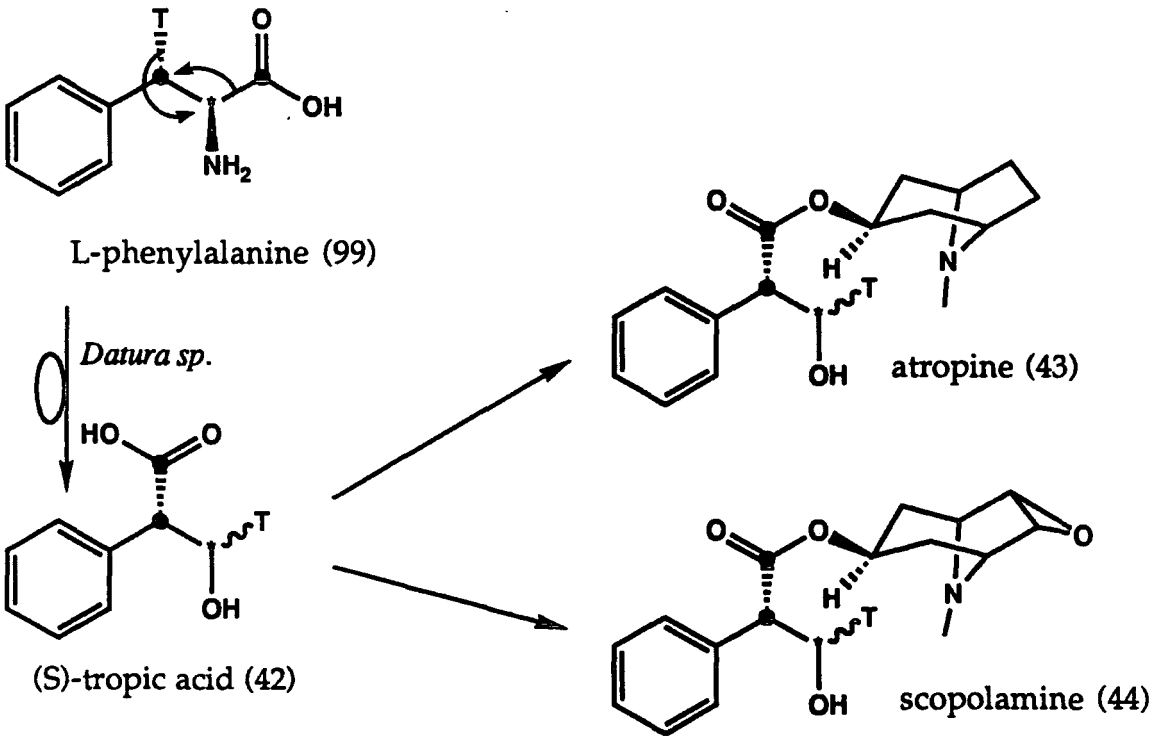


Fig. 1.404. Vining's proposed mechanism for the ring expansion of an acyl tetramic acid to form the 2-pyridone moiety of tenellin (1) by *B. bassiana*

Precedence for the possible operation of pathway A has been provided by Leete and co-workers who have shown that L-phenylalanine is subject to intramolecular rearrangement[71] to (S)-tropic acid (42) during the biosynthesis of the tropane alkaloids atropine (43) and scopolamine (44) in the Solanaceous plant genus *Datura* (Fig. 1.405). Feeding experiments with labelled L-phenylalanines using *D. innoxia* and *D. stramonium* showed that the carboxyl group of phenylalanine migrates from C-2 to C-3 to form the branched carbon skeleton of tropic acid (42) (see chapter 3, section 3.21).

The observation of concomitant migration of hydrogen[72,73] from the 3-position of phenylalanine to the hydroxyl bearing carbon of tropic acid raises the possibility that the rearrangement occurs by a radical induced mechanism mediated by a co-enzyme B_{12} dependent mutase activity. A good example of this relatively rare type of biological rearrangement occurs in the metabolism of (S)-methylmalonyl CoA (83a) to succinyl CoA (45a) (Fig. 1.406).



Scheme 1.405. (S) Tropic acid (42) and the biosynthetic origin of atropine (43) and scopolamine (44) from L-phenylalanine (99).

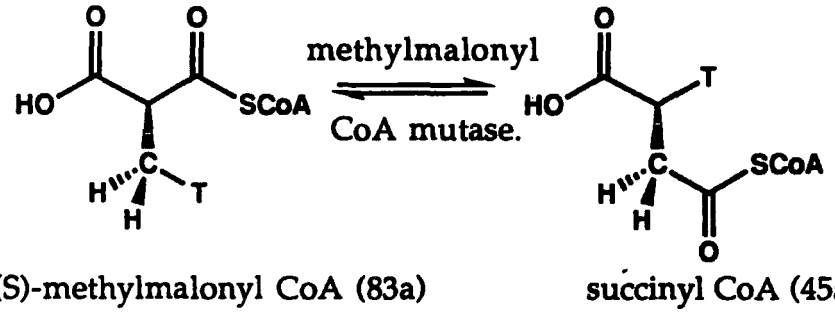


Fig. 1.406. A 1-2 carboxyl shift mediated by co-enzyme B_{12}, the conversion of (S)-methylmalonyl CoA (83a) to succinyl CoA (45a).

Tenellin (1) can be placed confidently and firmly within the broader group of secondary metabolites derived jointly from a polyketide and an amino acid moiety. The polyketide side chain is typical of fungi, being derived from acetate and methionine rather than acetate and propionate as in the bacterial systems.

Whilst the feeding experiments carried out on tenellin have elucidated the primary metabolites utilised *in vivo* to form its structural skeleton, no information has been obtained concerning intermediates or mechanisms of biosynthesis. As with tenellin (1), the biosynthesis of very many of the other polyketides remains shrouded in mystery at a level intermediate between primary precursors (acetate, propionate, methionine etc.) and the fully assembled metabolites. Tenellin (1) therefore offers an ideal system to study the biosynthesis of not only the interesting 2-pyridone systems, but also the polyketides in the wider sense and fungal polyketides more specifically.

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Chapter 2.

Polyketides and the sidechain of tenellin.

2.1 Polyketides and the link to primary metabolism
- fatty acid biosynthesis.

2.2 Fatty acid biosynthesis.

2.21 Unsaturated fatty acids as the starting point for secondary metabolite biosynthesis.

2.3 Polyketide biosynthesis.

2.31 General notes, the relationship between polyketides and the fatty acids.

2.32 Simple precursors.

2.33 Orsellinic and 6-methylsalicylic acids.

2.34 Dehydrocurvularin.

2.35 The macrolides.

2.36 Genetic aspects of PKS.

2.4 Branching strategies.

2.5 Experimental investigation into the origin of carbon and hydrogen atoms during the biosynthesis of the polyketide sidechain of tenellin (1).

2.51 Experimental investigation into the origin of the sidechain hydrogens.

2.52 Experimental investigation of the mode of methylation during the biosynthesis of tenellin (1).

2.53 Experimental investigation into the timing of chain methylations.

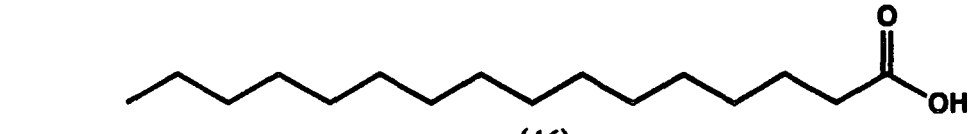
2.54 Discusion.

2.6 References.

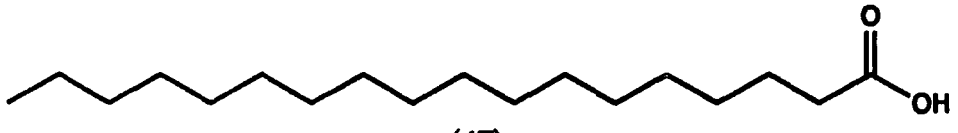
2.1 Polyketides and the link to primary metabolism - fatty acid biosynthesis.

Extensive biosynthetic and structural studies using a large variety of polyketides from many sources have indicated a close analogy between the biosynthesis of these molecules and the classic biosynthesis of the fatty acids[1]. Both classes are made up of linear chains of acetate (and propionate and butyrate in the case of the bacterial and marine polyketides and fungi where propionate appears as a starter unit).

As a class the fatty acids encompass the typical fully saturated compounds such as palmitic (46) and stearic (47) acids as well as the unsaturated examples such as oleic (48) and linoleic (49) acids. They are commonly found as triesters of glycerol and as such act as energy reserves for the cell. Phosphoglycerides in which one of the fatty acids is replaced by a phosphatidylcholine or related ester, make up the bulk of cell walls and membranes. Fatty acids also provide valuable starting materials for further metabolism.



(46)



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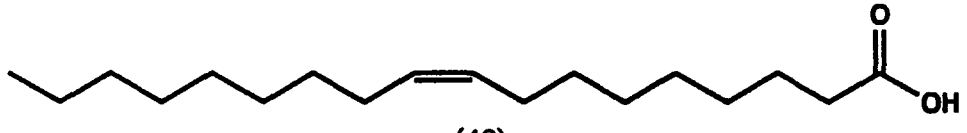

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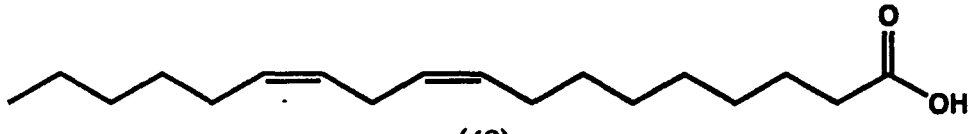
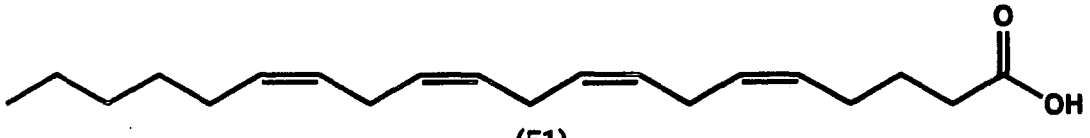


(49)



(51)

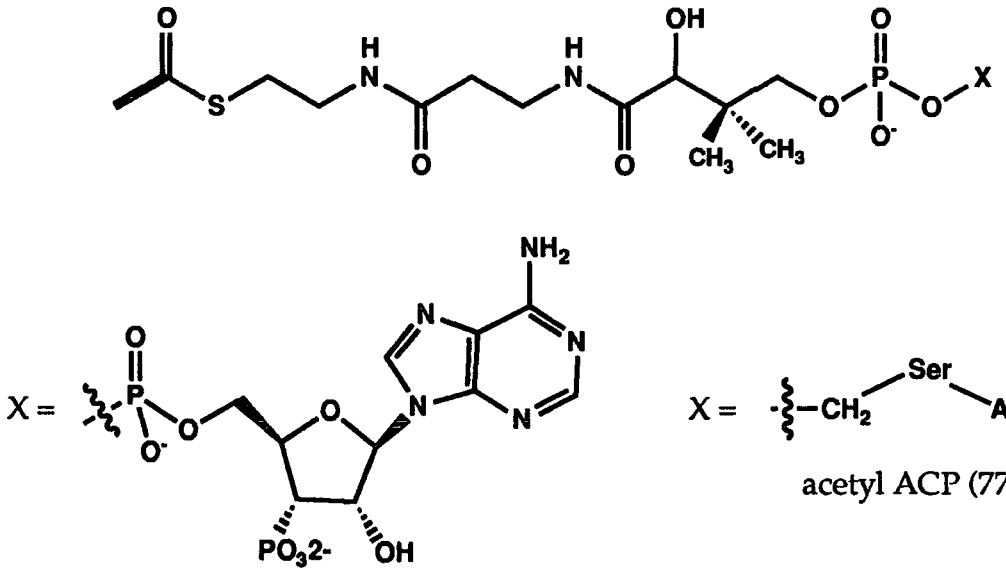
Palmitic acid (46), stearic acid (47), oleic acid (48), linoleic acid (49) and arachidonic acid (51).

The biosynthesis of fatty acids has been shown to occur on a highly cooperative set of enzymes or enzyme complex known as the fatty acid synthase (FAS)[2]. The exact nature of this FAS varies, and the types of FAS are classified with reference to the level of association between the constituent parts. In primitive systems there is a cluster of distinct enzymes each with a unique activity, this is termed a Type II FAS, and is typical of the plants and bacteria. In the more advanced cases a much higher level of association and cooperation is observed, for example the FAS in mammals consists of two identical multifunctional proteins, each containing all of the activities required for synthesising fatty acids, which must dimerise to form the active form of FAS. This highly integrated system is termed Type I FAS[3].

Between these extremes exist a variety of intermediate systems, each classified according to its own merits. Although the exact nature of the FAS complexes varies between organisms, the series of biochemical reactions leading to the fatty acids are identical[4].

2.2 Fatty acid biosynthesis.

The process (Scheme 2.202) starts when acetate (77), activated *in vivo* to the thiolester acetylcoenzyme-A (acetyl CoA) (77a), condenses with carbon dioxide to form malonyl CoA (82a). This important reaction is catalysed by acetyl-CoA carboxylase, a biotin dependent enzyme, and is essential for synthesis. Malonyl CoA (82a) provides the two carbon units used to build the even numbered chains typical of the fatty acids. The condensation occurs with retention of configuration at C-2 of malonate[5].



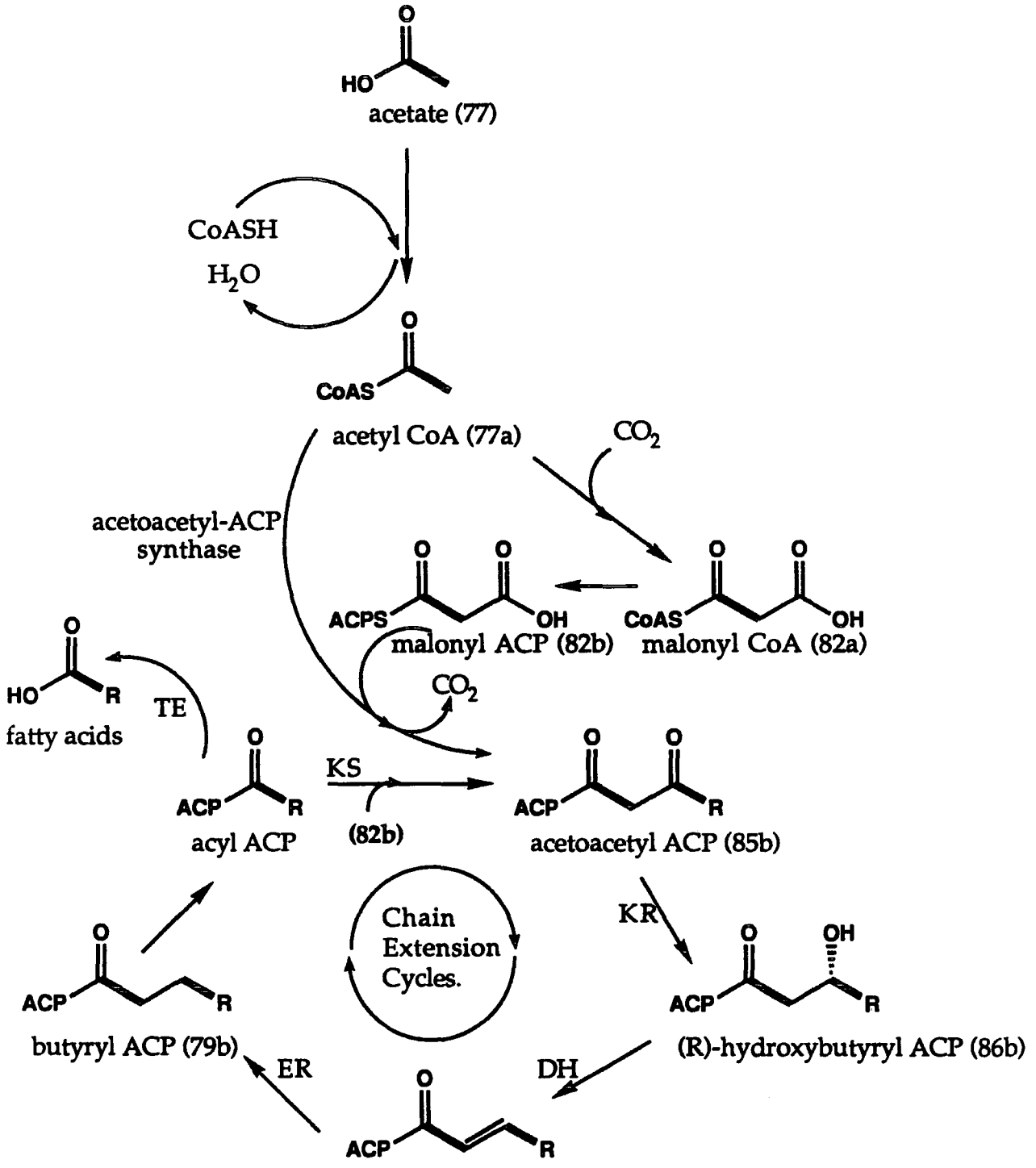
acetyl CoA (77a)

acetyl ACP (77b)

Fig. 2.201. Acetyl CoA (77a) and acetyl ACP (77b).

Malonyl CoA (82a) is transesterified onto an acyl carrier protein (ACP) where condensation occurs directly with the chain starter unit acetyl CoA (77a) to yield acetoacetyl ACP (85b). The enzyme which catalyses this step in *E. coli*, acetoacetyl-ACP synthase, has recently been discovered[6]. Concomitant decarboxylation, liberating the carbon dioxide originally added by acetyl CoA carboxylase, occurs during this condensation and it is this that provides the driving force for the reaction. This condensation has been shown to occur with inversion of configuration at C-2 of malonate[7].

The acyl carrier protein (ACP) now holds the growing chain during biosynthesis. The acyl group is bonded as a thiolester to a phosphopantetheine prosthetic group, similar to that of CoA, attached to the ACP.

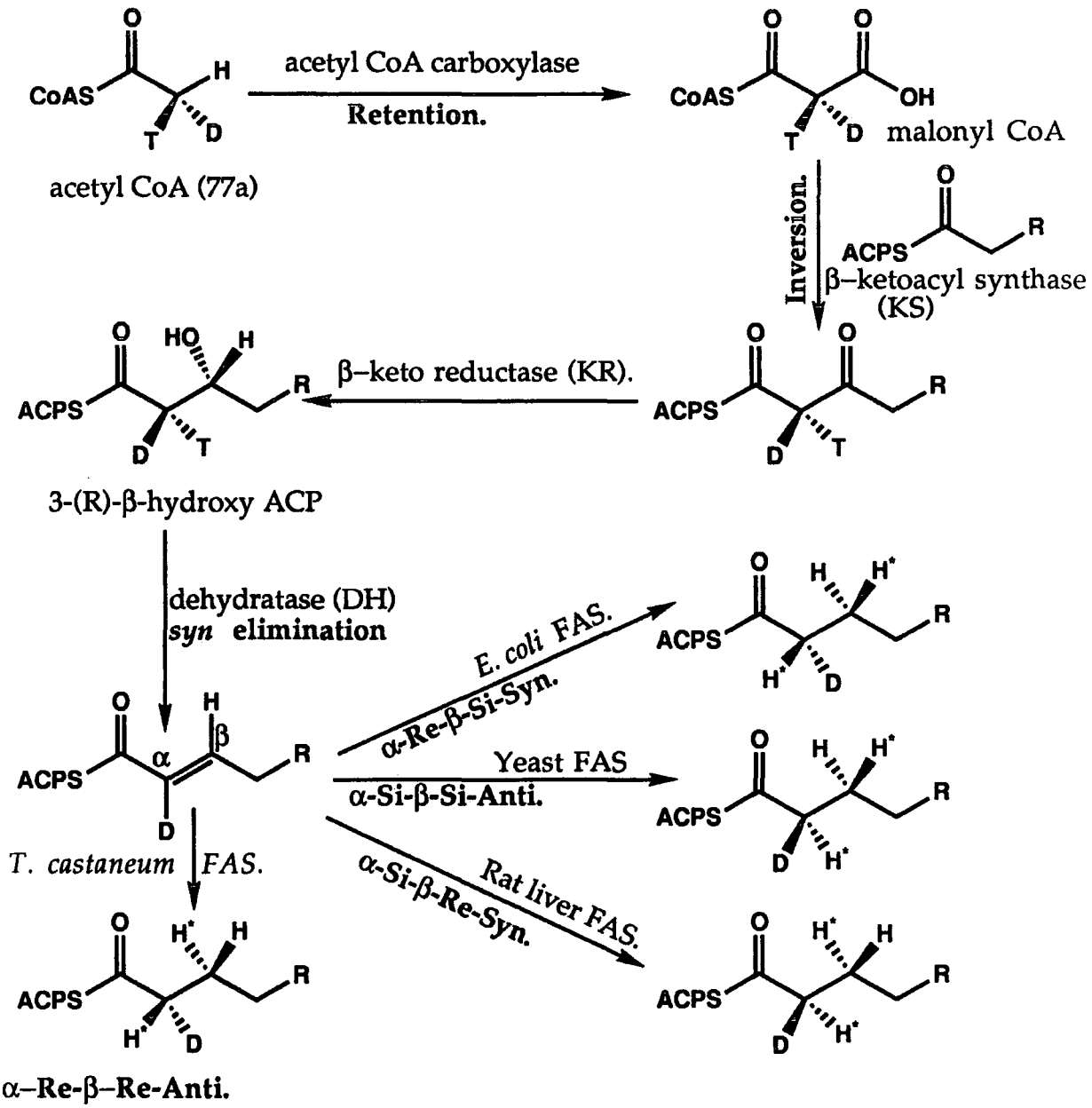


CoA = co-enzyme A, ACP = acyl carrier protein.
KS = β-keto acyl synthase, KR = β-keto reductase.
DH = dehydratase, ER = enoyl reductase.
TE = thiolesterase.

Scheme 2.202. Fatty acid biosynthesis.

The acetoacetyl ACP (85b) thus formed is sequentially reduced by a series of enzymes. Firstly by β–ketoacyl dehydogenase (KR) to yield (R)-3-hydroxybutyryl ACP (86b), subsequently dehydrated by *syn* elimination to crotonyl ACP (87b) by a dehydratase (DH) activity. Full reduction is effected by an enoyl reductase (ER) utilising NADPH as the co-factor to yield butyryl ACP (79b). The stereoselectivety of this reaction is known to vary depending on the organism concerned[8]. For instance *E. coli* FAS mediates syn addition of hydrogen to the double bond such that protonation occurs on the Re face at the α–carbon, whilst rat liver FAS mediates syn addition of hydrogen on the opposite face[9]. The FAS from yeast reduces the double bond by anti addition of hydrogen from the α–Si-β–Si faces[10]. Recently the fourth mode of hydrogen addition, α–Re-β–Re, has been observed in plant (dyers thistle, *Carthamus tinctorius*) and insect (flour beetle, *Tribolium castaneum*) systems[11]. The stereochemical course of FAS is summarised in Scheme 2.203.

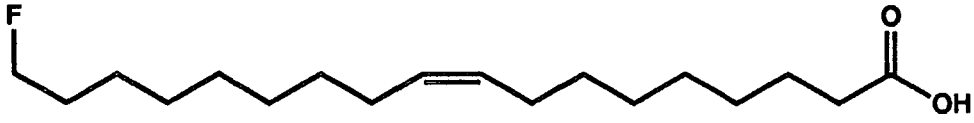
Further condensation with malonyl CoA extends the chain length by two carbons and another cycle of reductions and dehydration furnishes hexanoyl ACP (80b). The cycle of condensations followed by full reductions is repeated until the fully formed fatty acid is released from the FAS complex by a thiolesterase (TE). In the case of the C_{16} fatty acid palmitic acid (46) seven cycles (including the initial priming reaction with acetyl CoA) are required to condense the seven molecules of malonyl CoA with the acetyl CoA starter unit. In this way the fully saturated fatty acids are formed from a head to tail arrangement of acetate units. Carbon dioxide used to activate acetyl CoA to malonyl CoA is regenerated. The major product of FAS is the C_{16} acid palmitate (46).



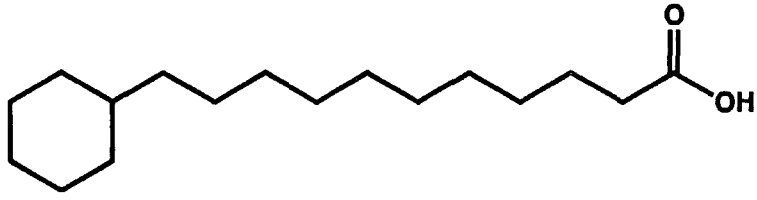
Scheme 2.203. Stereochemical course of fatty acid biosynthesis, showing the four modes of H_2^* addition by enoyl reductases.

A few fatty acids are known which do not conform to the rigidly linear, non functionalised even carbon-numbered products of FAS. These examples are most usually explained by the incorporation of a non-acetate starter unit. Since the first unit of the chain need not be activated to a malonyl functionality (as must all subsequent units), specific carboxylases are not required and a certain degree of flexibility of substrate specificity can occur in some systems. Thus the novel ω-fluorooleic acid (52)[12] arises from the utilisation of fluoroacetate (53) as the starter unit in seeds of the southern African plant *Dichapetalum toxicarium,* whilst the cyanobacterial ω-cyclohexyl (*Bacillus acidocaldarius*)[13] and ω-cycloheptyl (*Bacillus*

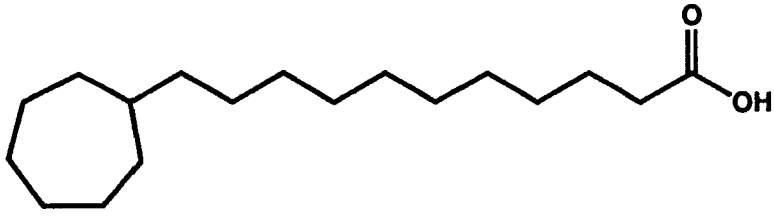
cycloheptanicus)[14] undecanoic acids (54a,b) arise from the employment of cyclohexyl and cycloheptyl carboxylic acids as starter units. In the case of *B. acidocaldus* the FAS was shown to have a wide substrate specificity for the starter unit, utilising a varied selection of linear, branched and alicyclic short chain fatty acids that were presented to a whole cell system, as starter units for fatty acids[13].



(52).



(54a).



(54b).

ω–Fluorooleic acid (52), ω–cyclohexyl (54a)
and ω–cycloheptyl (54b) undecanoic acids.

The acyl carrier protein (ACP) from *Escherichia coli* FAS has recently been isolated, and the sequence of its seventy seven constituent amino acid residues determined[15]. The three dimensional structure of the polypeptide has thus far proved difficult to obtain by crystallographic methods, but a method combining nmr distance measurements with computerised energy minimisation calculations has yielded valuable information about the shape of this protein. The most interesting feature is the presence of a hydrophobic cleft stretching along the top surface of the protein, terminating in a serine hydroxyl residue at one end. This cleft is a possible site of residence of the growing acyl chain bound to the serine residue *via* the phosphopantetheine prosthetic group. The exact shape and length of this cleft may be importantly involved in directing the final length of the fully formed fatty acid and thus the point at which chain growth terminates and the fatty acid is released.

2.21 Unsaturated fatty acids as the starting point for secondary metabolite biosynthesis.

Palmitate (46) is subject to post assembly modification by various enzymes located in the endoplasmic reticulum which can lengthen or shorten the chain, by two-carbon units, or introduce unsaturation to produce the range of structures commonly found as constituents of fats and oils such as oleic (48), linoleic (49) and linolenic acids (50)[16].

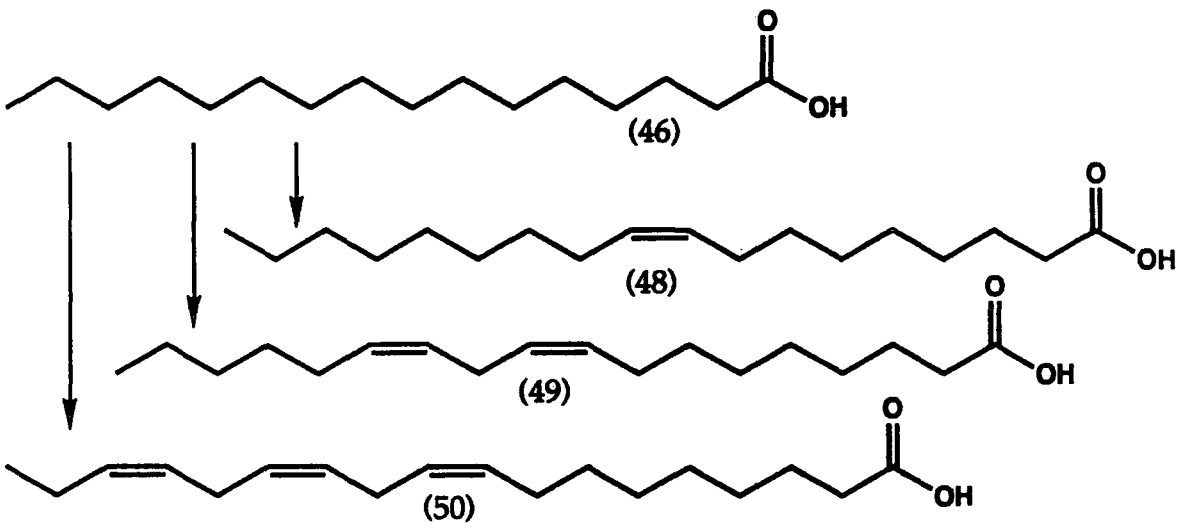


Fig. 2.211. Palmitic acid (46) is converted to a range of other fatty acids such as oleic acid (48), linoleic acid (49) and linolenic acid (50).

Mammals are devoid of a C-12 desaturase and are unable to synthesise linoleate (49) and linolenate (50). These substances are termed *essential fatty acids* and are a dietary requirement. They are necessary for the synthesis of the C_{20} tetraunsaturated compound arachidonic acid (51) and other poly-unsaturated fatty acids. These material are precursors for the highly important class of bio-active compounds known as the eicosanoids including the prostaglandins, the thromboxanes and the leukotrienes (Fig. 2.212).

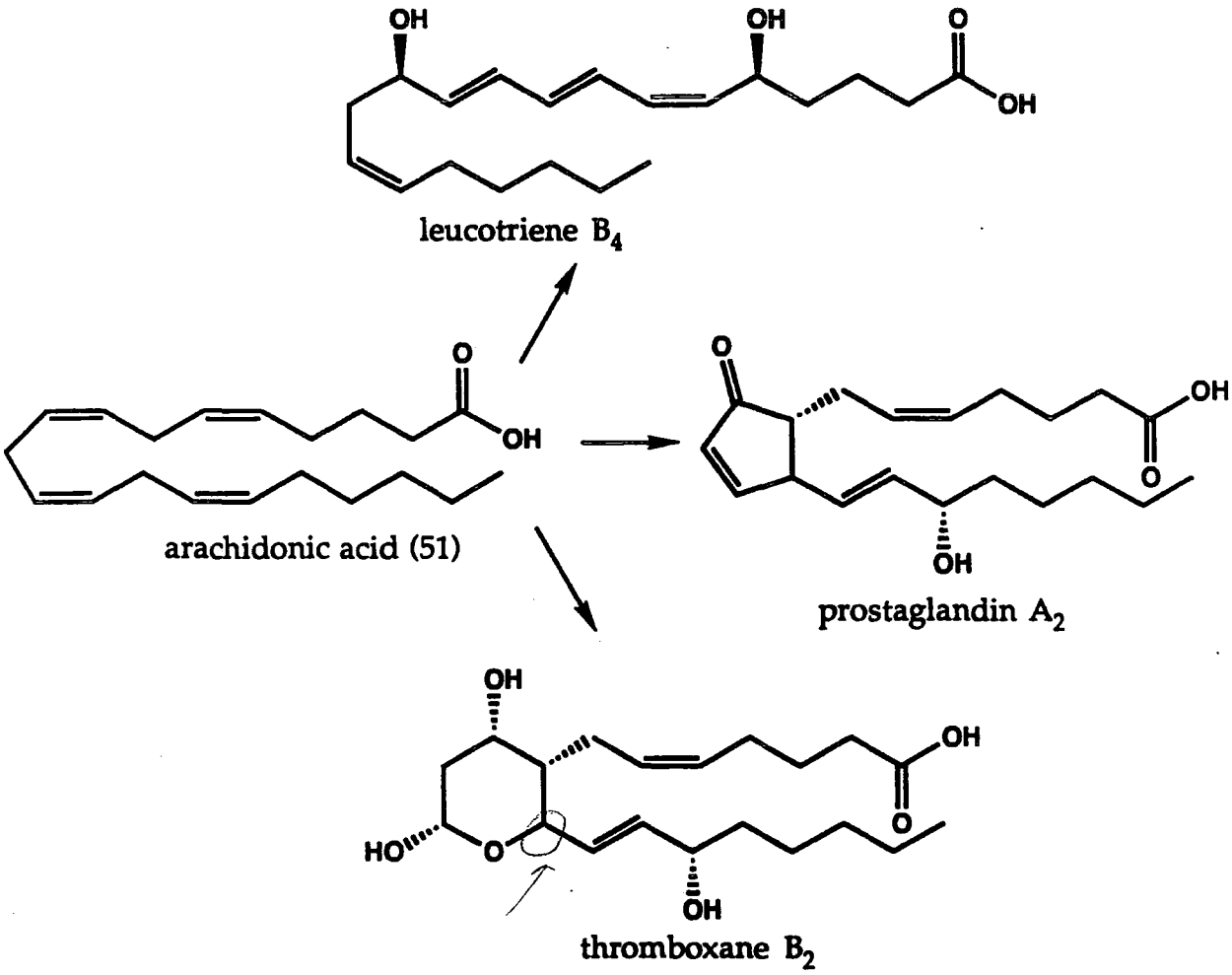


Fig. 2.212. The eicosanoid class of hormones are derived from arachidonic acid (51).

In many plant species, such as *Compositae* (daisy family) and *Umbelliferae* (carrot family), the unsaturated fatty acids serve as precursors to acetylenes and allenes, many of which are biologically active, or in some cases extremely toxic.

Unsaturation also serves as a starting point for hydroxylations, methylations and the formation of cyclopropane and cyclopropene rings as in the Sarcolaenaceae family of plants[17].

2.3 Polyketide biosynthesis.

Numerous biosynthetic studies have shown the close analogy between the biosynthesis of fatty acids by the classical mechanism discussed above and the biogenesis of the polyketides[1]. Traditionally the approach of the chemist has been to probe polyketide biosynthesis by utilising appropriate isotopically labelled substrates in feeding experiments, and subsequently detecting the fate of the label in the fully formed metabolite. More recent work has focused on the genetic aspects of polyketide biosynthesis, especially in delineating the enzymes responsible for each PKS transformation. Taken in tandem these two approaches have led to an increased understanding of the processes and mechanisms of polyketide biosynthesis. General conclusions obtained by these studies and three representative examples are presented here. Together they demonstrate many of the factors which have so far been discovered.

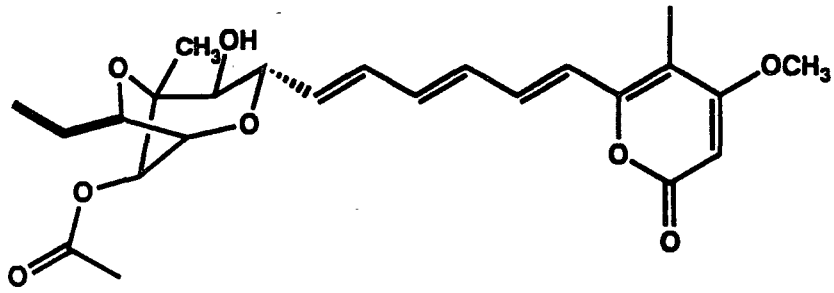
2.31 The relationship between polyketides and the fatty acids.

Very many studies on the biosynthesis of both fungal and bacterial polyketides have reinforced the observation that polyketide biosynthesis parallels FAS. Most studies have concentrated on tracing the primary units of PKS such as acetate and malonate, propionate and methylmalonate, butyrate and ethylmalonate and in a few cases isobutyrate and other short chain fatty acids.

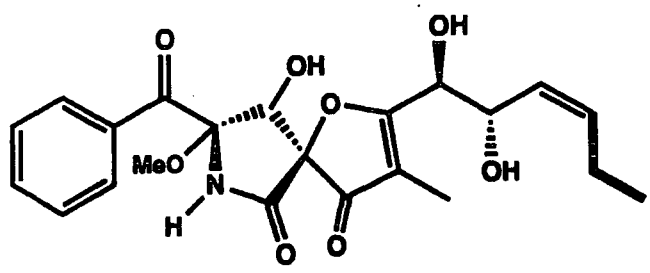
2.32 Simple precursors.

The flexibility in the choice of starter unit for PKS mirrors that observed in FAS, so that a variety of non acetate units are observed in this position. The fungal metabolites aurovertin-B (36b) (*Calcarisporium arbuscula*)[18] and pseurotin-A (16) (*Pseudeurotium ovalis* Stolk)[19] incorporate propionate (78) as the starter unit whilst the bacterial metabolite piloquinone (55) (*Streptomyces pilosus*)[20] uses isobutyrate (118), derived efficiently from L-valine (91), as the starter unit. The anthraquinone averantin (56)[21], a precursor to aflatoxin-B_1 (57) produced by a blocked mutant of *Aspergillus parasiticus*, has been shown to incorporate an intact hexanoate group (80) as a starter unit. The α–amino acid cysteine (93) has been postulated as the starter unit in the marine sponge metabolites latrunculins A and B (58a,b)[22] produced by *Latrunculia magnifica*. In a study utilising *Streptomyces parvalus*[23] it was found that novel analogues of the usual metabolite

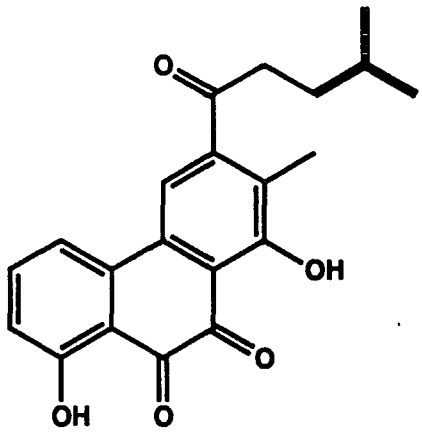
manumycin (59) containing alternative starter units could be produced when the culture broth was supplemented with high concentrations of suitable carboxylic acids.



(36b)



(16)



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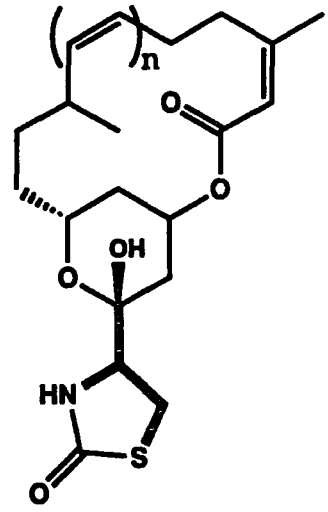


latrunculin-A, n=2, (58a).
latrunculin-B, n=1, (58b).

Fig. 2.321. Aurovertin-B (36b), pseurotin-A (16), piloquinone (55) and latrunculins-A and B (58a,b).

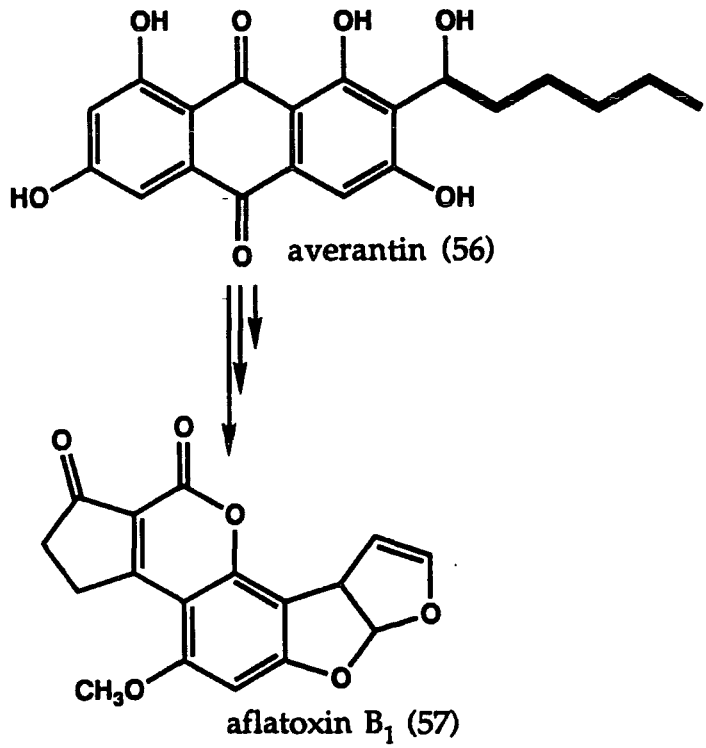
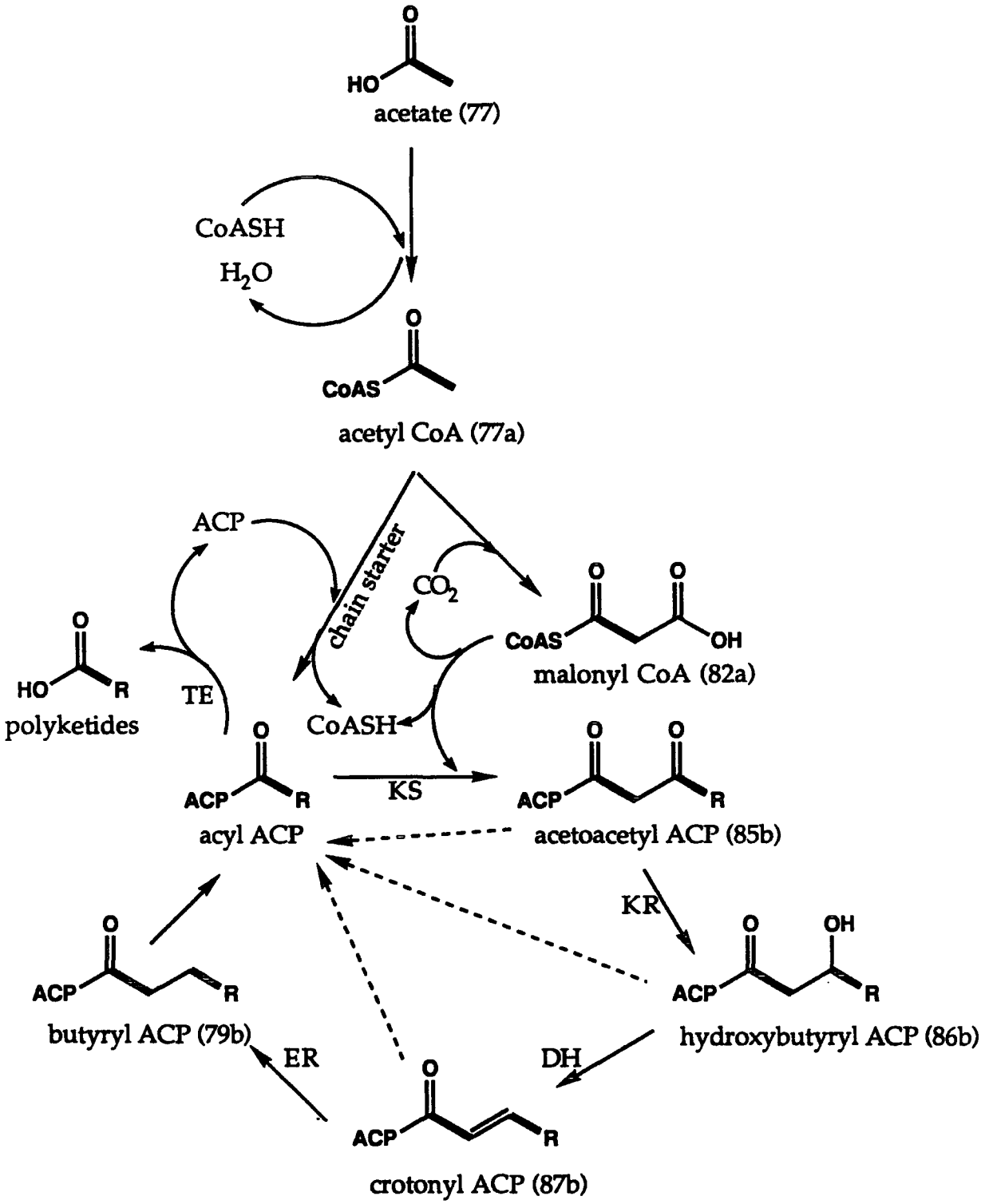


Fig. 2.322. Averantin (56), a precursor to aflatoxin-B_1 (57), incorporates hexanoate as a starter unit.

Malonyl CoA (82a) is the chain extending group in FAS, and indeed this group is used for the same purpose in PKS. In addition to malonyl CoA (82a) however, it has been observed that methylmalonyl CoA (83a) and ethylmalonyl CoA (84a) thiolesters can also be incorporated in a head to tail manner to build a branched carbon backbone in bacterial and marine organisms but not in fungi (see section 2.4).

In contrast to the complete reduction of β–keto functionality in fatty acid synthesis by the cycle of β–keto-reductase (KR), dehydratase (DH) and enoyl reductase (ER) activities, the level of reduction of the β–keto functionality in polyketide synthesis is variable. The cycle for chain elongation is envisaged as being very similar to that for FAS, with similar activities contained within the polyketide synthase (PKS). The major difference beween PKS and FAS lies in the selective editing of the reduction and dehydration steps. To maintain the analogy between FAS and PKS the model for PKS has been modified so that it is postulated that intermediates in the cycle 'loop back' (dashed arrows in Fig. 2.323) to the start of the cycle after the appropriate oxidation level has been reached, missing out later enzymic activities to leave correct functionality intact as the chain grows.



CoA = co-enzyme A, ACP = acyl carrier protein.
KS = β-keto acyl synthase, KR = β-keto reductase.
DH = dehydratase, ER = enoyl reductase.
TE = thiolesterase.
---------- PKS 'edited' pathways.

Fig. 2.323. Polyketide biosynthesis.

Thus a keto functionality, originally present at the β-position, is introduced into the growing chain if the acetoacyl intermediate is passed directly back to the beginning of the cycle before the operation of ketoreductase (KR), dehydratase (DH) or enoyl reductase (ER). Similarly hydroxyl and alkenic functionalities are introduced by jumping to the start of the cycle after β–ketoreductase (KR) and dehydratase (DH) activities respectively. In combination, the variability of chain starter and extender units and the variability in level of reduction at carbons derived from C-1 positions of acetate or propionate etc, produces the observed vast diversity in structure of the polyketides. Post assembly modification, as discussed in chapter 1, further diversifies this group of secondary metabolites.

2.33 Orsellinic and 6-methylsalicylic acids.

The PKS systems responsible for the biosynthesis of two of the simplest polyketides, orsellinic acid (60) and 6-methylsalicylic acid (25) have been thoroughly investigated and are well understood. The compounds are structurally very similar, differing only by the presence of an extra hydroxyl group in orsellinic acid (60).

Orsellinic acid (60) is one of the simplest polyketides, this simplicity led to it being one of the first of the class to be shown to be derived from head to tail condensation of acetate units[24].

The biosynthesis of orsellinic acid (60), has been investigated using orsellinic acid synthase (OAS) isolated from strains of *Penicillium cyclopium* and *Penicillium madriti* which produce this metabolite[25]. It was found that the acid could be synthesised in a cell-free system consisting only of acetyl CoA (77a), malonyl CoA (82a) and OAS suspended in aqueous buffer. These results can be rationalised by analogy to FAS, the acetyl (77) starter unit being extended by three rounds of condensation with malonyl CoA (82a), with concomitant loss of three equivalents of carbon dioxide, to form the poly-β-keto intermediate (88) (Fig. 2.331). Stepwise reduction of the β-keto functionality, which is the inevitable fate of these groups during FAS, does not occur however, and a tetra-β-keto structure is built up on the synthase. The close proximity of keto and acidic methylene functionalities is possibly stabilised by metal chelation. Cyclisation of the tetraketide, followed by two enolisations, yields orsellinic acid (60). Jordan has very recently shown in an elegant series of experiments[26], in contradiction of the earlier results of Floss *et al.*,[27] that removal of hydrogen during enolisation is stereospecific, and thus cyclisation must be under enzymic control. This type of chemistry has been proven reasonable *in vitro*, by the synthesis of the

analogous linear triketone methyl ester. The triketone methyl ester was efficiently cyclised to give methyl orsellinate after treatment with silica gel[28].

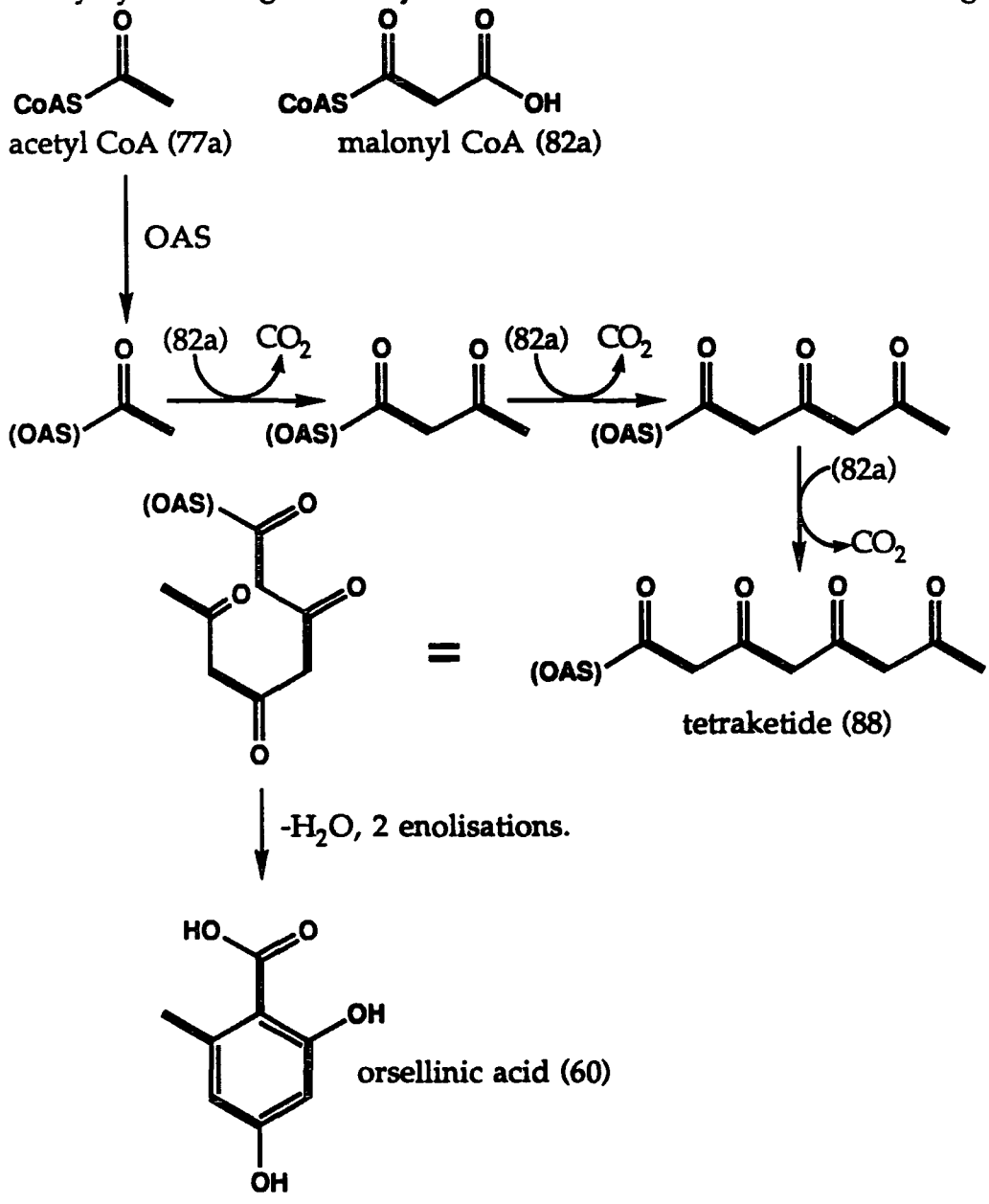


Fig. 2.331. Orsellinic acid (60) biosynthesis.

The enzyme system responsible for the biosynthesis of the tetraketide 6-methylsalicylic acid (25) (6-methylsalicylic acid synthase, 6MSAS) from acetate (77) and malonate (82) has also been isolated and purified[29]. In contrast to orsellinic acid (60), the biosynthesis of 6MSA (25) from acetyl CoA (77a) and malonyl CoA (82a) requires the presence of NADPH. The absence of this co-factor leads to the unreduced triketide metabolite triacetic acid lactone (61) (TAL), which is also produced by FAS when devoid of NADPH[30].

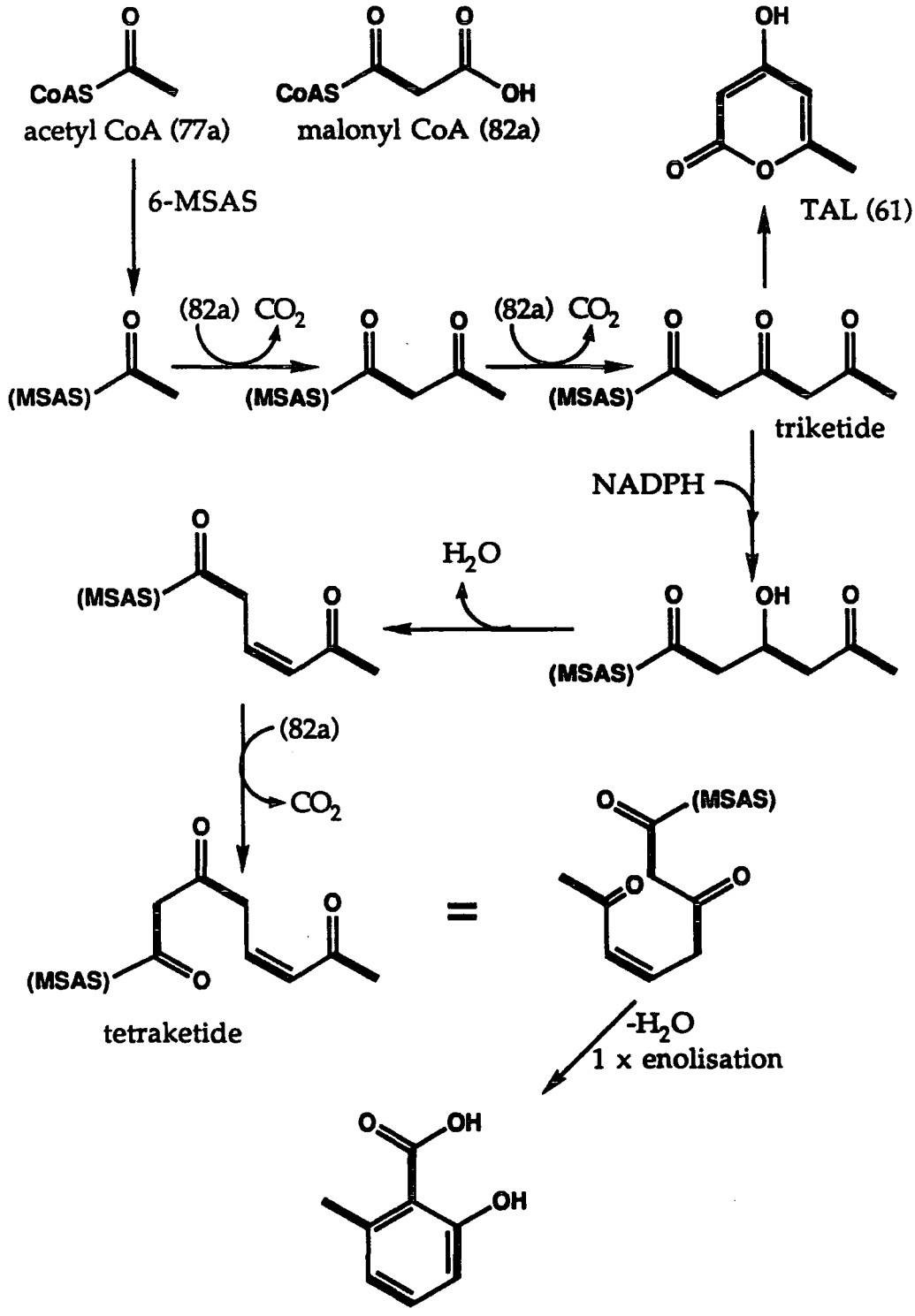


Fig. 2.332. The biosynthesis of 6-methylsalicylic acid (25).

The formation of 6MSA from an acetate starter unit and three malonate extender units parallels that of orsellinic acid, but is more complex due to the reduction and subsequent dehydration of the keto functionality arising from the first round of malonate extension. The timing of this reduction has been determined to occur at the tri-acetic acid level, before the final chain extension with malonyl CoA (82a). This modification, rendering a *cis* double bond in the pre-cyclised intermediate represents the simplest example of 'programming', i.e. the control of timing of functional group modification in PKS systems.

More recent work to increase the understanding of 6-MSAS has focused on the mechanism of aromatisation. Feeding experiments utilising tri- and mono-deuteroacetate, labelled with ^{13}C at C-1, determined that hydrogen removal during aromatisation was stereospecific[31,32]. These studies were confirmed by a series of experiments utilising malonates, rendered chiral by the incorporation of ^{13}C and ^{2}H[33], which determined the absolute stereospecificity of hydrogen removal[34,35]. This stereospecificity is a powerful indication that hydrogen loss is mediated enzymatically. The stereochemical course of the dehydrations leading to the aromatisation of 6MSA (25), mirrors that recently shown for orsellinic acid (60).

2.34 Dehydrocurvularin.

In contrast to orsellinic acid (60), the fungal octaketide dehydrocurvularin (30), isolated from the plant pathogen *Alternaria cinerariae,* is more heavily reduced. The origin of the carbon skeleton, as well as some of the pendant oxygen and hydrogen atoms has been determined to be from acetate (fig 2.341)[36]. The variation in oxidation level of carbons derived from C-1 of acetate in dehydrocurvularin (30) requires the operation of a reductase activity at these positions. Notionally the reduction could occur after the formation of a polyketo chain analogous to, but longer than, the intermediates in orsellinic acid (60) and 6-MSA (25) biosynthesis. Alternatively the possibility of a processive mechanism exists where reduction occurs as chain growth progresses, much as in FAS.

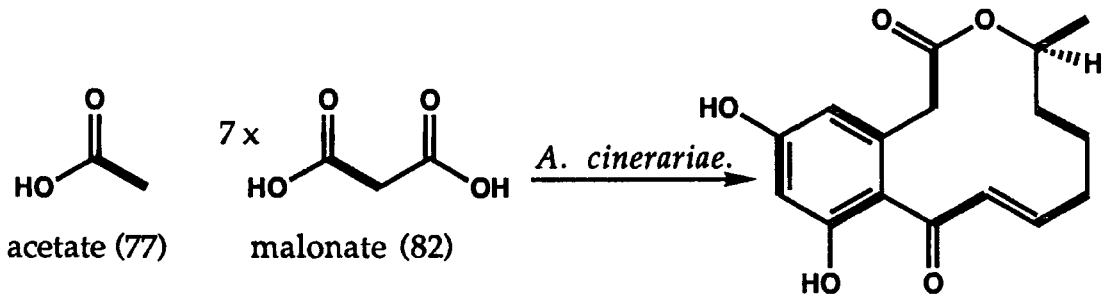


Fig. 2.341. Dehydrocurvularin (30) is an octaketide.

Convincing evidence for the operation of a processive mechanism in the biosynthesis of polyketides has been obtained by Cane[48] and Hutchinson[51] in Actinomycetes, whilst in fungal systems Vederas *et al*[37] have also been able to show that a processive mechanism is operative during the biosynthesis of dehydrocurvalarin by synthesing doubly ^{13}C-labelled di- (30a) and tetra-ketide (30b) putative precursors. When these compounds were incubated with a mutant of *A. cinerariae* which was incapable of oxidising fatty acids, dehydrocurvularin (30) was obtained which was found to be enriched in ^{13}C at sites corresponding to the position of label in the supplemented material[37].

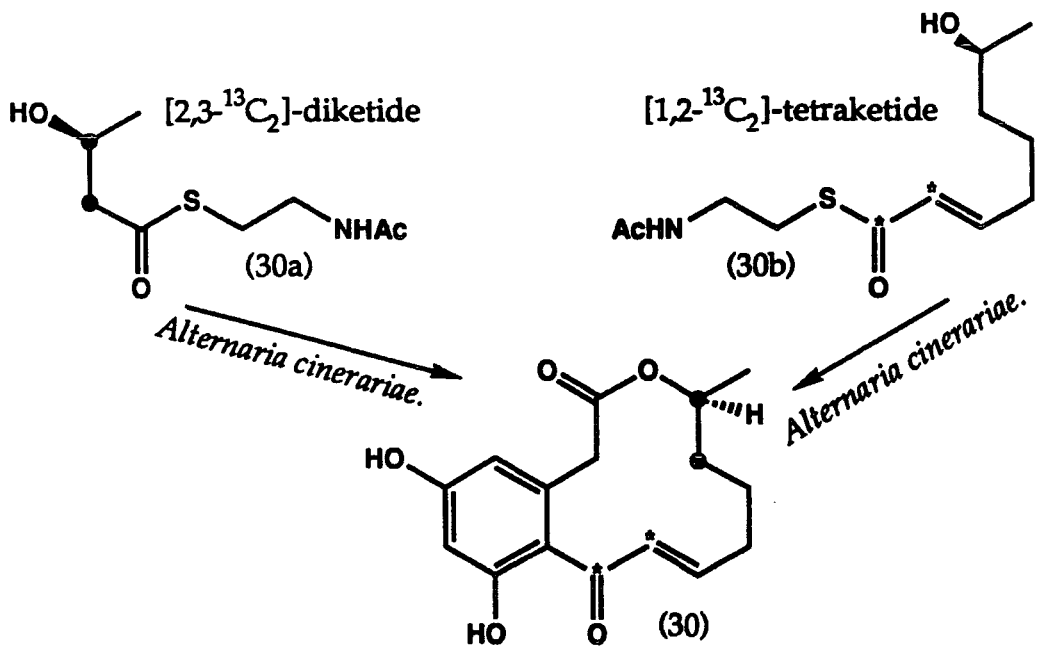
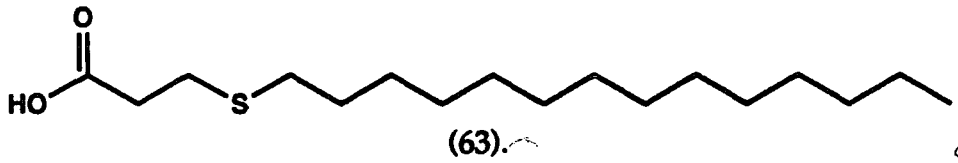


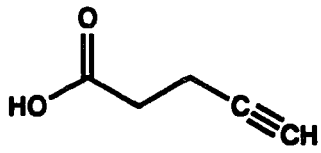
Fig. 2.342. The reduced di- (30a) and tetra-ketides (30b) are incorporated intact into dehydrocurvularin (30).

The use of doubly labelled material in these experiments highlights an important factor which has to be addressed when feeding more complex precursors to whole cell systems. It is generally found that the cellular mechanism of β-oxidation[38], the process responsible for the catabolism of fatty acids, results in very extensive degradation of carbon chains of four cabons and longer to acetate and propionate. Labelled materials are no exception, and the resulting labelled acetate (77) and propionate (78) can be used in the *de novo* synthesis of the secondary metabolite under investigation. This process usually results in a uniform distribution of label throughout the metabolite, and indeed this was the case when these precursors were fed to wild type *A. cinerariae.*

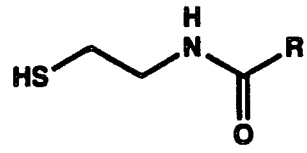
In order to overcome these problems a strategy was adopted to cut the level of operation of β-oxidation in the cell to a minimum. Firstly a uv-mutant of the wild type organism was generated. The mutant selected was unable to utilise fatty acids as a carbon source. Secondly this mutant was utilised for feeding experiments in a high glucose replacement medium so that glucose, and not fatty acids, was the main source of carbon for the cell. Thirdly the known β-oxidation inhibitor 4-pentynoic acid (62) was supplemented to the cells at the same time as the labelled substrates.



(63).



(62).



(122).

The β-oxidation inhibitors 3-(tetradecylthio)propionic acid (63) and 4-pentynoic acid (62), and the CoA mimic N-acylcysteamine (122).

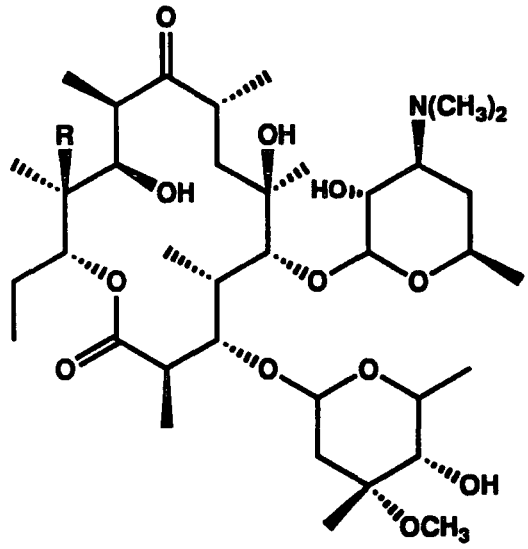
Labelling carbons at both ends of a bond effectively labels the bond itself, and this strategy was used to monitor the substrates used in this study. If the bond in question were cleaved, it would be statistically unlikely for the labels to come together again because of the dilution of labelled acetate (77) and propionate (78) by unlabelled material *in vivo*. The observation of dominant ^{13}C-^{13}C vicinal coupling in the ^{13}C nmr spectrum of the recovered dehydrocurvularin (30) for carbons corresponding to labelled carbons of the supplemented substrates was the evidence required to substantiate incorporation of the intact substrates.

Additionally the substrates were administered as N-acetylcysteamine (NAC) thiolesters to aid trans-esterification onto the PKS system. NAC thiol esters have been used extensively for this purpose, and it is often found that incorporation of labelled substrate is only possible when the substrates are activated in this way. The NAC portion of the thiol ester is thought to mimic the SCoA moiety of acyl CoAs and ACPs *in vivo*. N-Octanoyl cysteamine thiolesters have also been used with success for this purpose[39], presumably the longer alky chain increases the lipophilicity of the thiolester.

Together these four strategies resulted in a 12% incorporation of the diketide precursor (30a) and a 2% incorporation of the tetraketide precursor (30b) into dehydrocurvularin (30). In a further study[40], Vederas *et al* were able to observe up to 70% intact incorporation of doubly labelled tetraketides (30b) into dehydrocurvularin (30) produced by wild type *A. cinerariae* in conjuction with the powerful β-oxidation inhibitor 3-(tetradecylthio)propanoic acid (63)[41].

2.35 The macrolides.

The economically important pair of macrolide antibiotics erythromycins A and B (26a,b) (*Saccharopolyspora erythraea*) consist of fourteen-membered lactone rings substituted at two hydroxyl positions by sugars.



R=OH erythromycin-A (26a).
R=H erythromycin-B (26b).

The parent aglycone, 6-deoxyerythronolide-B (6-dEB) (26c), is a highly reduced polyketide derived from propionate (78) as a starter unit extended by six methylmalonate (83) units[42]. The utilisation of methylmalonate (83) as an extender unit ensures the introduction of methyl groups at alternate positions around the ring. These methyl groups are ultimately derived from C-3 of propionate (78) which is activated in bacterial systems to methylmalonyl CoA (83a) by a propionyl carboxylase activity analagous to the acetyl CoA carboxylases discussed for other systems above (Fig. 2.351).

Feeding experiments with propionates doubly labelled [1-^{13}C, 1-$^{18}O_2$] have been used to show that oxygens at carbon positions C-1, 3, 5, 9, 11 and 13 are also derived intact from propionate[42].

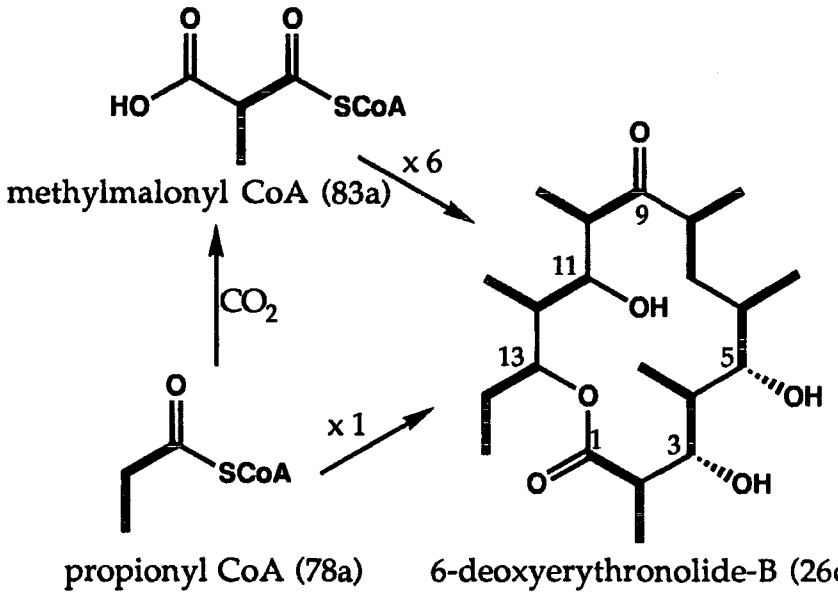


Fig. 2.351. The biosynthesis of the erythromycin skeleton 6-deoxyerythronolide-B (26c) involves the condensation of propionyl CoA (78a) unit with six methylmalonyl CoA (83a) units.

The macrolide class of polyketides has been studied extensively to further increase the knowledge about the mechanisms of chain extension. In the case of erythromycin (26) itself, extensive details about the post-assembly functionalisation of the complete aglycone, 6-deoxyerythronolide-B (6-dEB) (26c), have been determined by isolating partially assembled metabolites from the fermentation broth of mutants of the producing organism *Saccharopolyspora erythraea*. No intermediates between propionyl CoA (78a) and the fully assembled macrolactone ring have ever been isolated however[42].

Good evidence for the operation of a processive mechanism in the assembly of the macrolides comes from the discovery of a tetraketide in the fermentation broth of a mutant of *Streptomyces fradiae* which usually produces the sixteen membered macrolide tylosin (38)[43]. This tetraketide corresponded to a proposed precursor to tylactone (37), the aglycone of tylosin (38)[44].

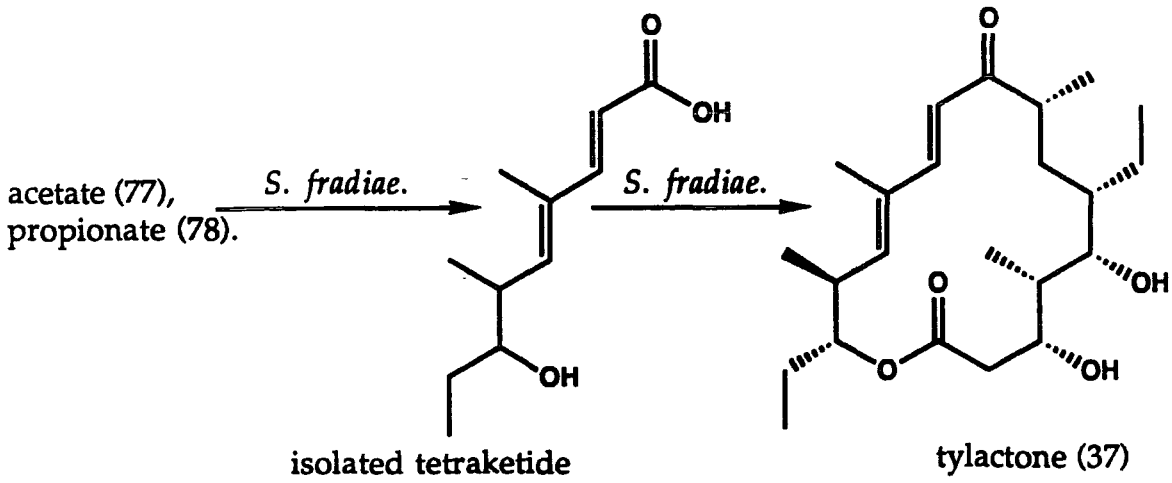
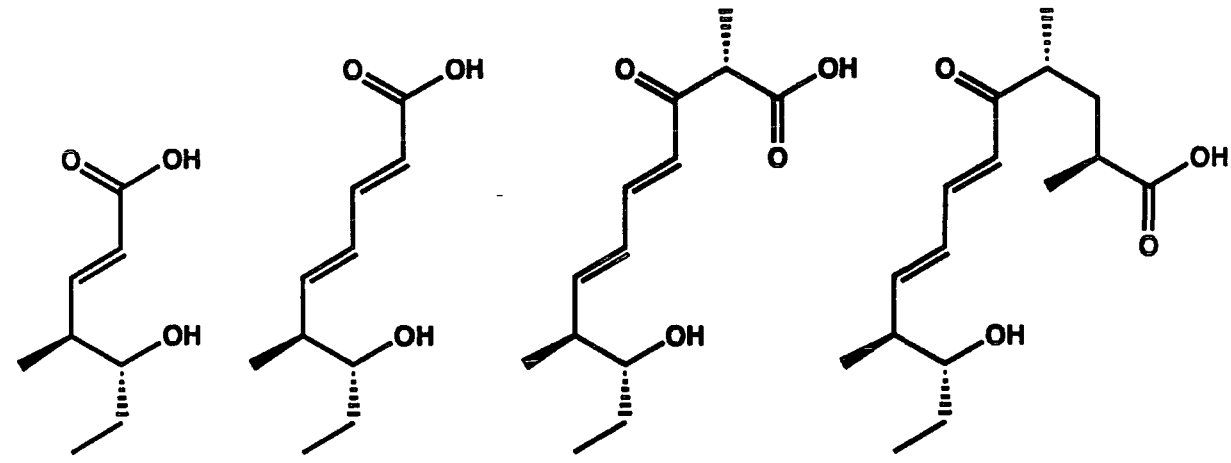
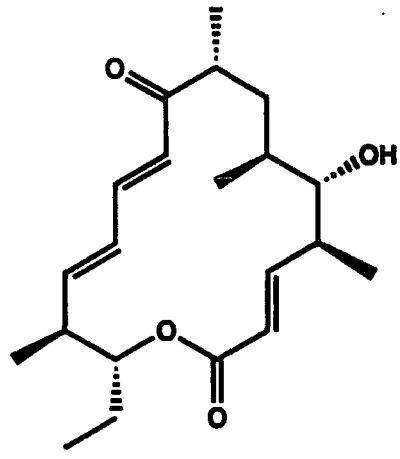


Fig. 2.352. A fully formed tetraketide fragment of tylactone (37) has been isolated from a mutant of the tylactone (37) producing organism *Streptomyces fradiae.*

In one further case triketide, tetraketide, pentaketide[45] and hexaketide[46] intermediates to the sixteen membered macrolide mycinamicin (64) have been isolated from a mutant of the mycinamicin (64) producing strain *Micromonospora griseorubida* disabled in its ability to produce the complete metabolite. Not only did the structures of these fragments accurately correspond to the postulated intermediates of a processive mechanism, but the stereochemistry of each chiral centre of each respective fragment also matched identically the corresponding centre in protomycinolide IV (65), the aglycone precursor to mycinamicin (64) itself[47].



triketide. tetraketide. pentaketide. hexaketide.



protomycinolide IV (65)

Fig. 2.353. The four polyketides isolated from the mycinamicin producing strain *Micromonospora griseorubida,* and their structural relationship to protomycinolide IV (65).

The accumulating evidence in favour of a processive mechanism during the biosynthesis of the macrolides is backed up by biosynthetic feeding experiments utilising each of the three macrolides discussed above, erythromycin-A (26a), tylosin (38) and mycinamicin (64). In the case of erythromycin-A (26a), a doubly ^{13}C-labelled diketide was successfully incorporated into the macrolide without prior degradation by β-oxidation, when it was activated to an NAC thiolester[48]. In a separate experiment the same substrate, this time labelled with ^{13}C and ^{2}H was also incorporated when supplemented to *S. erythraea* as a NAC thiol ester[49]. A singly labelled NAC thiolester activated diketide precursor to mycinamicin (64) was incorporated into the macrolide skeleton, and intact incorporation was judged by the specific enhancement of a single peak in the ^{13}C nmr

spectrum[50]. Diketide and triketide singly ^{13}C-labelled precursors were similarly incorporated intact into tylactone (37) when supplemented to the fermentation broth as NAC thiolesters[51].

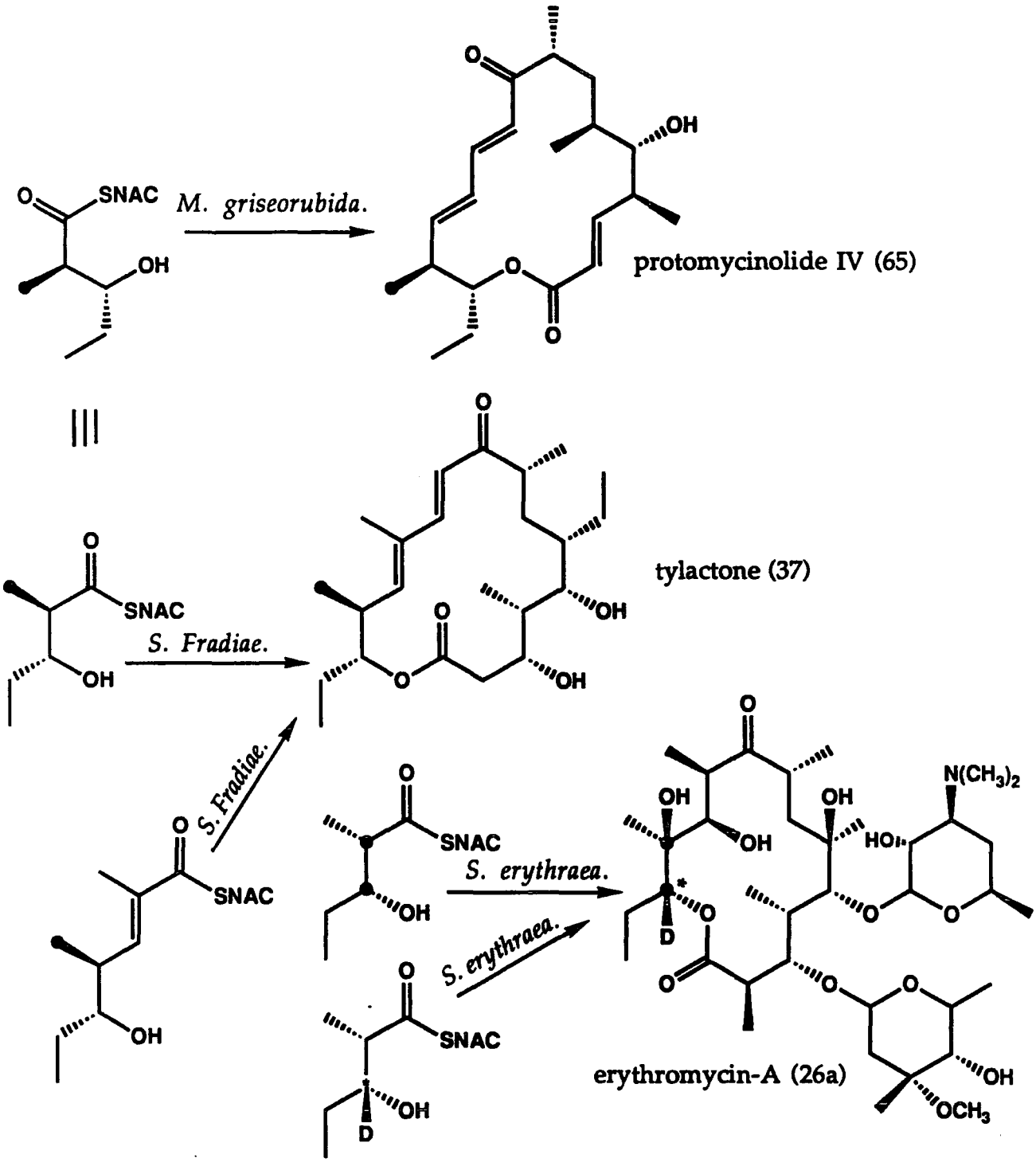


Fig. 2.354. The incorporation of N-acetylcysteamine (NAC) thiolesters of labelled di- and tri-ketides into mycinamicin (64), tylactone (37) and erythromycin-A (26a).

Together, the results from feeding experiments with the simple precursors (acetate (77), propionate (78) etc), the successful incorporations of di- and tri-ketides into mycinamicin, tylactone and erythromycin and the discovery of PKS intermediates in a limited number of systems lend significant support for the theory that PKS closely resembles FAS in the stepwise modification of oxidation level at C-1 derived carbons in a processive manner as the chain grows. The similarity is strongest in the mode of assembly between the growing acyl chain and a malonyl CoA thiolester. Both FAS and PKS show some flexibility in the choice of starter unit. In FAS generally only malonyl CoA serves as the chain propagator, contrasting with malonyl and both methyl-, and ethylmalonyl CoA thiolesters in PKS. The striking difference between the two systems, however, is the variable nature of the oxidation level at the β-position of the growing acyl chain *left intact* in PKS.

Results of the numerous feeding experiments probing PKS, whilst illuminating aspects of the assembly, are inherently unable to shed light on the control mechanisms governing the level and degree of oxidation at the β-position of the growing acyl chain. In the case of FAS, complete enzyme systems capable of synthesising fatty acids have been isolated and characterised, in particular the rôle of the acyl carrier protein has been investigated and the protein itself has been characterised by n.m.r. techniques. PKS enzyme systems have been less tractable to this type of investigation and so investigators have approached the problem of PKS characterisation from a radically different direction.

2.36 Genetic aspects of PKS.

The study of the enzyme systems that synthesize both the fatty acids and the polyketides has proceeded classically by two approaches, feeding experiments with labelled substrates using whole cell and cell free systems, and the direct isolation of the enzyme systems themselves. A third method has relied on the generation of mutants, by interference with genetic material, of organisms leading to systems that are disabled in one or more aspects of enzymic transformation. The accumulation of different precursors to the metabolite under study can give clues to the order of assembly of simple precursors, and in the case of the macrolide antibiotics such as erythromycin (26), have provided valuable information about the steps leading to the active form of the antibiotic from the unfunctionalised products of the polyketide synthase (PKS).

Erythromycin (26) has proved one of the most heavily studied polyketides in this respect. Whilst feeding experiments with *Saccharopolyspora erythraea* have identified the carbon skeletal origin to be from seven units of propionate (78) and blocked mutant studies have identified 6-dEB (26c) as the first free intermediate[42], the paucity of information about mechanistic steps between these extremes has been glaring.

The PKS system generating 6-dEB (26c) has resisted attempts at isolation, but the genes coding for this set of enzymes have been located and recently sequenced. Leadlay and co-workers initially found one large open reading frame (orf) located about 12kB away from the erythromycin resistance gene (ermE)[52]. The exceptionally large protein (3178 amino acid residues) coded for by this gene was deduced to contain nine activities when the amino acid sequence was compared to the sequences of individual FAS proteins.

Katz and co-workers have discovered two more similarly sized orfs, containing nine and ten activities respectively, at positions adjacent to the orf reported by Leadlay[53]. These three orfs, each capable of being expressed as a single giant protein, were found to contain all of the activities necessary for the *de novo* synthesis of 6-dEB from propionyl CoA and methylmalonyl CoA. Each of the three proteins was found to contain the necessary functionality to perform two condensations between a growing acyl chain and methylmalonyl CoA and the appropriate number of reduction and/or dehydration steps.

Each orf was deduced to code for a giant protein made up of two biosynthetic modules, each module was responsible for a chain extension

and an appropriate number of transformations to render the correct functionality at the β-keto position of the growing acyl chain. Each module carries an acyl carrier protein which holds the growing acyl chain for that chain extension cycle, and an acyl transferase activity responsible for transesterifying the growing chain onto the ACP. Staunton has coined the term 'cassette'[54] to describe a group of modules that are bound together, in this case each orf corresponds to one cassette, and each cassette contains two modules (Fig. 2.361).

Orf 1 contains the first cassette of two modules. Module 1 has an acyl transferase (AT) to transfer propionyl CoA onto an acyl carrier protein (ACP), also coded for on this protein. There is also a β-keto synthase (KS) to perform the first condensation with methylmalonyl CoA and another acyl transferase to transfer this β-keto acyl residue onto a second ACP where the first β-keto reductase activity (KR) performs reduction to an alcohol. A further set of ACP and KS, AT, and KR activities are located on this protein, constituting module 2, for the second condensation/reduction cycle.

Similarly the second open reading frame codes for a single protein containing the next cassette of two modules, each with its own ACP and AT units. The lack of a KR region in module 3 ensures the preservation of the keto group, whilst the presence of ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains in module 4, ensures full reduction after this chain elongation step.

The third open reading frame (the orf discovered by Leadlay) contains the modules necessary for the final two condensations and reduction cycles, with two more ACP units, in addition to a thiolesterase activity in module 6, responsible for lactonisation and the release of the completed 6-dEB (26c).

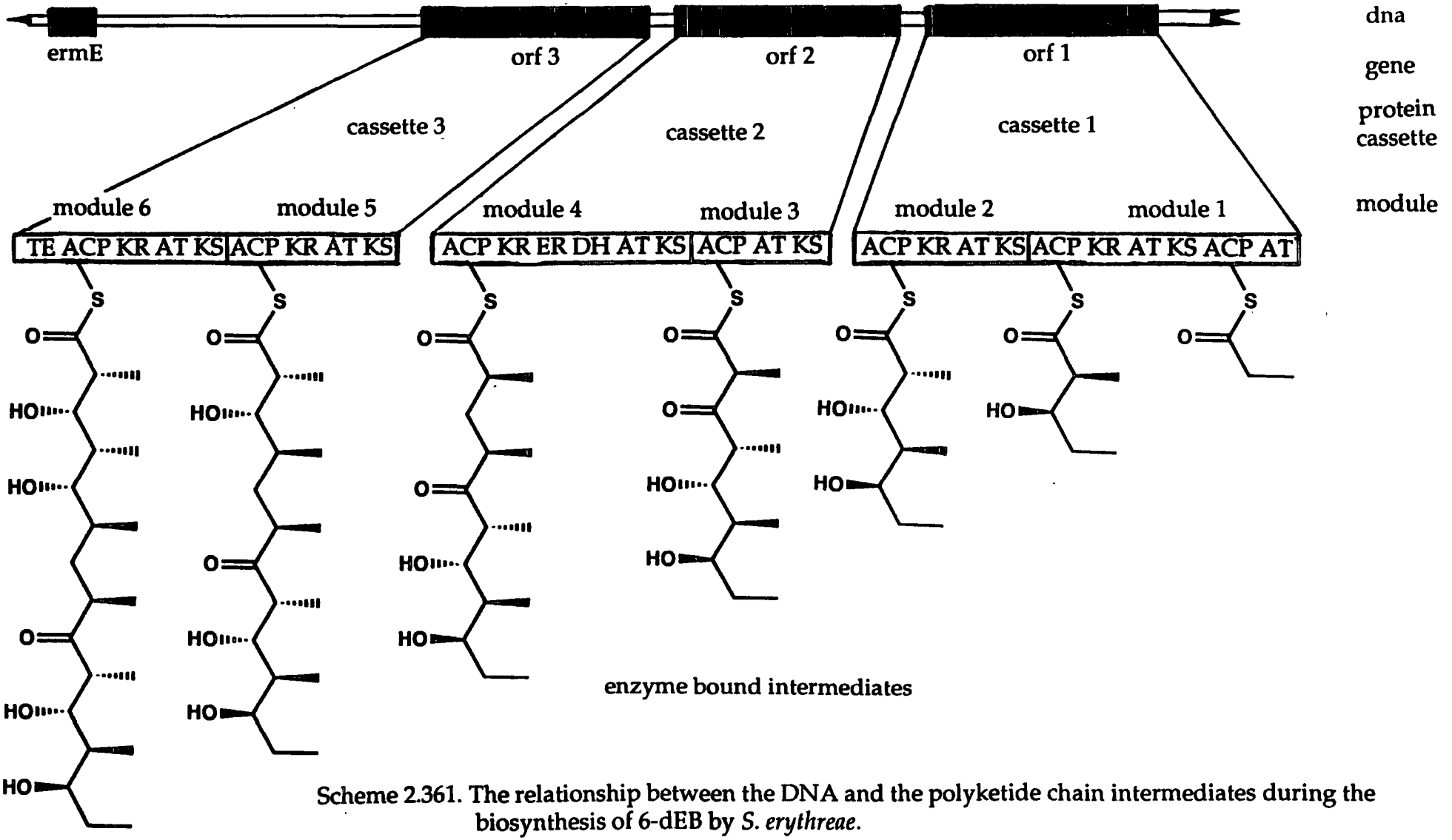
These findings are in full agreement with results obtained from feeding experiments with labelled precursors. They show that the level of oxidation at sites derived from C-1 of propionate is controlled by the *presence or absence* of the relevent activities (KR, DH and ER) in each of the six modules.

Katz and co-workers set about verifying their conclusions by generating a mutant of *S. erythraea* in which the DNA base sequence corresponding to KR of module 5 was selectively deleted from the gene (orf 3). They predicted that this deletion should have the effect of leaving intact a keto group at C-5 of the erythromycin skeleton, leading to the new metabolite 5,6-dideoxy-3-mycarosyl-5-oxoerythronolide-B (26d). Indeed this compound was found to be the major product of this mutant. Furthermore they went on to demonstrate that the acyl transferase (AT) catalytic activities are responsible in some measure for the specificity in the choice of starter unit and chain

extender units.

The discovery that discreet activities are responsible for each and every step of erythromycin (26) biosynthesis, and that no less than seven ACPs are specified, one for the starter unit and one for each subsequent chain elongation step, goes somewhat beyond the model proposed for PKS as a system closely analagous to FAS. The almost baroque complexity found in the proteins responsible for erythromycin (26) biosynthesis is however entirely necessary, explaining how the control processes making choices for starter unit and chain extender operate, and also how the level of oxidation at each C-1 derived position is determined.

It is expected that the type of enzyme system responsible for the assembly of the erythromycin aglycone is also responsible for the assembly of the tylactone (37) and mycinamicin (64) lactone rings. The discovery that each protein of the enzyme system contains modules, each module programming for one cycle of condensation and reduction, helps to explain the structural and stereochemical homologies found through and between the different classes of polyketides[55]. It is easy to visualise how genetic cassettes coding for proteins, containing the entire machinery for assembly of individual stretches of polyketide chain, could be disseminated through a genus, family or order of organisms. The genetic use of cassettes could be a way of preserving polyketide stretches of biological activity, especially in systems of the complexity of the macrolides and polyethers where many of the structural homologies have been observed[55].



Scheme 2.361. The relationship between the DNA and the polyketide chain intermediates during the biosynthesis of 6-dEB by *S. erythreae*.

2.4 Branching strategies.

A significant difference has been observed between the biosynthesis of fungal and bacterial polyketides containing pendant methyl (and in the case of some bacterial metabolites, ethyl) groups. As discussed above for the macrolide antibiotics, bacterial PKS systems effect branching of the polyketide backbone by the utilisation of methylmalonyl (or ethylmalonyl) CoA extender units as appropriate. It has been observed however that in fungal systems chain methylations occur by the reaction between the carbon chain and the S-methyl group of L-methionine[56]. The divide in mechanistic approaches between the bacteria and the fungi is almost complete, with only rare indiscretions. One example is the incorporation of a single methionine derived pendant methyl group into the bacterial metabolite ambruticin-S (66) (*Polyangium cellosum*) which also incorporates propionate groups *via* methylmalonate (83)[57].

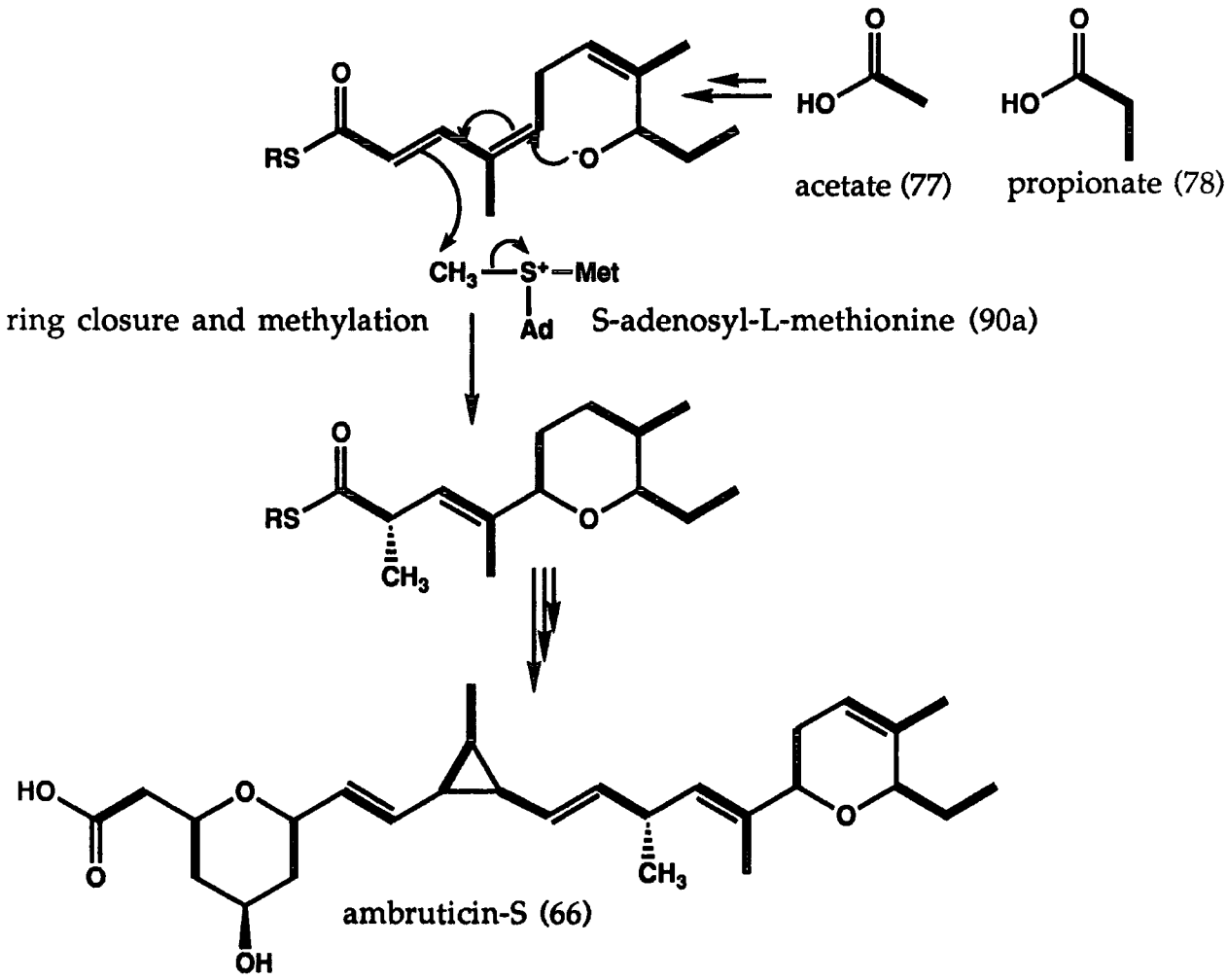


Fig. 2.401. Biosynthesis of ambruticin-S (66) from acetate (77), propionate (78) and L-methionine (90).

One possible explanation (Fig. 2.401) for this observation is that methylation accompanies ring closure of the terminal pyran ring. This would imply that the methylation event is an adornment and not a feature of polyketide chain building *per se.*

The bacterial strategy ensures that the growing chain is appropriately functionalised as each malonyl unit is added, and so the timing of chain branch addition is not in dispute. In the case of the fungi however the timing of chain methylation has not been determined and there is a dearth of information concerning this point.

The operation of a processive mechanism during the assembly of fungal polyketides has been comprehensively demonstrated for the case of dehydrocurvularin (30) elaborated by *Alternaria cinerariae* (section 2.24). Although this metabolite does not contain pendant methyl groups, it would be expected that a very similar process could operate in the cases of branched chain metabolites such as tenellin (1) and cubensic acid (24). Methylation of a growing polyketide chain, with the chain extender being derived from acetate and the pendant methyl group being derived from methionine, can be envisaged to occur by two mechanistically distinct routes.

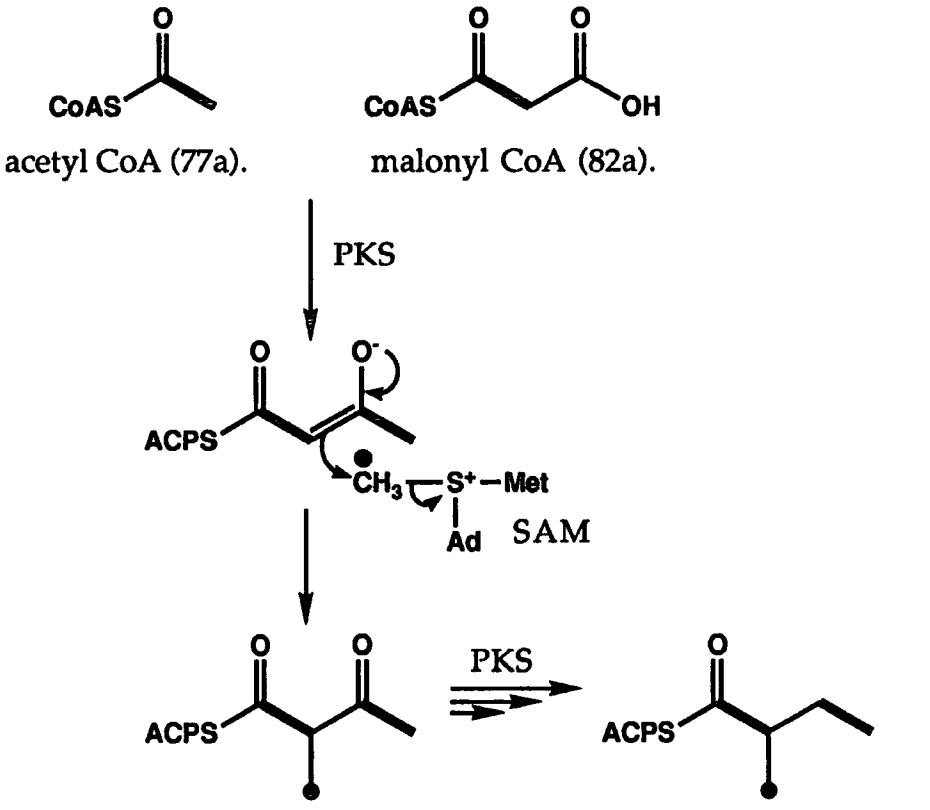
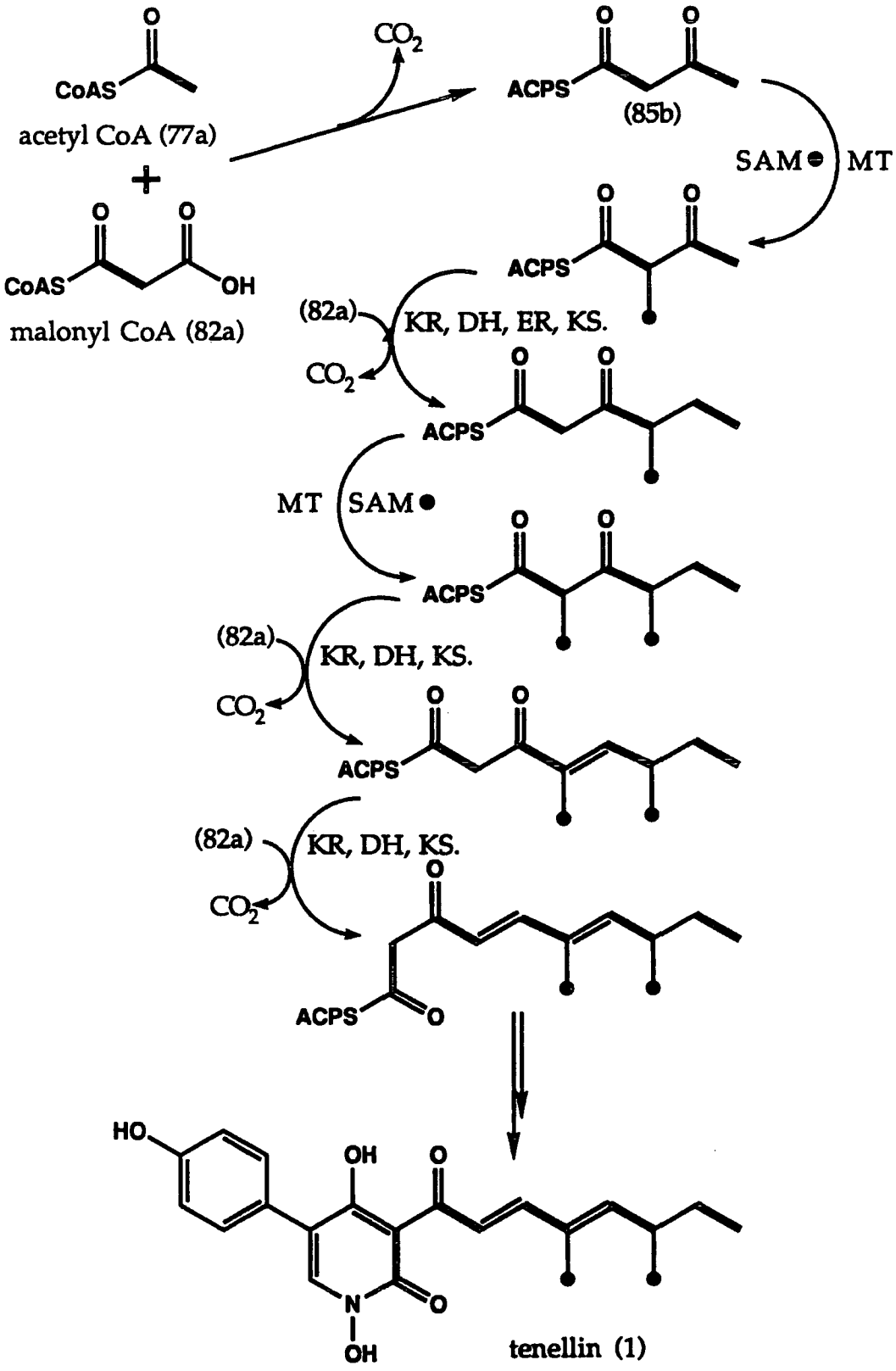


Fig. 2.402. Modification of PKS to enable methylation by SAM.



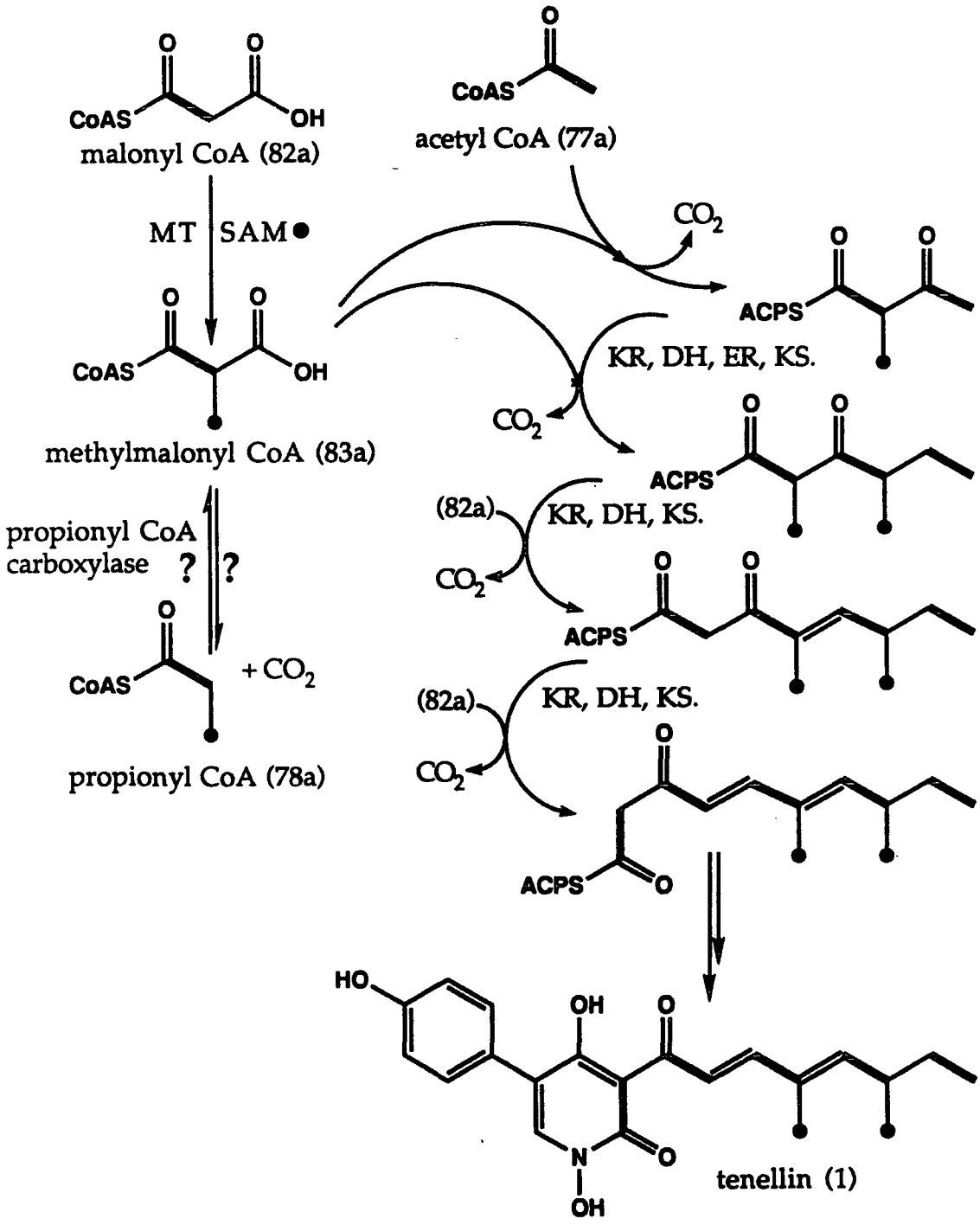
Scheme 2.403. Possible intermediates to the polyketide sidechain of tenellin(1), where methylation occurs as an integral part of PKS.

It can be envisaged that the methylation event occurs as an integral part of the PKS system (Fig. 2.402). The β-keto acyl group formed by chain extension with malonyl-CoA would then attack the methyl group of S-adenosyl methionine (SAM), methylating the growing chain directly. The usual programmed cycle of β-keto reduction would then render the correct oxidation level at this position before the methylated chain could be extended by another round of malonyl-CoA condensation.

In the case of tenellin (1) (Scheme 2.403) this type of mechanism would require that the first chain building condensation between the acetyl starter unit and malonyl CoA (82a), forming an acetoacetyl residue (85a), would be followed by methylation with a SAM (90a) dependent methyltransferase (MT) and a full round of β-keto reduction affording a 2-methylbutyryl (108) residue. The second condensation and methylation would be followed by only β-keto reduction and dehydration. The next two chain extensions with malonyl CoA would not be accompanied by a MT, so that the complete pentaketide with two appended methyl groups would be the product of the PKS. Methylation of the growing chain in this way would involve the participation of a methyltransferase activity as an *integral* part of the PKS system.

There is limited experimental evidence to support the operation of such a methylation strategy. It has been shown that the methylation event during the biosynthesis of 5-methylorsellinic acid (67) occurs by the electrophilic attack of a methyl group of SAM onto a complete tetraketo chain *before* cyclisation to form the aromatic ring[58]. Other electrophilic moieties such as isoprenyl groups, have also been implicated in electrophilic attack at an activated methylene during polyketide biosynthesis. In these and other such systems, however, alkylation occurs after complete assembly of the polyketide chain, rather than as part of a processive assembly.

In an alternative scenario the methyltransferase activity would be *separate* from the PKS. Methylation of malonyl CoA (82a), generated by the familiar carboxylation of acetyl CoA (77a), would furnish methylmalonyl CoA (83a). Methylmalonyl CoA could then be used as the chain extender unit, as is the case in bacterial PKS systems. For tenellin (1) (Scheme 2.404) this would mean that in addition to the acetate starter unit, the first two extender units would be methylmalonyl CoA (83a), the second two being malonyl CoA (82a). The reduction steps would follow as before.



Scheme 2.404. Intermediates during the biosynthesis of the polyketide sidechain of tenellin (1) from SAM (90a) and acetate(77): the possible use of methylmalonate as a chain extender.

The generation of methylmalonyl-CoA (83a) in this case differs from the bacterial systems where the branched chain extender unit is generated by the carboxylation of propionyl-CoA (78a) by a specific propionyl CoA carboxylase. This carboxylase is presumed missing from the fungi because of the observation that propionate (78) is incorporated into fungal polyketides only as a starter unit in a few rare cases, and is not involved mid-chain.

The presence of methylmalonyl-CoA (83a) in fungal systems, however, is evinced by the dual pathways leading to the propionate starter unit of the aurovertin (36) group of metabolites in the fungus *Calcarisporium arbuscula*. In one pathway incorporation of propionate (78) as an intact starter unit is observed, whilst in an additional pathway the starter group is derived from L-methionine (90) and acetate (77). These observations can be rationalised in two ways. Vleggaar[18] supposed that two independant pathways *were* operating, one where propionate acts as a direct starter unit, the other where an acetyl starter unit is cleaved from the terminus of a methylated polyketide chain. The observations may also be explained by a *unifying* mechanism involving the formation of methylmalonyl-CoA (83a) from acetate, activated to malonyl-CoA (82a), and L-methionine (90). *In vivo* decarboxylation of methylmalonyl-CoA (83a) would furnish propionyl-CoA (78a) thus generated from acetate (77) and L-methionine (90) (Scheme 2.404). Circumstantial evidence for methylmalonate generation from propionate in the fungus *Pseudeurotium ovalis* was gathered by Tamm who observed deuterium wash-out from [2-^{13}C, 2-$^{2}H_2$]-propionate during the incorporation of propionate as a starter unit in pseurotin-A (16)[19].

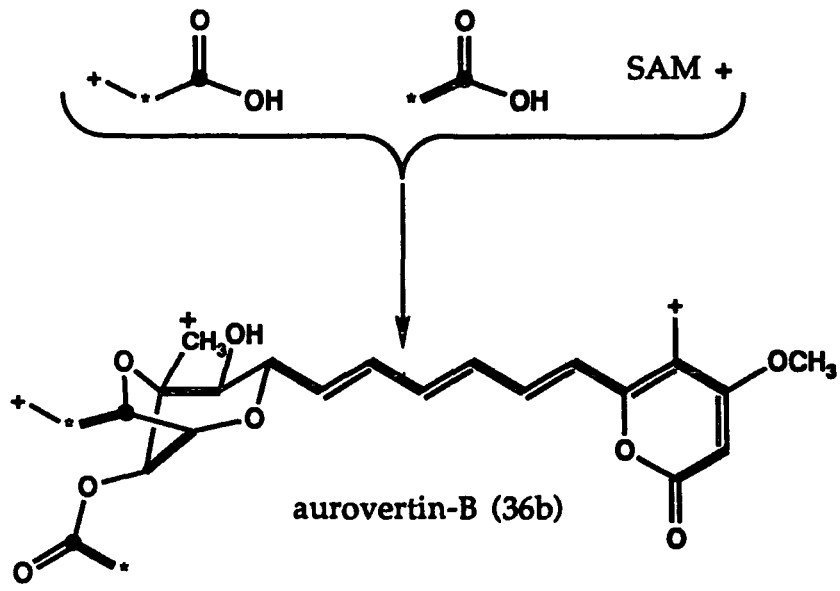


Fig. 2.405. The dual incorporation of propionate, acetate and methionine into aurovertin-B (36b).

Clearly in the case of tenellin (1) these two possibilities are distinguished by the intermediacy of methylmalonate (83) in one pathway and its absence from the other.

2.5 Experimental investigation into the origin of carbon and hydrogen atoms during the biosynthesis of the polyketide sidechain of tenellin (1).

2.51 Experimental investigation into the origin of the sidechain hydrogens.

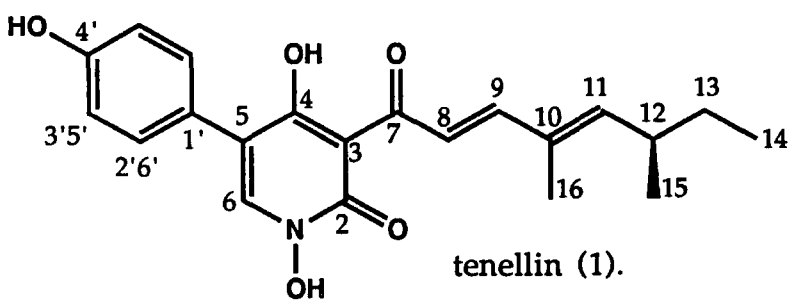
Vining *et al.* demonstrated the acetate origin of the sidechain carbons of tenellin (1) by a series of feeding experiments utilising singly and doubly labelled acetates[59]. In an extension of this work the origin of side-chain hydrogens was investigated by supplementing cultures of *B. bassiana* with sodium [1-^{13}C, 2-^{2}H$_3$]-and [2-^{13}C, 2-^{2}H$_3$]-acetates[60] immediatly prior to tenellin (1) production.

Tenellin (1) enriched at carbon positions 2, 7, 9, 11 and 13 (Table 2.513 for ^{13}C nmr peak assignments) was obtained when *B. bassiana* was supplemented with sodium [1-^{13}C, 2-^{2}H$_3$]-acetate to a concentration of 14.5mM. In addition to the carbon enhancements (Fig. 2.514), the presence of deuterium in the terminal methyl group was indicated by the presence of β-shifted peak enhancements upfield of carbon position 13 (Fig. 2.515). Upfield β–shifts were not observed for either C-7 or C-11, indicating that deuterium is probably not present at C-8 or C-12. However *downfield* β–shifts have been observed[61] for keto-functionalised reporter nucleii, and in the case of C-7 it was thought possible that either a very small, or even zero, β–effect could be operating. In order to be able to conclusively determine whether deuterium was present at C-8 an experiment was performed to observe α–shift effects.

A complementary pattern of carbon enrichment in the sidechain of tenellin (1) was observed when *B. bassiana* was cultured with sodium [2-^{13}C, 2-^{2}H$_3$]-acetate to a concentration of 14.5mM, enrichments (Fig. 2.514) being observed for carbon positions 3, 8, 10, 12 and 14. A large α–shifted multiplet was observed upfield of C-14 indicating the presence of deuterium at this site. Simultaneous proton and deuterium decoupled ^{13}C nmr revealed the presence of four methyl species, CD_3, CD_2H, CDH_2 and CH_3 (Fig. 2.516, Table 2.517). The incorporation of $^{13}CH_3$ was estimated by calculation of peak enhancement in comparison with the unlabelled peak corresponding to C-16. The dual decoupling experiment also revealed that no deuterium was retained at carbon positions 12 or 8.

RUN ON May 14 92
SOLVENT DMSO

OBSERVE C13
Frequency 100.577 MHz
Spectral width 25000.0 Hz
Acquisition time 1.199 sec
Relaxation delay 5.000 sec
Pulse width 10.0 usec
Ambient temperature
No. repetitions 4096
DECOUPLE H1
High power 38
Decoupler continuously on
WALTZ-16 modulated
Double precision acquisition
DATA PROCESSING
Line broadening 1.4 Hz
Gaussian apodization 0.700 sec
FT size 131072
Total acquisition time 7.1 hours



tenellin (1).

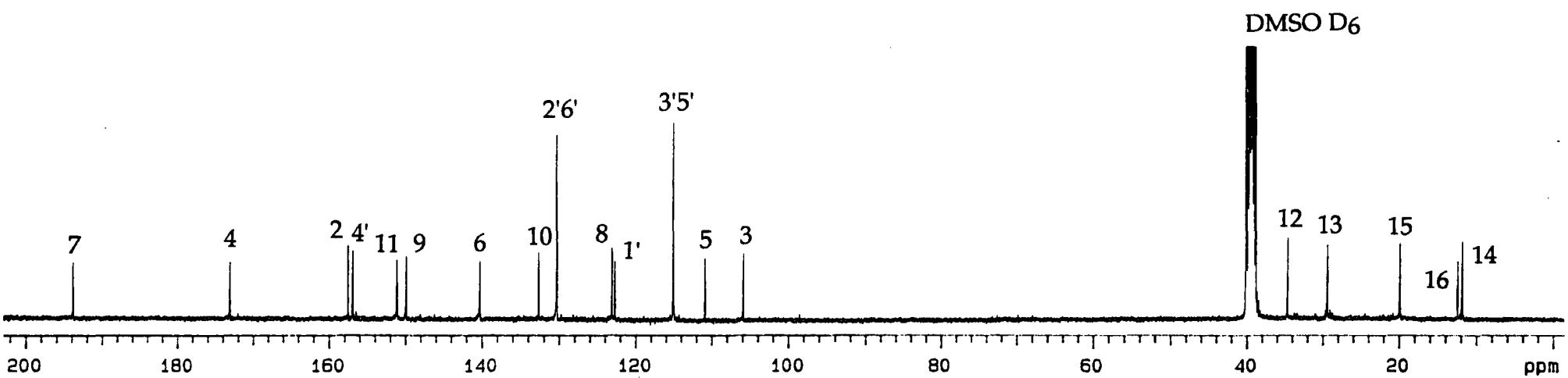
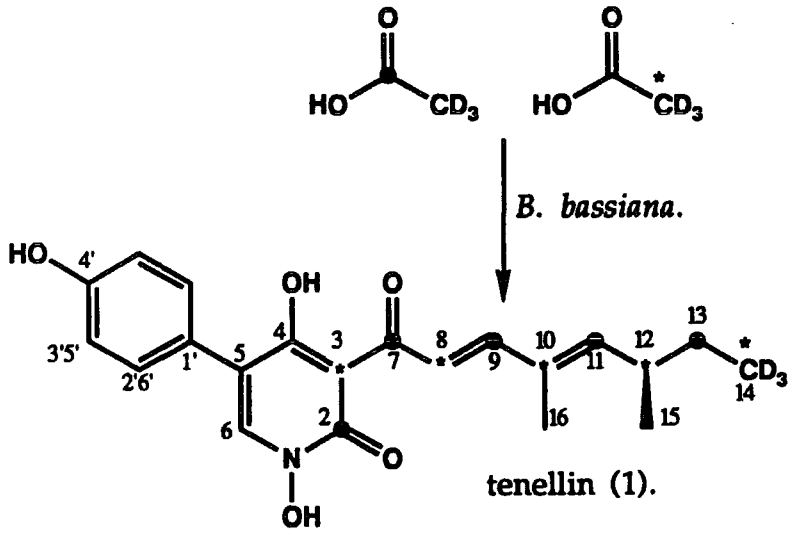


Fig. 2.511. ^{13}C nmr spectrum of tenellin (1).



tenellin (1).

Fig. 2.512. Pattern of incorporated labels from tenellin (1) supplemented with [1-^{13}C, 2-^{2}H$_3$] and [2-^{13}C, 2-^{2}H$_3$] acetates.

Carbon Nº.	δC (DMSO-D6)	Type.	% enrichment from [1-^{13}C] acetate	% enrichment from [2-^{13}C] acetate
2	157.5	CON	0.75	-
3	105.9	C	-	2.24
4	173.1	COH	-	-
5	110.8	C	-	-
6	140.3	CH	-	-
7	193.8	CO	0.80	-
8	123.1	CH	-	1.85
9	149.9	CH	0.82	-
10	132.6	C	-	2.51
11	151.1	CH	0.81	-
12	34.7	CH	-	2.59
13	29.4	CH_2	1.64	-
14	11.8	CH_3	-	2.48
15	19.9	CH_3	-	-
16	12.4	CH_3	-	-
1'	122.7	C (Ph)	-	-
2'6'	130.3	CH (2xPh)	-	-
3'5'	115.0	CH (2xPh)	-	-
4'	156.9	COH (Ph)	-	-

Table 2.513. ^{13}C nmr peak assignments for tenellin (1).

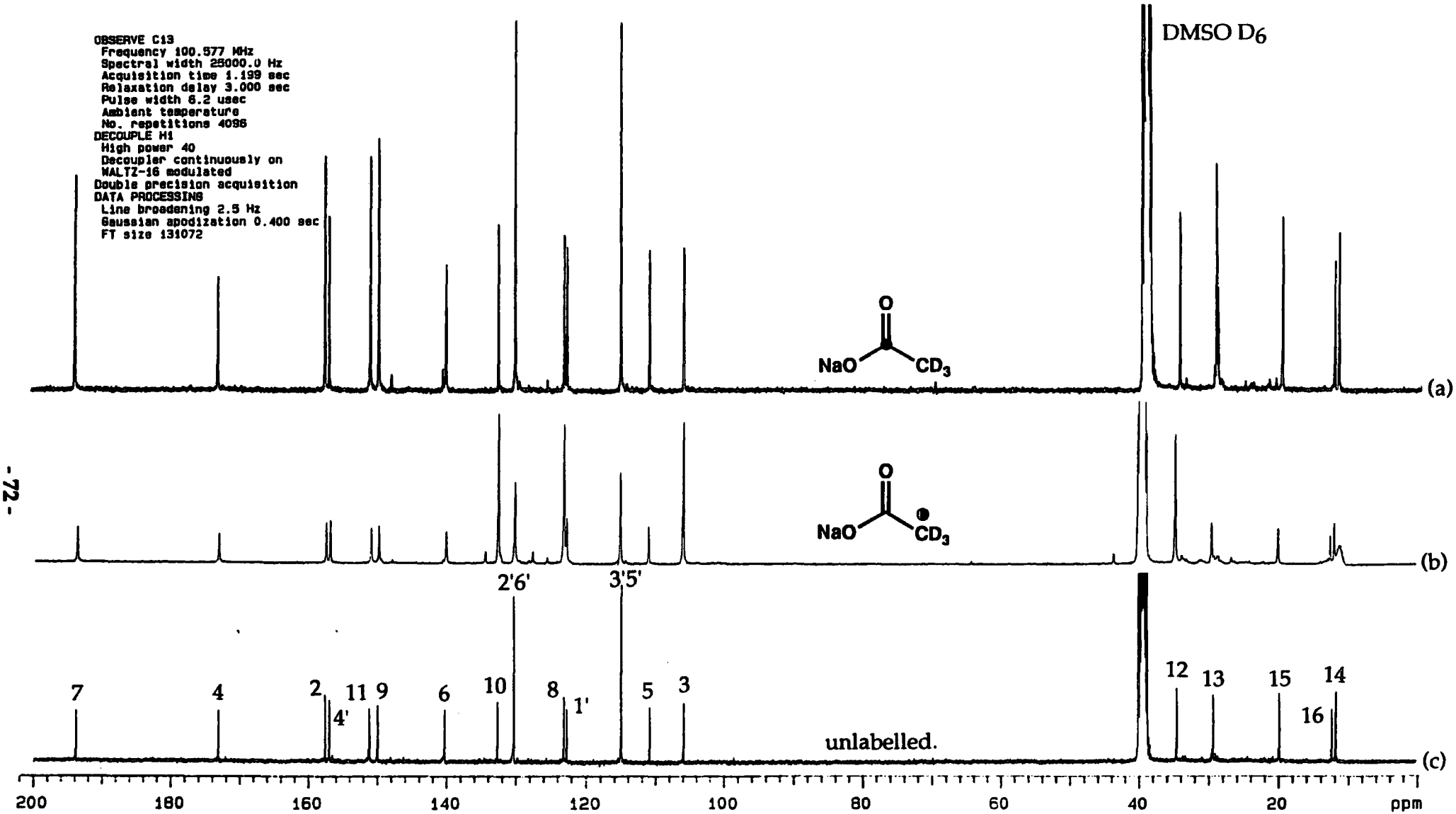


Fig. 2.514. ^{13}C nmr spectra of tenellin (1) obtained from *B. bassiana* cultures (a) supplemented with sodium [1-^{13}C, 2-$^{2}H_3$]-acetate, (b) supplemented with sodium [2-^{13}C, 2-$^{2}H_3$]-acetate, (c) unfed.

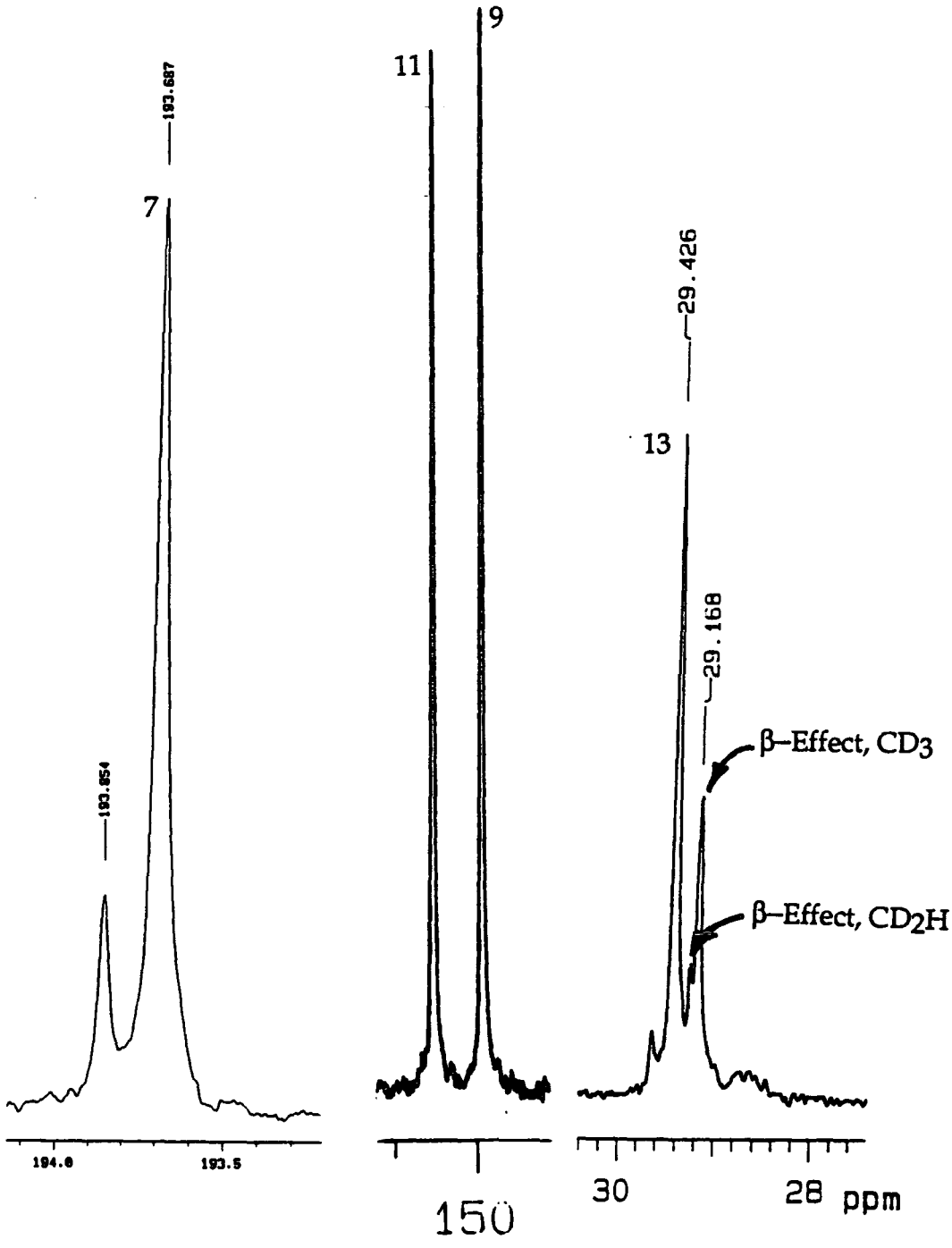


Fig. 2.515. Expansions of peaks corresponding to tenellin (1), obtained from *B. bassiana* cultures supplemented with sodium [1-^{13}C, 2-^{2}H$_3$]-acetate, corresponding to positions 7, 11, 9 and 13.

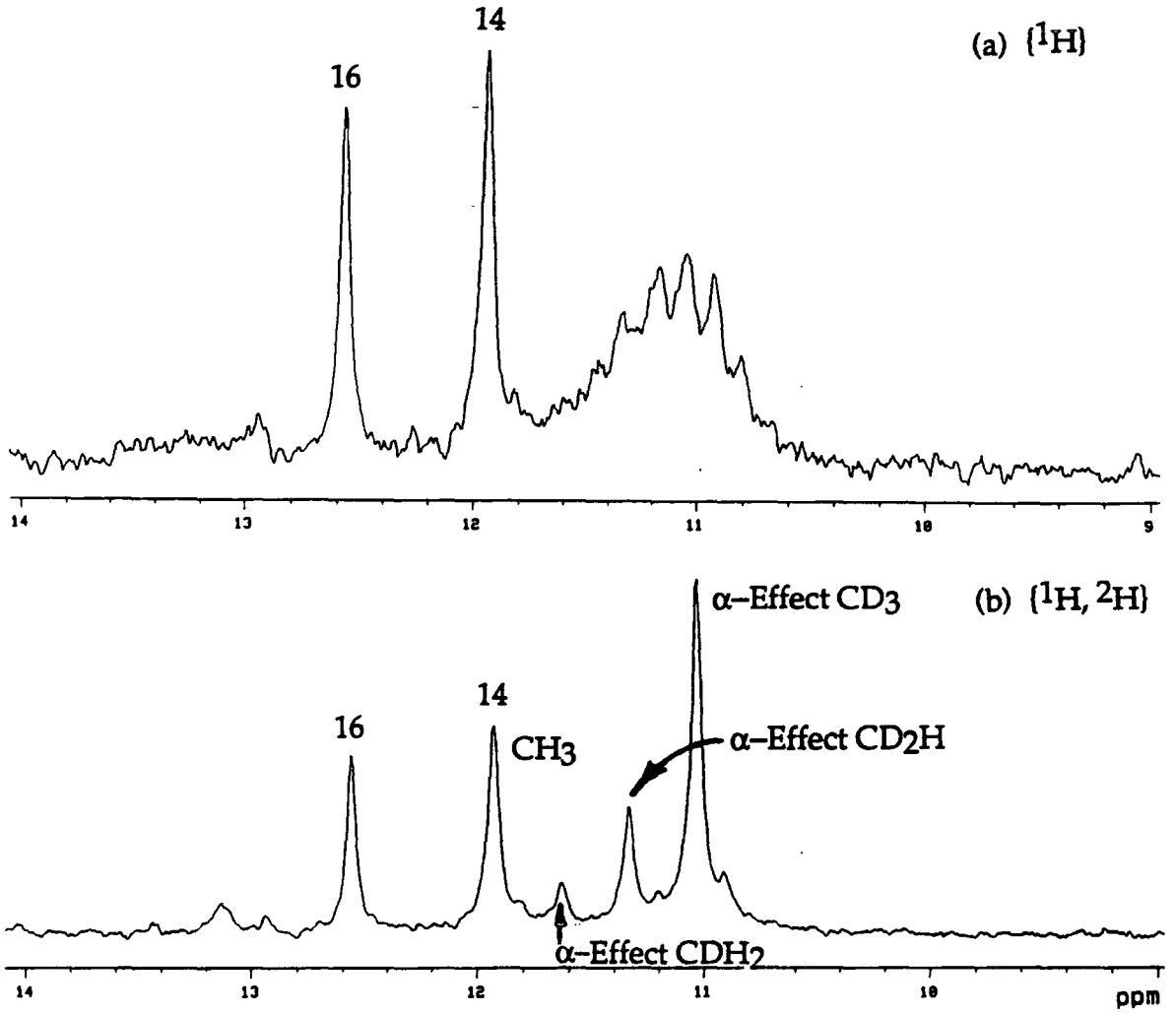


Fig. 2.516. ^{13}C nmr spectra of tenellin (1) obtained from *B. bassiana* cultures supplemented with sodium [2-^{13}C, 2-$^{2}H_3$]-acetate, (a) proton decoupled only, (b) dual proton and deuterium decoupled.

$^{13}CH_3$ %	$^{13}CH_2D$ %	$^{13}CHD_2$ %	$^{13}CD_3$ %
4.3	7.2	21.7	66.6

Table 2.517. Distribution of deuterium labelled ^{13}C species in the terminal methyl group of tenellin (1) obtained from [2-^{13}C, 2-$^{2}H_3$]-acetate.

Of the three acetate C-2 derived carbons which carry hydrogen in tenellin (1), only the terminal methyl group was observed to incorporate significant deuterium from [2-^{13}C, 2-$^{2}H_3$]-acetate. The loss of some deuterium from the acetate starter group indicates an equilibrium between acetyl CoA (77a) and malonyl CoA (82a) *in vivo*. If the mechanism of polyketide synthesis involved in the construction of the tenellin sidechain closely resembles that of FAS then no deuterium would be expected at C-12, as observed, because of the intermediacy of a C-12-C-13 double bond. However deuterium could be expected at C-8 of tenellin (1) where incomplete reduction has occurred. The clear ^{13}C enrichment at this position, but lack of deuterium, can however be explained by extensive exchange of the acetoacyl intermediate with the medium, from both [1-^{13}C, 2-$^{2}H_3$] and [2-^{13}C, $^{2}H_3$] acetates.

In an attempt to obtain further information on the side chain protons, experiments were carried out utilising partially deuterated medium. Tenellin (1) obtained from a broth of *B. bassiana* cultured in a medium containing 50% D_2O was examined by ^{13}C nmr in the hope that solvent donated hydrogen positions could be observed. That all hydrogen bearing carbons of tenellin (1) had incorporated deuterium from the medium was evident by the observation of extensive line-broadening caused by α-effects and coupling, as well as multiple β-effects (Fig. 2.518). This result is not unexpected since all of the starting materials for tenellin (1) must have been biosynthesised from deuterium containing species. In order to attempt to circumvent this problem, non-deuterated acetate and methionine were added to a broth of *B. bassiana* containing 50% D_2O immediately prior to tenellin (1) production. A similar result was obtained, with widespread deuterium incorporation, and it was not possible to unambiguously show the positions of media donated protons in the sidechain of tenellin (1) by this method.

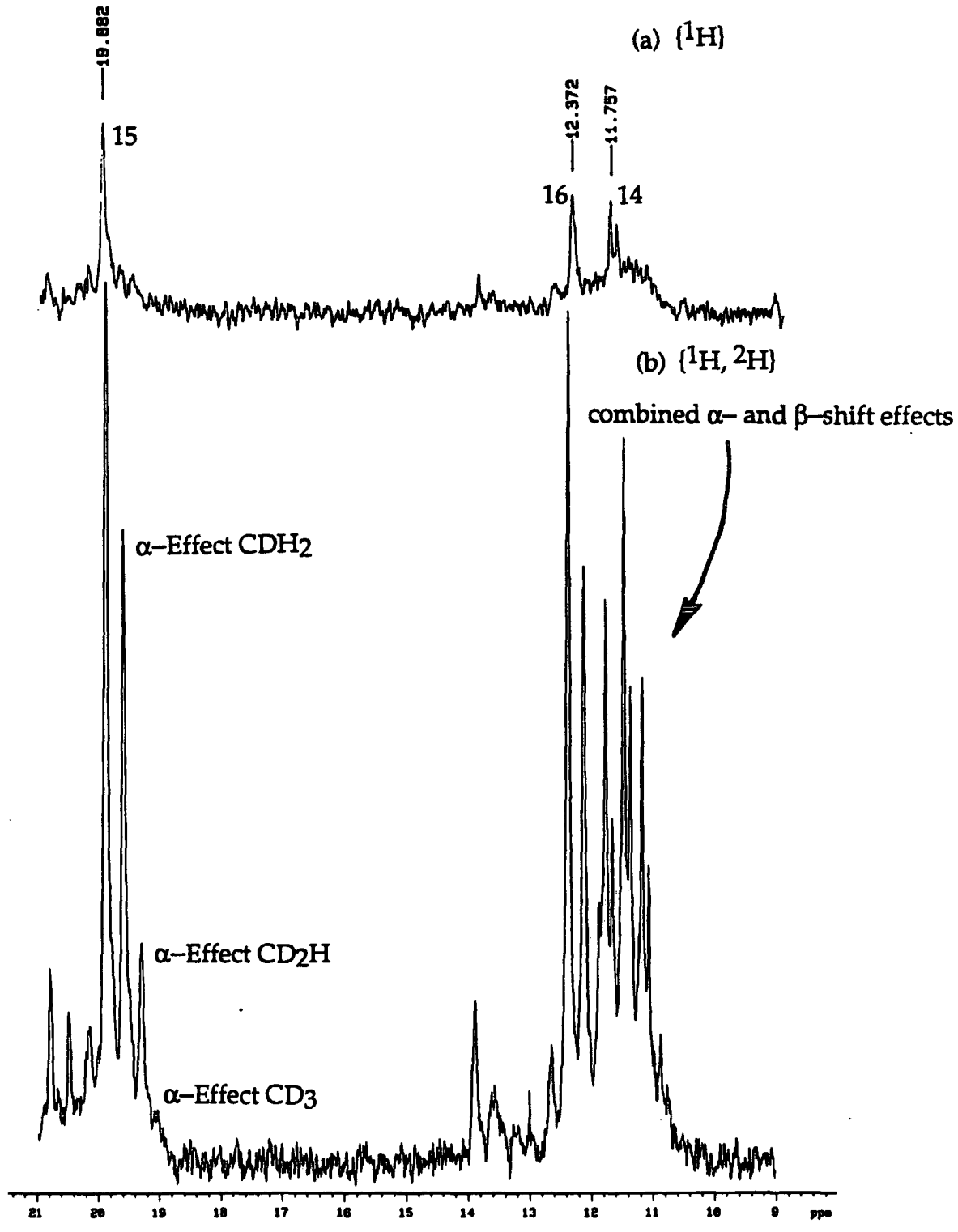


Fig. 2.518. Methyl region of ^{13}C nmr spectra of tenellin (1) obtained from *B. bassiana* cultured in 50% D_2O, (a) proton decoupled only, (b) dual proton and deuterium decoupled.

2.52 Experimental investigation of the mode of methylation during the biosynthesis of tenellin (1).

In a preliminary experiment cultures of *B. bassiana* were supplemented with [*S-methyl* -$^{13}C^{2}H_3$]-L-methionine to a concentration of 2.4mM just prior to tenellin (1) production. After four days, crude tenellin (1) was obtained and examined by high field ^{13}C nmr under two different sets of conditions. In the {^{1}H}-^{13}C spectrum the presence of incorporated label into tenellin (1) was evinced by the observation of large multiplets approximately 1ppm upfield from the unlabelled positions of the two pendant methyl groups C-15 and C-16 (Fig. 2.521). That a large proportion of the original deuterium was also present was indicated by the apparent ^{13}C-D couplings and the α-shifted positions of the labelled peaks.

When the sample was examined under the same conditions but with additional broad band deuterium decoupling, {^{1}H, ^{2}H}, the multiplets were observed to collapse to singlets (Fig. 2.522). Very high enrichment levels (~ 8%, compared to 1-2% for acetate) for the methyl group of [*S-methyl*-$^{13}C^{2}H_3$]-L-methionine were calculated by the measurement of relative peak heights. The magnitude of the α–shift for each enriched methyl position (approximately 1ppm), and the lack of resonances corresponding to partially deuterated methyl groups, showed that all three of the original deuteriums of the labelled precursor were incorporated intact during the methylation event.

The retention of deuterium indicates that methylation can not proceed by a mechanism involving either the transfer of a methylene group from a sulphur ylide[62] or by the intermediacy of exo-methylene groups or cyclopropanes as in the biosynthesis of 24-propylidenecholesterol[63] in the marine alga *Chrysoderma mucosa*. The retention of all three deuterium atoms is consistent with the attack of an activated methylene group on SAM and is in agreement with other fungal polyketides where methionine donates a methyl group to a carbon centre.

These results are consistent with both of the scenarios for methylation of tenellin (1), by methionine, outlined in section 2.5. In order to attempt to delineate between these two possible routes, a series of experiments was devised to probe the possible involvement of methylmalonate (83) during the biosynthesis of the polyketide sidechain of tenellin (1).

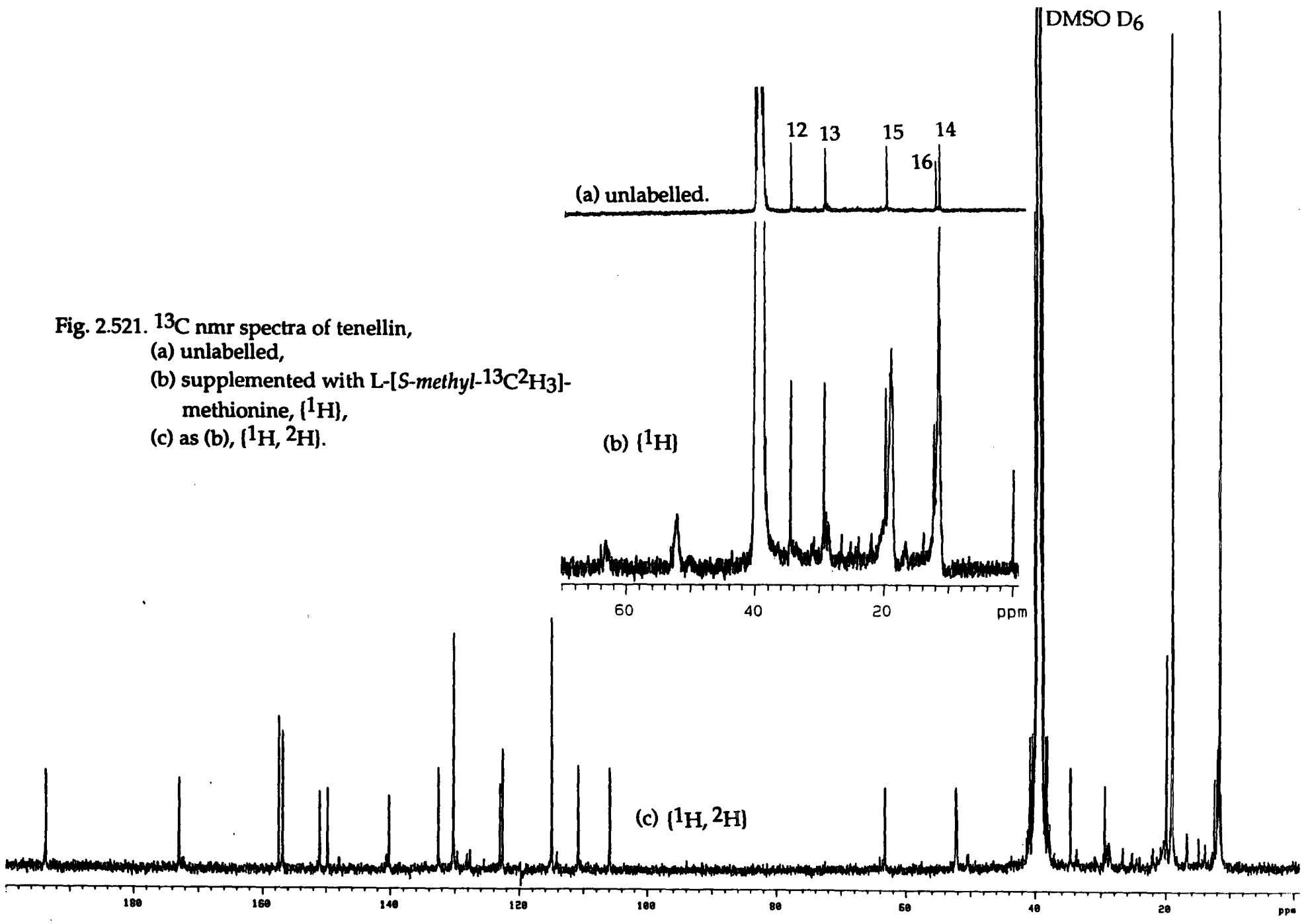


Fig. 2.521. ^{13}C nmr spectra of tenellin,
(a) unlabelled,
(b) supplemented with L-[*S-methyl*-$^{13}C^{2}H_3$]-methionine, {^{1}H},
(c) as (b), {^{1}H, ^{2}H}.

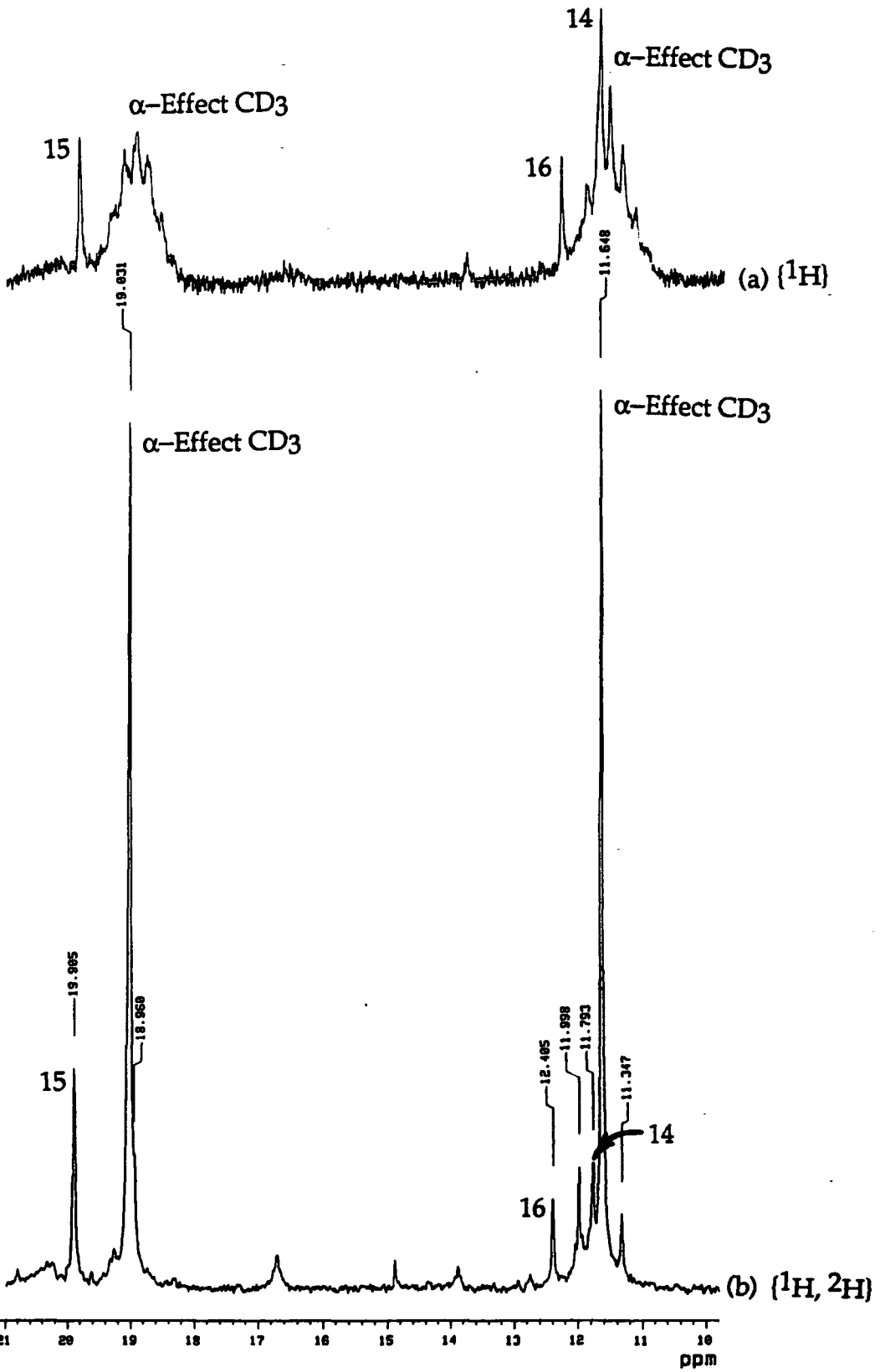
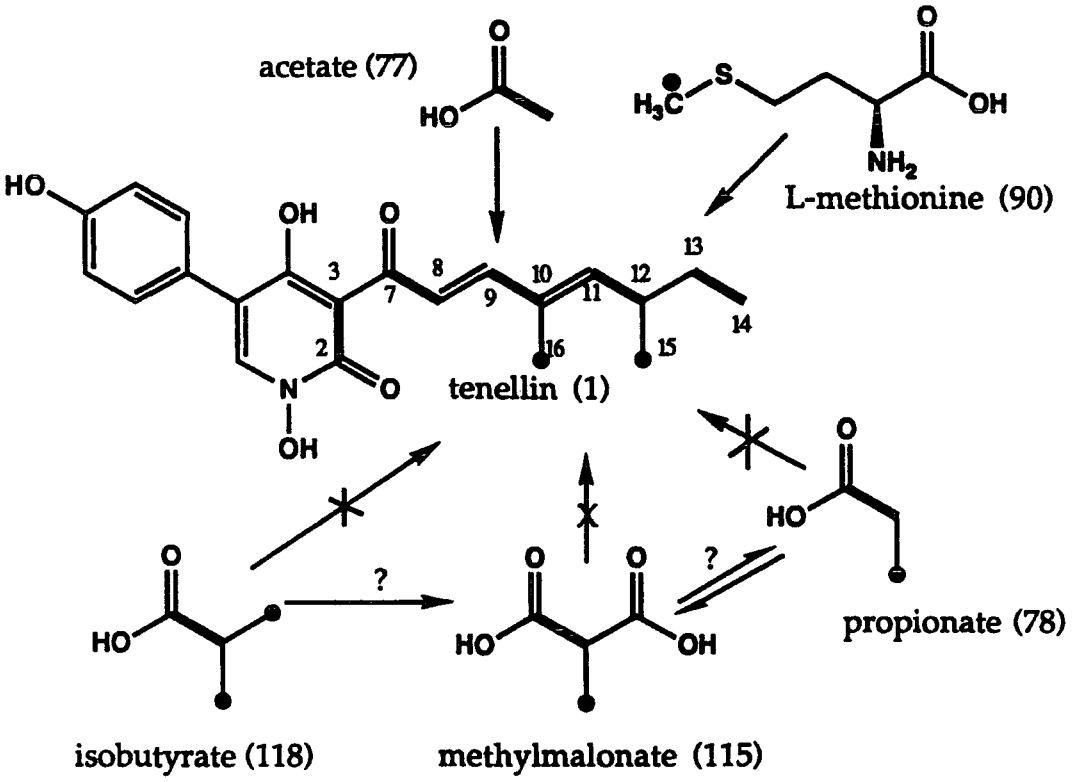


Fig. 2.522. ^{13}C nmr spectra of tenellin obtained from *B. bassiana* supplemented with L-[*S-methyl*-$^{13}C^{2}H_3$]-methionine, (a) proton decoupled, (b) proton and deuterium decoupled.

In an initial set of experiments the possibility that propionate (78) could provide the pendant methyl groups, *via* methylmalonate (83) as in the bacterial systems, was rejected. A fermentation broth of *B. bassiana* supplemented with sodium [1-^{13}C]-propionate (78) to a concentration of 12.9mmol, produced tenellin (1) which after ^{13}C nmr analysis contained no observable carbon enrichments.

The lack of propionate involvement could be due to the absence of a specific propionyl-CoA carboxylase which would be required to produce methylmalonate (83) in the fungal systems. Methylmalonate (83) however can be supplied *in vivo* by a number of other metabolic pathways. Direct methylation of malonate (82) by a SAM (90a) donated methyl group is one possible process, but methylmalonate (83) is also generated during the catabolism of valine (91) *via* isobutyrate (118)[64] and by the direct isomerisation of succinate (45) (chapter 1, section 1.4).

In order to establish the intermediacy, or otherwise, of methylmalonate (83) during the assembly of the polyketide side-chain of tenellin (1), the following experiments were performed. Firstly [*methyl* -^{13}C]-methylmalonate (115) was synthesised (chapter 5, section 5.03) and supplemented to fermentation broths of *B. bassiana* (9.2mM and 23.5mM) immediately prior to tenellin (1) production. In a second experiment, cultures of the producing strain were supplemented with [3,3'-$^{13}C_2$]-sodium isobutyrate (118) to a concentration of 10mM, (synthesis chapter 5, section 5.03) also prior to tenellin (1) production. Tenellin (1) obtained from these experiments was examined by ^{13}C nmr but no significant enrichment of carbons was observed in either case. In order to increase the sensitivity of the experiment doubly labelled diethyl [2,3-$^{13}C_2$] -2-methylmalonate (115a) (kindly supplied by T. J. Simpson) was supplemented in a third experiment to a concentration of 8.4mM. The incorporation of intact [2,3-$^{13}C_2$]-2-methylmalonate (115a) would be expected to lead to the observation of ^{13}C-^{13}C coupling in the ^{13}C nmr spectrum of tenellin (1), but no such coupling was observed.

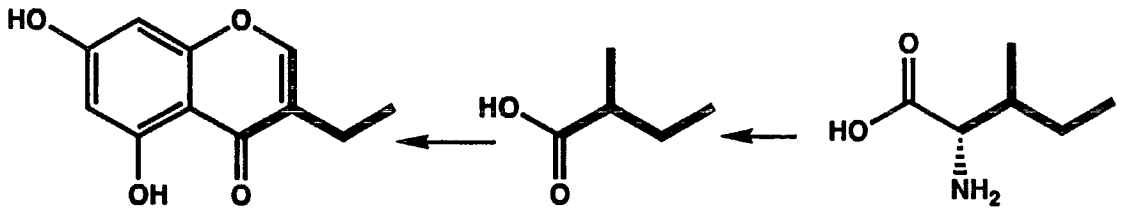


Scheme 2.523. Propionate, methylmalonate and isobutyrate are not involved in tenellin (1) biosynthesis.

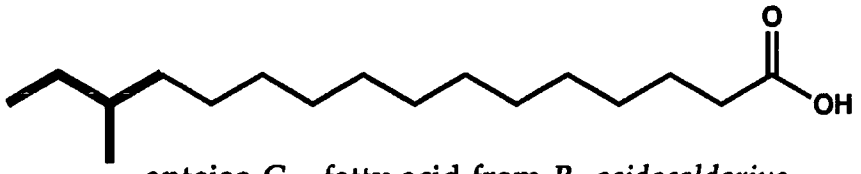
2.53 Experimental investigation into the timing of chain methylations.

Clearly the operation of a PKS system utilising methylmalonate in chain extension during the biosynthesis of the methylated side-chain of tenellin (1) would appear to be unlikely, and methylation may well be intimately connected with a processive method of polyketide construction. The involvment of a methyltransferase activity coupled to the PKS in the processive assembly of the sidechain would require early intermediates to tenellin to be methylated. In the this processive hypothesis, methylation of the growing chain would occur at the β-keto stage, immediately after chain extension by malonate and prior to the cycle of reductions. If this were the case for tenellin (1), the intermediates involved in a processive mechanism leading to the polyketide moiety of tenellin (1) are those depicted in Scheme 2.403. The product of the first condensation, methylation and round of reductions would be an enzyme bound 2-methylbutyrate (108) residue.

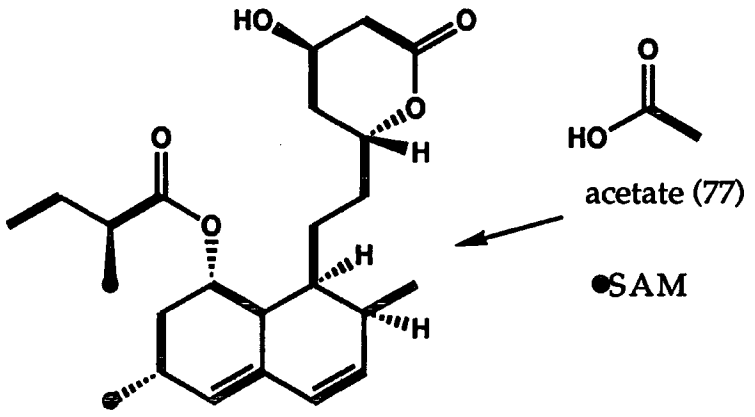
Evidence for the involvement of 2-methylbutyrate (108) as a building unit for fatty acid or polyketide synthesis is scant. It is known, however, that 2-methylbutyrate (108), derived from the *in vivo* breakdown of isoleucine (94), forms the the initial diketide residue of the aromatic plant pentaketide lathodoratin (68)[65] biosynthesised by *Lathyrus odoratus* (sweet pea), and the branched carboxylic acid has also been utilised as a starter unit for fatty acid synthesis by *Bacillus acidocaldarius* when supplemented to this organism[13]. 2-Methylbutyrate (108) has been more closely linked to polyketide synthesis by the observation that the 2-methylbutyryl acyl group of the fungal polyketide mevinolin (69) (*Aspergillus terreus*) is derived, not from isoleucine catabolism, but efficiently from acetate and methionine[66]. It is therefore plausable that 2-methylbutyrate (108) could be an intermediate to the sidechain of tenellin (1).



lathodoratin (68) 2-methylbutyrate (108) isoleucine (94)



anteiso-C_{17} fatty acid from *B. acidocaldarius*



mevinolin (69)

Fig. 2.531. Occurrence of 2-methylbutyrate (108) in lathodoratin, the anteiso series of fatty acids in *Bacillus acidocaldarius* and mevinolin (69).

In order to test this hypothesis a number of ^{13}C and ^{2}H labelled 2-methylbutyrates were synthesised (chapter 5, section 5.02) and administered to growing cultures of *B. Bassiana*. The use of a strategy to limit the effects of possible β-oxidation of this substrate was also adopted in a number of experiments. The results of the feeding experiments are summarised in Table 2.532. It was found in control experiments that high concentrations of the N-propionylcysteamine thiolester (110c,d) severely inhibited normal cell growth, and the maximum tolerable concentration for this material was determined to be 5.0mM. Similar experiments determined a tolerable concentration of the β–oxidation inhibitor 4-pentynoic acid (62) to be approximately 1.0mM.

The labelling patterns of molecules labelled with adjacent ^{13}C and ^{2}H labels (110c) were designed to facilitate the observation of incorporation of *intact* 2-methylbutyrate units into the polyketide chain. Incorporation of

these groups should lead to an observed β-shift of the enriched resonance in the ^{13}C nmr of tenellin (1). In order to increase the sensitivity of the experiments, $^{13}CD_3$ labelled precursors were also synthesised and supplemented to *B. bassiana*. The intact incorporation of one of these groups should lead to the observation of an α-shifted enriched peak, shifted by up to 1ppm from the unlabelled resonance. It was hoped that comparison of ^{13}C spectra of tenellin obtained under standard $\{^1H\}$ conditions and under simultaneous proton and deuterium decoupled conditions, $\{^1H, ^2H\}$, would enable the observation of low (<0.5%) carbon enrichments.

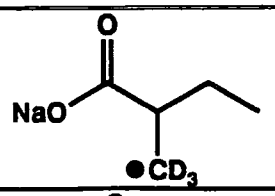
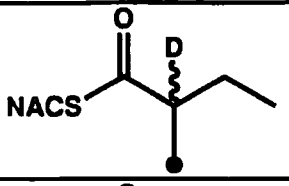
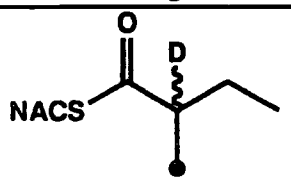
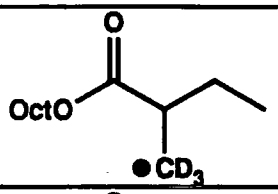
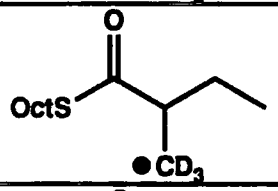
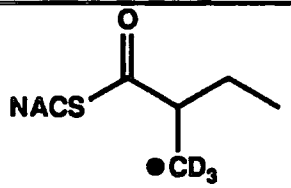
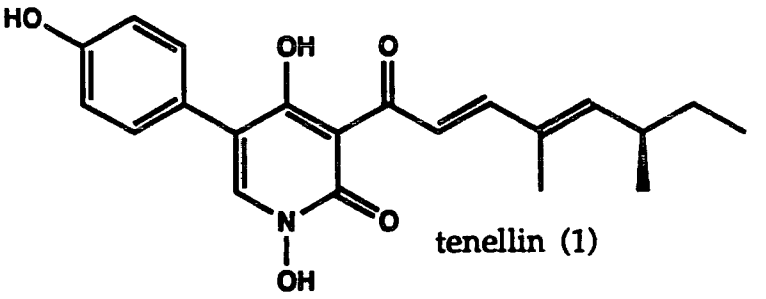
Substrate.	N$^{o.}$	Feeding conditions and final concentration	Crude tenellin yield mg/l	% incorporation.	Tenellin enriched carbon positions.
	(109d)	Single feeding to 9.4mM	750	-	no enrichments.
	(110c).	Pulse fed over 3 days to 16.0mM.	-	-	-
	(110c).	Single supplementation to 5.0mM in the presence of β-oxidation inhibitor.	500	-	no enrichments.
	(112d).	Single supplementation to 5.0mM.	-	-	-
	(111d).	Single supplementation to 3.4mM.	900	-	no enrichments.
	(110d).	Single supplementation to re-suspended cells in glucose replacement medium with β–oxidation inhibitor (1.2mM), to 5.0mM.	300	-	no enrichments.

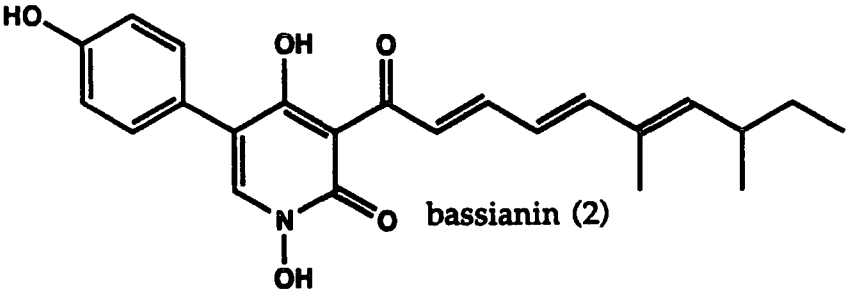
Table 2.532. Summary of 2-methylbutyrate feeding experiments with *B. bassiana*.

The uniform failure of any of the 2-methylbutyrates administered in this series of experiments to become incorporated into the polyketide side-chain of tenellin (1) may indicate that methylation of the chain occurs by some mechanism not considered here. Alternatively the 2-methylbutyrates may be unable to intercept the PKS system and be taken up by it. Numerous possibilities exist to account for this inability, not least the operation of efficient catabolic processes degrading these short-chain fatty acids.

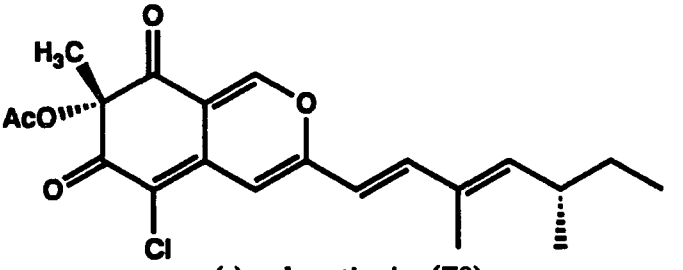
Other more subtle factors may also account for the inability of the 2-methylbutyrates to be taken up by the PKS system. If it were assumed that the PKS system of tenellin (1) resembled that of erythromycin (26) in as much as the activities for PKS were arranged in 'cassettes', that is a system consisting of a few multifunctional proteins, with each individual enzyme system responsible for, say, two condensation and modification sequences, then it may only be possible to intercept the fungal PKS system between 'cassettes'. It is plausible to imagine that the first enzyme 'cassette' involved in the biosynthesis of tenellin (1) would take an acetyl CoA starter unit and perform the first condensation with malonyl ACP, methylate at the β-keto stage, perform a full round of reductions, add the second chain extending malonate, methylate a second time and reduce the keto functionality to an enoyl system, all as enzyme bound species. The formed ACP-bound doubly methylated triketide would then be passed to a second cassette responsible for the third and fourth chain extensions, this time without methylation steps. This idea gains credance in the light of the existance of other fungal polyketides possessing the exact same moiety, especially bassianin (2) which would be expected to share much of the hardware responsible for polyketide synthesis with its shorter sibling tenellin (1), as well as for example the (-)-sclerotiorin (70) family[67,68], the postulated putative precursor to (-)-betaenone[69] (27), and the the oxidatively modified alternaric acid (71)[70]. Very many other examples are known of a doubly methylated triketide unit at the beginning of a polyketide chain where only the oxidation level differs from tenellin (1). Examples include funiculosin (5), citreomontanin (32) and radiclonic acid (39) amongst others. A doubly methylated triketide occurs as an O-acyl moiety in lunatoic acid-A (72)[71] elaborated by the fungus *Cochliobolus lunata*.



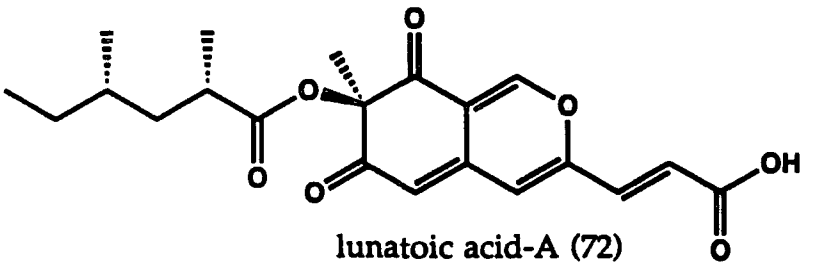
tenellin (1)



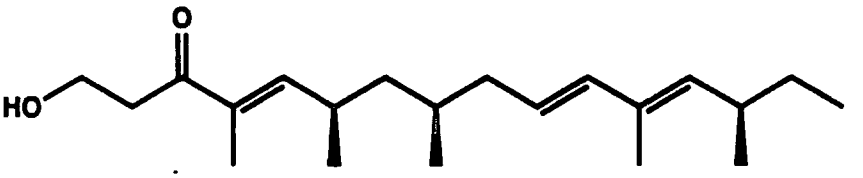
bassianin (2)



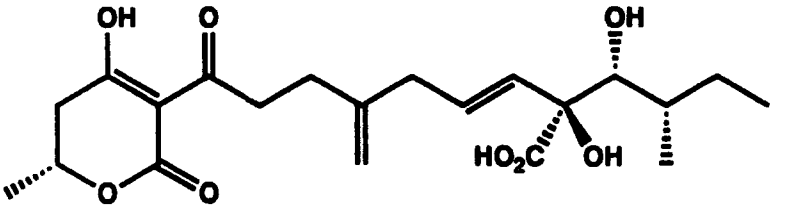
(-) sclerotiorin (70)



lunatoic acid-A (72)



postulated precursor to (-)-betaenone-B (27)



alternaric acid (71)

Fig. 2.533. Metabolites bearing doubly methylated triketide groups.

This idea was tested by the synthesis (chapter 5, section 5.10) and administration of the triketide ethyl [1-^{13}C]-E-2,4-dimethylhex-2-enoate (148b) to a growing culture of *B. bassiana* in a preliminary experiment. The labelled precursor was pulse fed over three days to a final concentration of 3.9 mM. Tenellin (1) obtained after seven days incubation was examined by ^{13}C nmr, however no carbon enrichment at C-9 or any other carbon position was apparent.

In order to overcome the possibility that the ethyl ester was consumed by efficient β-oxidation, the N-propionylcysteamine thiolester of [1-^{13}C]-E-2,4-dimethylhex-2-enoate (150b) was also synthesised (chapter 5, section 5.10) and supplemented to *B. bassiana* in conjunction with the β–oxidation inhibitor 4-pentynoic acid (62). A final concentration of 3.0 mM was achieved by pulse feeding the thiol ester over three days as tenellin (1) production progressed. The β–oxidation inhibitor was added to a concentration of 1.0 mM. Tenellin obtained after 5 days fermentation was examined by ^{13}C nmr, however no carbon enhancements were observed. An ethereal extract of the aqueous medium was analysed by t.l.c and showed no sign of the supplemented triketide thiolester.

2.54 Discussion.

Feeding experiments with sodium [1-^{13}C, 2-^{2}H$_3$]- and [2-^{13}C, 2-^{2}H$_3$]-acetates have shown that the sidechain of tenellin is a pentaketide, incorporating an intact acetate group containing three deuteriums as a starter unit, but not incorporating deuterium at any other position. All positions were heavily labelled when tenellin (1) was obtained from *B. bassiana* cultured in 50% D_2O. Equilibrium between acetate and malonate *in vivo* could be observed as evinced by low levels of deuterium wash-out from the terminal methyl position. In contrast no deuterium was lost from the methyl group of L-[*S-methyl* -^{13}C^{2}H$_3$]-methionine, intact incorporation of ^{13}CD$_3$ groups into tenellin (1) being observed. This is consistent with the nucleophilic attack of an activated methylene group on S-adenosyl methionine (SAM).

The possibility that methylmalonate is utilised as a chain extender during the biosynthesis of the sidechain of tenellin (1) was rejected because of the failure to incorporate the known precursors to methylmalonate, sodium [1-^{13}C]-propionate and sodium [3,3'-^{13}C$_2$]-isobutyrate, as well as sodium [*methyl* -^{13}C]-and [2,3-^{13}C$_2$]-methylmalonate itself. Methylation is therefore likely to involve a SAM dependent methyltransferase and be intimate with the PKS. However positive evidence for the operation of a processive mechanism involving methylation of the growing chain at a β-

keto stage is also lacking due to the failure of numerous feeding experiments utilising [1-^{13}C] labelled monomethylated diketides and dimethylated triketides. These more complex putative precursors may be subject to efficient β-oxidation, even when strategies were used to limit this process. These compounds were rarely detectable in the medium a few days after supplementation, and instances where recovery of labelled material was possible were usually from unhealthy cultures. β-Oxidation of the labelled di- and tri-ketides used in this study would afford labelled propionate, which was shown not to play a part in tenellin (1) biosynthesis, and so detection of label in tenellin (1) by incorporation of β-oxidation products is unlikely, and in fact was never observed. It may prove worthwhile using the more powerful β-oxidation inhibitors investigated recently by Vederas *et. al.* in order to press these experiments further.

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Chapter 3.

Phenylalanine metabolism and the biosynthesis of the pyridone moiety of tenellin.

3.1 Phenylalanine metabolism.

The α–amino acid L-phenylalanine (99) is often found as a constituent part of plant and microbial secondary metabolites. The ready availability of both enantiomers of this amino acid in ^{13}C and ^{14}C labelled forms, as well as in ^{2}H and ^{3}H stereospecifically labelled forms[1] has meant that its incorporation into a number of metabolites is well studied. The use of these materials has allowed the fate of individual phenylalanine protons to be traced, and has led to an understanding of the metabolic processes operating on phenylalanine *in vivo* (Fig. 3.101).

Phenylalanine is synthesised *in vivo* by the reversible amination of phenylpyruvate (95)[2]. L-Phenylalanine (99) is for example in rapid equilibrium by way of phenylpyruvate (95) with D-phenylalanine (99) in the cytochalasin-D (18) synthesising fungus *Zygosporium masonii*. Enolisation of the α-ketoacid lead to the rapid exchange of the benzylic protons[3]. A similar process was found to be operating during the biosynthesis of cyclopenin and cyclopenol (21a,b)[4]. In these two cases the intermediacy of phenylpyruvate (95) explained the observation that both D and L forms of the amino acid are incorporated into the metabolites. Other processes affecting phenylalanine have been shown to occur more stereospecifically. For example ammonia elimination catalysed by L-phenylalanine ammonia lyase (PAL) occurs by the specific removal of the *3-pro-S* hydogen and the amino group, to yield *trans*-cinnamic acid (89)[1]. Kirby *et al.* have also shown that the *3-pro-R* proton is subject to stereospecific exchange with retention of configuration during the biosynthesis of gliotoxin (75)[5] in the fungus *Trichoderma viride*.

Phenylalanine (99) is also frequently *p*-hydroxylated to provide an additional source of tyrosine (98), and the enzyme systems responsible are known to be widespread in the microfungi, plants and higher animals, but largely absent from other species[6].

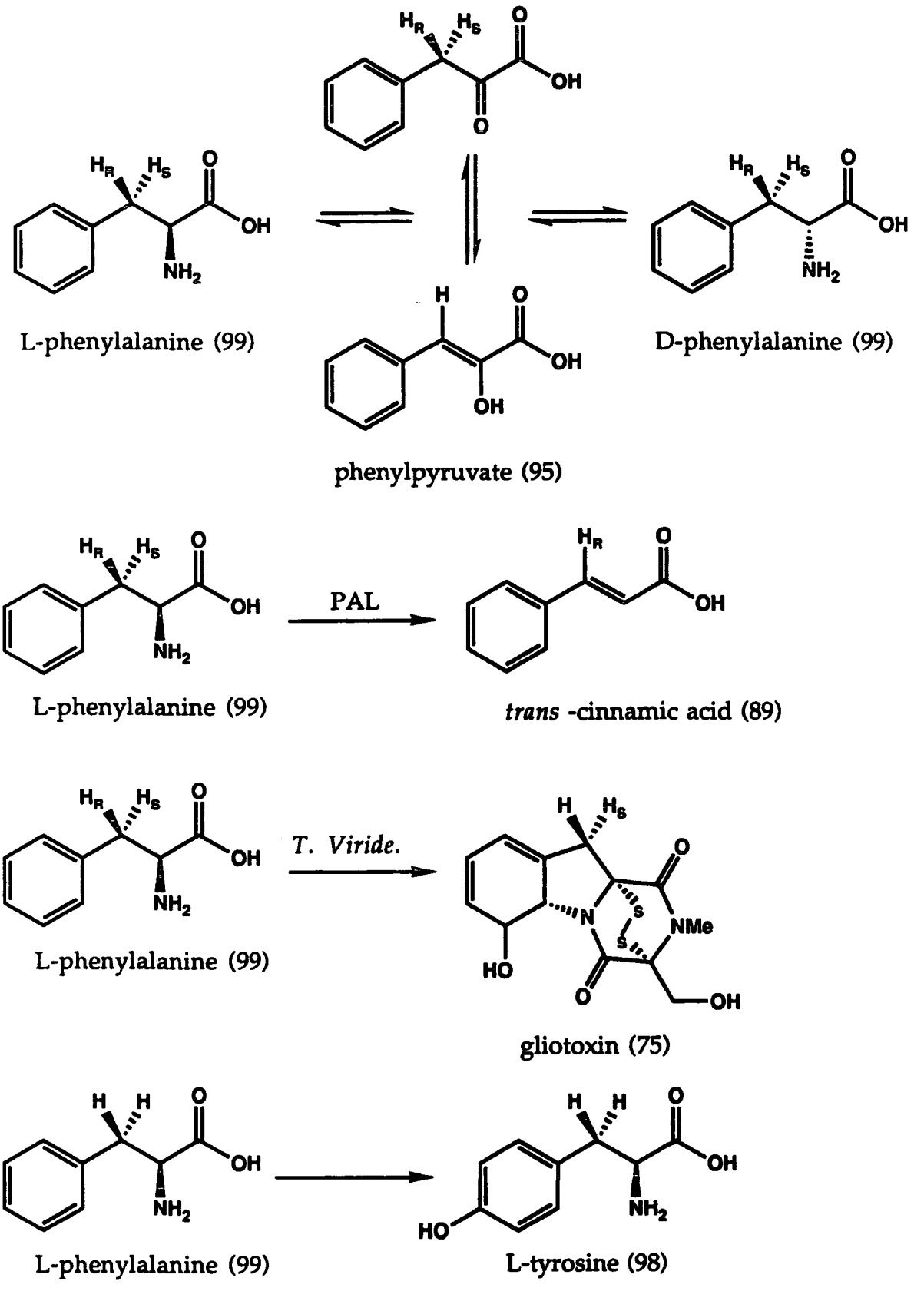


Fig. 3.101. Some aspects of L-phenylalanine (99) metabolism.

3.2 L-Phenylalanine undergoes a rearrangement during the biosynthesis of tenellin (1) and tropic acid (42).

The most intriguing feature of tenellin (1) biosynthesis is the formation of the six-membered 2-pyridone ring from an *intact* L-phenylalanine unit and the terminal acetate residue of a polyketide chain[7]. The same precursors account for the 2-pyridone moiety of ilicicolin-H (6)[8], and would seem the most likely candidates for the origin of the 2-pyridone rings of bassianin (2) and leporin-A (7)[9]. The sidechain of harzianopyridone (9) has been shown to be polyketide in nature (acetate and methionone derived)[10], but the origin of the rest of the pyridone ring has not been conclusively determined, although the amino acid aspartate has been implicated. Visual inspection of the structure of funiculosin (5) would suggest that this too could be derived from a polyketide chain and an unusual amino acid residue, perhaps derived ultimately from phenylalanine (99) or tyrosine (98). (*N.B.* The origin of these metabolites differs from the origin of two of the fungal 2-pyridones, viridicatin (8a) and viridicatol (8b), which have been shown to be products of the metabolism of the cyclic *bis*-aminoacids cyclopenin (21a) and cyclopenol (21b)[11,12]. respectively.)

Rearrangement of the amino acid moiety, formally by a 1-2 carboxyl shift, is necessary to form the requisite structural framework of the six-membered pyridone ring. Vining *et al.* showed that this rearrangement is intramolecular in the case of tenellin (1). The supplementation of [1,3-$^{13}C_2$]-phenylalanine to a fermentation broth lead to the isolation of tenellin (1) labelled specifically at carbon positions 4 and 5 (Fig. 3.211)[13]. The retention of the amino nitrogen from phenylalanine during tenellin (1) biosynthesis was also shown by a ^{15}N feeding experiment. L-Phenylalanine (99) was also shown to be a better precursor to tenellin (1) than L-tyrosine (98), suggesting that aryl hydroxylation occurs after condensation with the polyketide chain.

Two different mechanistic explanations have been advanced to account for this observed intramolecular rearrangement of phenylalanine during tenellin (1) biosynthesis (Fig. 1.402). In a pathway postulated by Vining *et al.* the expansion of a five membered ring tetramic acid precursor, formed by the condensation of L-phenylalanine (99) and a polyketide chain was considered, whilst we theorised[14] an alternative scenario which involved the direct rearrangement of phenylalanine (99) prior to condensation, to provide the carbon framework necessary to form the six-membered 2-pyridone.

3.21.Direct rearrangement of phenylalanine.

Although the existence of an L-phenylalanine mutase activity is without precedent in fungi, Leete has shown that L-phenylalanine is subject to apparent intramolecular rearrangement, by 1-2 carboxyl shift, during the biosynthesis of the tropic acid (42) moiety of the alkaloids atropine (43) and scopolamine (44) (these two metabolites were previously known as hyoscyamine and hyoscine respectively[15]) in the Solanaceous[16] family of plants. These two compounds are pharmacologically important smooth muscle relaxants, and account for the toxicity of the common plants[17] Deadly nightshade (*Atropa belladonna*), Henbane (*Hyoscyamus niger*, incidentally the plant used by Dr. Crippen to murder his wife, by poisoning, in 1910[18]) and Thornapple (*Datura stramonium*) among others.

Leete showed that the three sidechain carbons of tropic acid (42) are derived from the three propanoid carbons of phenylalanine using DL-phenylalanine labelled with ^{14}C at carbon positions 1, 2, and 3[19,20]. The rearrangement was shown to be intramolecular by a feeding experiment with DL-[1-^{14}C, 1,3-$^{13}C_2$]-phenylalanine which resulted in the alkaloids containing tropic acid with adjacent ^{13}C labels[21]. Unlike the case of tenellin (1), the L-phenylalanine nitrogen is not retained, and is repaced by an hydroxyl functionality in tropic acid (42).

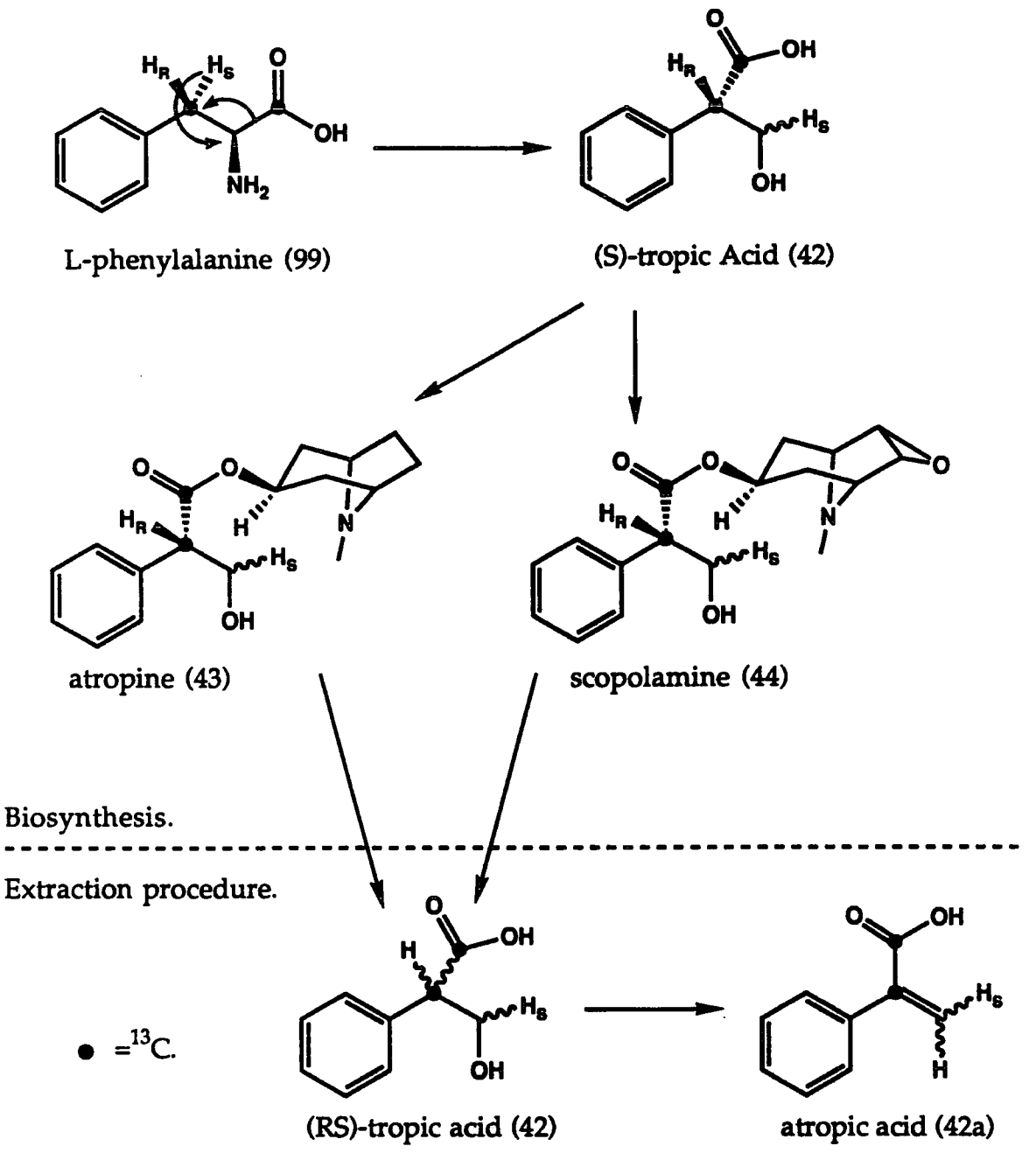


Fig. 3.211. Summary of biosynthesis and extraction procedure of tropic acid (42).

In order to probe the stereochemistry of the carboxyl shift, Leete supplemented growing *Datura stramonium* and *D. innoxia* plants with a mixture of equal amounts of the four stereoisomers of [1-^{14}C, 3-^{3}H]-phenylalanine[22]. The scopolamine (44) and atropine (43) isolated from the plants contained almost all of the supplemented tritium. When the alkaloids were hydrolysed, using barium hydroxide solution, tropic acid was afforded which contained 59% of the original tritium. Tritium loss during this hydrolysis step accompanied epimerisation at the benzylic position[23]. Exactly half of the original tritium was found to be present on the

hydroxymethylene carbon (C-2 of phenylalanine) of the tropic acid by its conversion to atropic acid (42a). Thus the carboxyl shift giving rise to the (S)-tropic acid (42) of atropine (43) and scopolamine (44), is accompanied by back migration (vicinal interchange) of a proton from the benzylic carbon of phenylalanine to the hydroxymethylene carbon of tropic acid.

The studies of Haslam *et al.* [24] are complementary to those of Leete. The supplementation of the two individual stereoisomers of L-[2-^{14}C, 3-^{3}H]-phenylalanine to *Datura stramonium* plants in *separate experiments* gave rise to scopolamine (44) and atropine (43) which were not used for activity measurements. The alkaloids were hydrolysed instead with sodium hydroxide solution to give tropic acid (42). When L-[2-^{14}C, 3-*R*-^{3}H]-phenylalanine was administered, tropic acid was obtained which contained 7-12% of the original tritium. Supplementation with L-[2-^{14}C, 3-*S* -^{3}H]-phenylalanine resulted in tropic acid (42) containing 65-70% of the original tritium. Since Leete showed that tritium is not lost in the biosynthesis of the alkaloids, but is lost mainly from the benzylic position during ester hydrolysis by epimerisation, these results essentially show the amount of tritium present on the hydroxymethyl carbon of tropic acid (42). It is thus the *3-pro-S* hydrogen of phenylalanine which migrates and is retained at the hydroxymethylene position, the *3-pro-R* hydrogen remains, but is washed out to a high extent during epimerisation. These conclusions were reinforced by a more rigorous repetition of Haslam's work by Leete[25].

Non-stereospecifically [1-^{14}C, 3-^{3}H]-labelled L-phenylalanine was also supplemented to a culture of *B. bassiana*.[7] The tenellin (1) obtained had lost 96.6% of the original tritium, leading Vining *et al.* to the conclusion that:

"The loss of at least 96% of the ^{3}H from [3-^{3}H]-phenylalanine during incorporation rules out any rearrangement mechanism involving a 1,2 hydride shift."

However since any hydride shift occurring during phenylalanine rearrangement as part of tenellin (1) biosynthesis would be expected to be stereospecific, the rearranged amino acid would be expected to possess tritium at either the *pro-R* or the *pro-S* position of the aminomethylene carbon. In the case of tenellin (1), one of these two hydrogens is lost to form the unsaturated pyridone ring, also probably stereospecifically. If this step were stereospecific and the hydrogen lost in this step corresponded to the hydrogen involved in the 1,2 shift, then no tritium would be expected to be retained in the fully formed tenellin (1) (there is a 50% chance of this). Thus the results of Vining do not necessarily rule out the operation of a hydride

shift during the biosynthesis of tenellin (1), and his assertation is flawed.

The vicinal interchange between C-1 and the 3-*pro*-R hydrogen of L-phenylalanine during (S)-tropic acid biosynthesis bears resemblance to the small group of 1-2 shifts catalysed by co-enzyme B_{12}, and in particular to the isomerisation between succinyl-CoA (45) and methylmalonyl-CoA (83a) (chapter 1, section 1.4) known to occur in the bacteria and mammals. Co-enzyme B_{12} catalysed rearrangements are characterised by the back-migration of a proton accompanying the movement of the major group[26]. If tropic acid arises by a co-enzyme B_{12} catalysed rearrangement of L-phenylalanine, then 3-amino-2-phenylpropionic acid (104) would be an intermediate.

In the case of tenellin (1) a similar process could be postulated, bearing in mind that a hydride shift has not necessarily been ruled out, again yielding 3-amino-2-phenylpropionic acid (104). In this case however there would be no replacement of the amine group by an hydroxyl, and the rearranged amino acid would condense with the terminal residue of the polyketide chain to generate the six membered ring of tenellin (1).

No intermediates between L-phenylalanine (99) and tropic acid (42) or tenellin (1) have so-far been identified, but a pathway unifying the biosynthesis of both metabolites would involve the intermediacy of 3-amino-2-phenylpropionic acid (104).

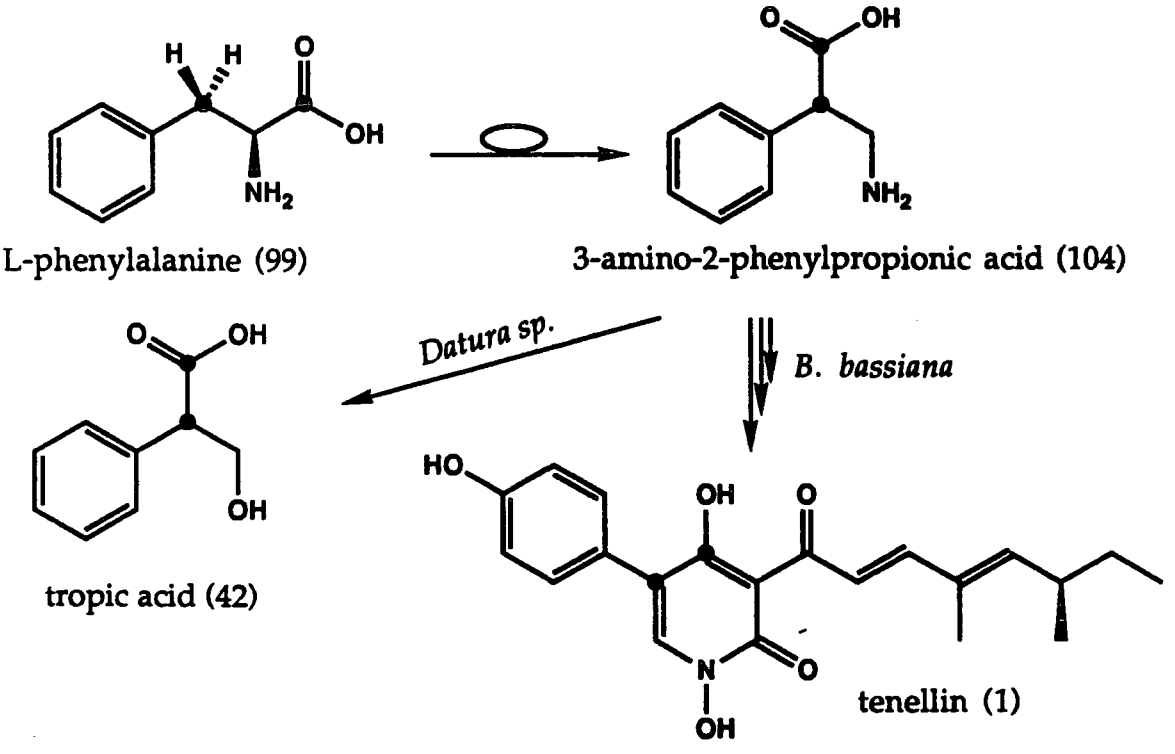


Fig. 3.212. The possible intermediacy of 3-amino-2-phenyl propionic acid (104) in tropic acid (42) and tenellin (1) biosynthesis.

To test this hypothesis 3-amino-2-phenylpropionic acid (104) was synthesised (chapter 5, section 5.01) carrying either [3-^{13}C] (104b) or [3-^{14}C] (104c) labels. The possible participation of this rearranged amino acid during the biosynthesis of tropic acid (42) from L-phenylalanine (99) was tested by the administration of [3-^{14}C]-labelled material (104c) to growing *Datura innoxia* plants. The amino acid was taken up in solution by the plants *via* a woollen wick threaded through the stems. After twenty eight days almost all of the radioactivity had been taken up, and the plants were harvested and the tropic acid alkaloids atropine (43) and scopolamine (44) extracted (as detailed in chapter 7) and found to contain a negligible amount of radioactivity.

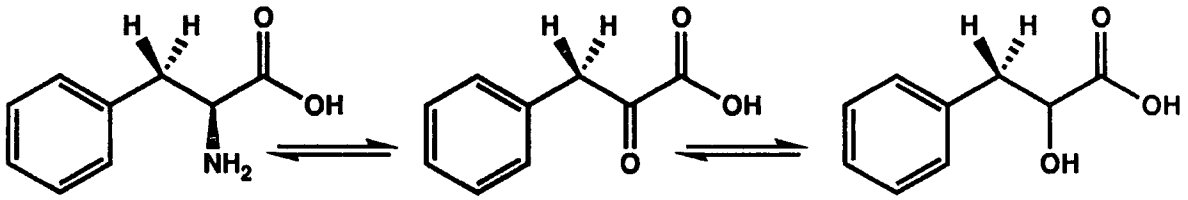
In the case of tenellin (1), [3-^{13}C]-3-amino-2-phenylpropionic acid (104b) was added to fermentation broths of *B. bassiana*, immediately prior to tenellin (1) production, to a final concentration of 2mM. A small amount of [UL-^{14}C]-L-phenylalanine (s.a 1.7 μCimmol^{-1}) was also added as an internal reference. The tenellin (1) obtained after fermentation for seven days had an absolute incorporation of ^{14}C of 3.4%. This is in agreement with the results of Vining[7], but examination of the ^{13}C nmr spectrum showed no observable incorporation of label from [3-^{13}C]-3-amino-2-phenylpropionic acid (104b). This experiment was repeated with a similar outcome. It remained possible that the exogenously added material could not penetrate the cells. This possibility was however eliminated after a three-day old *B. bassiana* culture was supplemented with unlabelled (104a) and L-phenylalanine (99) in equal amounts. Both amino acids were successfully detected by tlc analysis after several days from extracts of the washed cells.

The known co-enzyme B_{12} mutase enzymes which catalyse carboxyl shifts utilise CoA-thiolesters directly[26]. It might therefore be concluded that the CoA thiolester produced after rearrangement of L-phenylalanine-CoA may actually be an intermediate species, which is involved in the condensation with the polyketide chain during tenellin biosynthesis. A specific CoA thiolester synthase may not necessarily be present in *B. bassiana*, and if the exogenously added 3-amino-2-phenylpropionic acid (104) were not activated to the CoA thiolester it then would not be able to participate in tenellin (1) biosynthesis.

In order to test this possibility, syntheses (chapter 5, section 5.05) of the N-propionylcysteamine thiolesters of both L-phenylalanine (99) and 3-amino-2-phenylpropionic acid (104) were developed. In a preliminary feeding experiment the incorporations of [UL-^{14}C]-L-phenylalanine (99) and [UL-^{14}C]-L-phenylalanine (N-propionylcysteamine) thiolester (125a) into tenellin (1) were compared. The two substrates were added to fermentation

broths of *B. bassiana* just prior to tenellin (1) production at equivalent concentrations (3.0mM) and specific activities (1.0μCimmol^{-1}). Tenellin (1) obtained from these broths showed a specific incorporation of 3.66% when [UL-^{14}C]-L-phenylalanine (99) was the substrate and a specific incorporation of 3.69% when [UL-^{14}C]-L-phenylalanine (N-propionylcysteamine) thiolester (125a) was the substrate. The near identical results do not convincingly show that activation to a thiolester provides additional impetus to push the amino acid substrate into the 2-pyridone synthase activity, and it could be more reasonably concluded that the thiolester was efficiently hydrolysed before incorporation. For this reason the synthesis and supplementation of ^{14}C-labelled 3-amino-2-phenylpropionate (N-propionylcysteamine) thiolester (128) was not pursued further.

It would seem therefore that 3-amino-2-phenylpropionic acid (104) is not involved in either tenellin (1) or tropic acid (42) biosynthesis. In the case of tropic acid (42), other species suggest themselves as putative intermediates. Phenyllactate (96) and phenylpyruvate (95), known products of phenylalanine metabolism, may be a more direct source of the carbon framework which undergoes rearrangement. In the case of tenellin (1) the possibilities are more limited because of the retention of the amine nitrogen, and the intermediates of 2-pyridone ring formation during the biosynthesis of tenellin (1) may be more akin to the acyltetramic acids suggested by Vining.



L-phenylalanine (99) phenylpyruvate (95) phenyllactate (96)

Fig. 3.213. Phenylalanine, phenylpyruvate and phenyllactate are interconverted *in vivo*.

3.22 Ring expansion of an acyltetramic acid during tenellin biosynthesis.

Biosynthetic investigations into the tetramic acid family of natural products have revealed that the framework of the tetramic acid ring itself is derived from the amine nitrogen and carboxyl and α-carbons of an α-amino acid coupled to the two terminal carbons of a polyketide chain (Chapter 1, section 1.4). Thus the simplest member of the family, tenuazonic acid (14), is derived from isoleucine coupled to a C_4 diketide[27]. The more complex cytotoxin erythroskyrine (12) is derived from L-valine (91) with a polyunsaturated polyketide sidechain[28]. L-Phenylalanine (99) is observed as the intact amino acid moiety in pseurotin-A (16)[29] and the cryptotetramic acid cytochalasin-D (18) (Chapter 1, section 1.2).

The acyl tetramic acids occur in both fungi and bacteria and such precedent suggests a tetramic acid derived from L-phenylalanine and a pentaketide as a potential precursor to tenellin (1) in *B. bassiana*. A further set of enzymes would be required to modify such a tetramic acid to provide the 2-pyridone ring observed in tenellin (1).

Vining *et al.* suggested a ring expansion mechanism which incorporated oxidation of the aromatic ring of phenylalanine (99). The oxidation of the aryl nucleus affords a quinomethine structure, the re-aromatisation of which could provide some of the impetus for ring expansion (Chapter 1, section 1.4).

In order to ascertain the validity of this hypothesis an experiment was designed to test for the possible existance of a specific aryl *p*-hydroxylation activity in *B. bassiana*. Two shortchain acyl tetramic acids carrying in one case a benzylic group (131a), and in the other a *para*-hydroxyl benzylic (131b) moiety were synthesised (chapter 5, section 5.06) as model systems. It was hoped that (131a) would act as a substrate for the hydroxylation enzyme to give (131b). An aqueous solution of the sodium salt of 5(S)-3-acetyl-5-benzyl-2,4-dioxopyrrolidine (131a) was added to a fermentation broth of *B. bassiana* to a concentration of 1mM. After three days the fermentation broths were homogenised and filtered. The filtrate was acidified and extracted into diethyl ether, which was evaporated to provide a crude solution suitable for chromatographic analysis. Tlc analysis of this concentrated solution showed the presence of the exogenously added starting material (131a) in addition to tenellin (1) and other unidentified spots. One faint spot corresponded to an authentic sample of 3-acetyl-5-(*p*-hydroxybenzyl)-2,4-dioxopyrrolidine (131b). HPLC analysis suggested the presence of a very low amount of this *p*-hydroxylated material. The very low concentration in the extract of the

compound giving the peak that corresponded with the authentic *p* -hydroxylated material meant that further characterisation was not possible. An attempt was made to characterise other compounds present in the extract by separating them by preparative tlc, but the very low concentrations of these compounds precluded this.

To test for the possible intermediacy of an acyl tetramic acid during tenellin (1) biosynthesis directly, an acyl tetramic acid analogue of tenellin carrying a single ^{13}C label was synthesised (chapter 5, section 5.08). The highly polar nature of this acyl tetramic acid meant that purification by column chromatography, or recrystallisation, was not possible (chapter 5, section 5.08). However crude material added to cultures of *B. bassiana* was characterised first by ^{13}C nmr and mass spectrometry and shown to be free from traces of ^{13}C labelled phenylalanine or its derivatives. This crude acyl tetramic acid (139) was supplemented to a culture of *B. bassiana* to a concentration of 1.2mM. Tenellin (1) obtained from this culture after seven days, was examined by ^{13}C nmr which showed an enhancement of the resonance corresponding to C-4 (Fig. 3.221 and Fig. 3.222).

3.23 Discussion.

The results of the feeding experiments detailed above have shown that 3-amino-2-phenylpropionic acid (104) is not an intermediate during either tropic acid (42) or tenellin (1) biosynthesis. The labelled amino acid was taken-up by both the *D. innoxia* plants and *B. bassiana* fungal culture, but was not incorporated into either metabolite. No further evidence for possible intermediates to tropic acid (42) was gathered, but the shortening list of possible candidate molecules (cinnamate (89)[30], littorine[30], 1-carboxy-2-phenyloxirane[31], atropic acid (42a)[31] and 2-carboxyphenylacetaldehyde[31] have been shown not to be precursors to tropic acid (42)) means that phenyllactate (96), or phenylpyruvate (95), are becoming more likely direct precursors.

In the case of tenellin (1), it seems likely that an acyl tetramic acid is a precursor, however the evidence gathered to date is tentative. It remains possible that catabolism of the supplemented labelled tetramic acid (139) could afford [1-^{13}C]-phenylalanine, a known precursor to tenellin, *in vivo*. Further experiments using a doubly labelled acyl tetramic acid, with labels carried in both the amino acid and polyketide moieties, would show whether catabolism was an important factor in label incorporation. If the doubly labelled tetramic acid were a genuine precursor to tenellin (1) then the specific incorporation for each label should be identical.

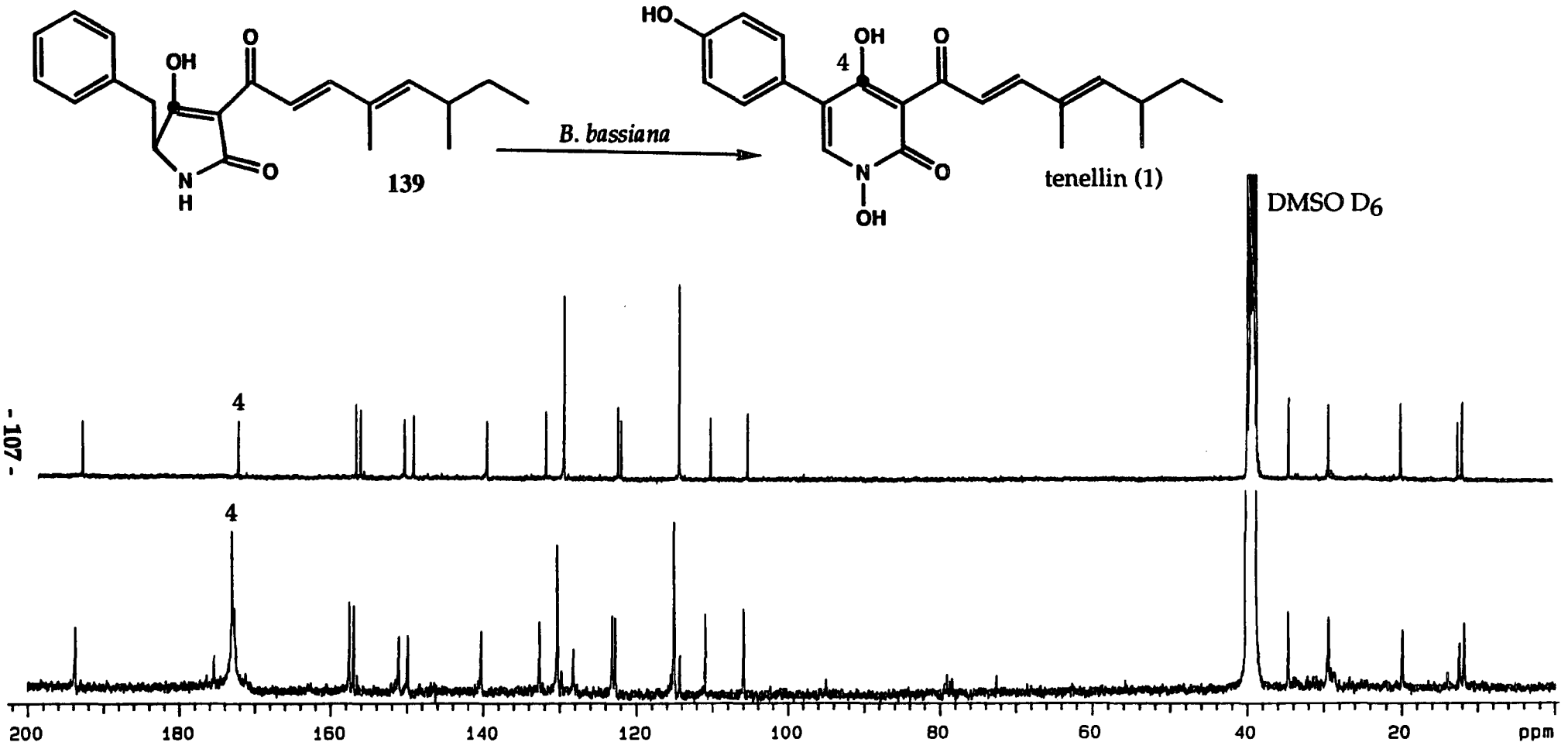


Fig. 3.221. ^{13}C nmr spectra of tenellin (a) unlabelled, (b) obtained from a culture of *B. bassiana* supplemented with the acyl tetramic acid (139).

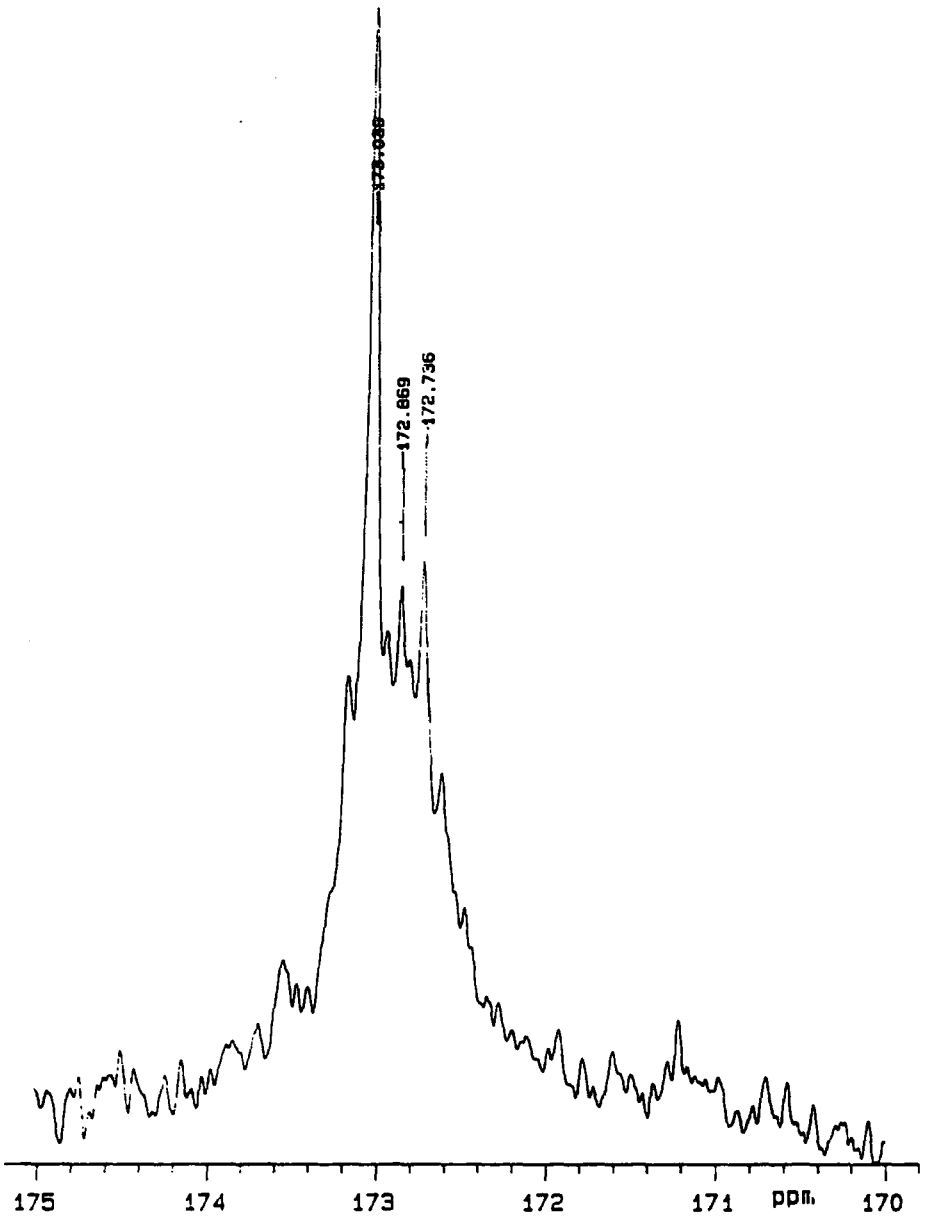


Fig. 3.222. Expansion of C-4 region of tenellin (1) obtained from *B. bassiana* supplemented with the acyl tetramic acid (139).

Vining suggested that the mechanism of tetramic acid ring expansion was coupled to oxidation of the phenyl ring, so that a quinomethine structure underwent rearrangement to form the observed six-membered pyridone ring of tenellin (1) (Chapter 1, section 1.4). This hypothesis, however, cannot be invoked to explain the origins of leporin-A (7). An alternative mechanism could rely on the generation a radical at the benzylic position of the tetramic acid, followed by ring closure of the homo-allylic system to form a fused cyclopropane. Homolytic bond fission and the abstraction of hydrogen would furnish the pyridone system. The radicals involved in this scenario are stabilised species.

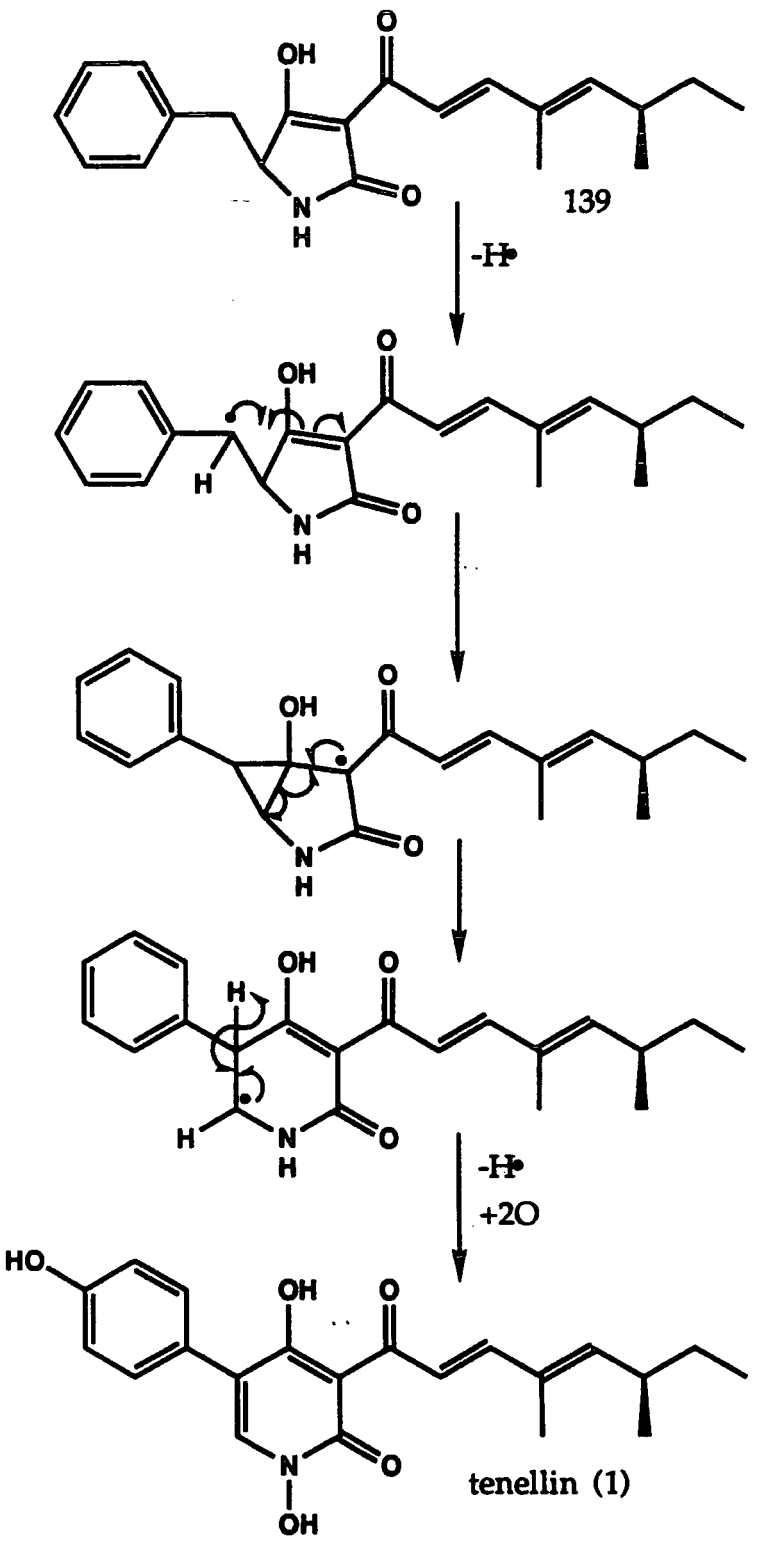


Fig. 3.231. Possible radical rearrangement of a benzyl tetramic acid to afford tenellin (1).

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Chapter 4.

The determination of the absolute configuration of tenellin.

4.1 The determination of the absolute configuration of tenellin (1).

Tenellin (1) possesses a single chiral centre at C-12 of the polyketide sidechain. Although the specific optical rotation of pure tenellin is known to be -44° (1% in acetone)[1], the absolute configuration at this position has not been determined. Two total syntheses of tenellin (1) have been achieved[2,3], but both were racemic. We therefore set out to determine the absolute configuration of tenellin (1) by a degradative procedure.

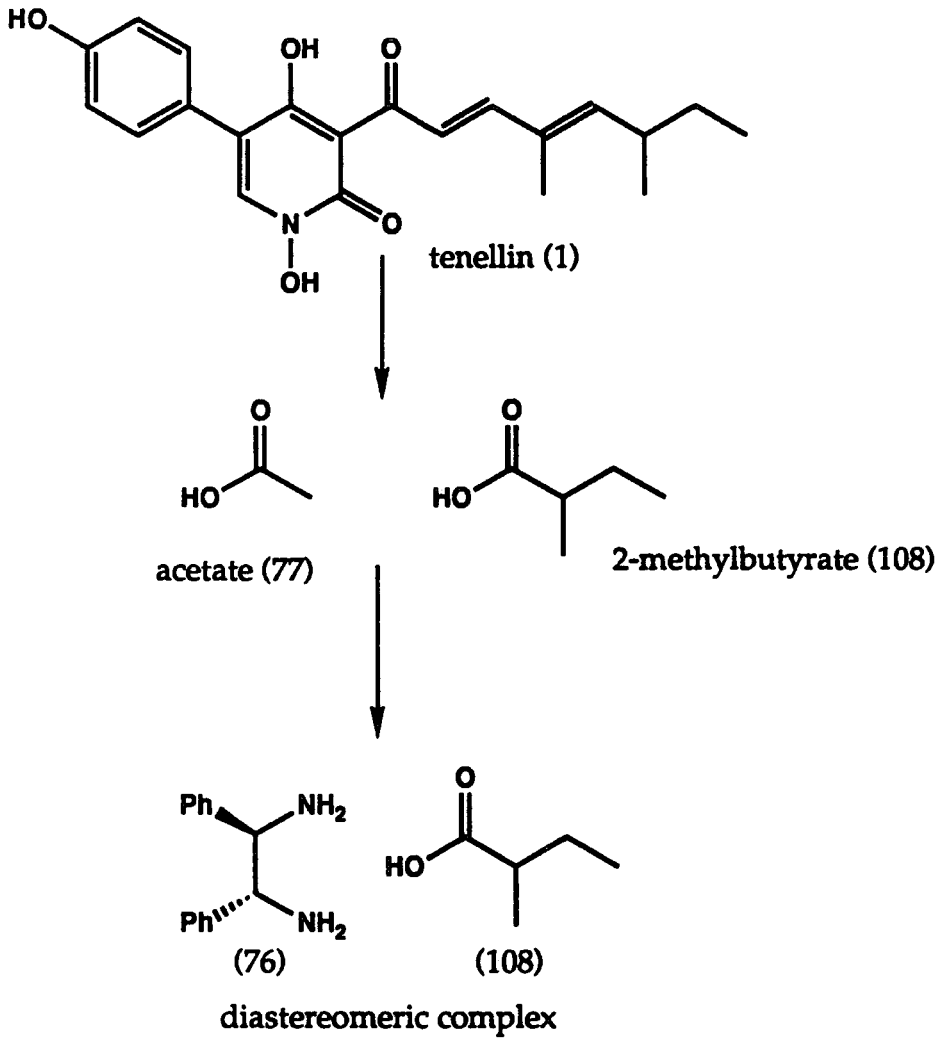


Fig. 4.101. Method for determining the absolute configuration of tenellin.

Crude tenellin (1), freshly obtained from approximately 1.5 litres of *B. bassiana* culture, was subjected to catalytic ruthenium tetroxide oxidation[4] using the improved method of Sharpless[5]. After three days of reaction when all colour had subsided, the free carboxylic acids were obtained as a mixture of their sodium salts. Acidification and lyophillisation afforded an aqueous solution containing only acetate (77) and 2-methylbutyrate (108) by ^{1}H and ^{13}C

nmr analysis. These acids were obtained as their sodium salts by freeze drying after the aqueous solution had been adjusted to pH8 with dilute NaOH.

The salts were treated with gaseous HCl under $CDCl_3$ and the resultant solution was used directly for nmr assay of the absolute stereochemistry of the 2-methylbutyrate (108). The method used was that of Parker[6] and is a direct way of determining the enantiomeric purity of α–substituted carboxylic acids, relying on the observation that chemical shift non-equivalence of the protons of chiral carboxylic acids can be induced when a diastereomeric salt complex is formed *in situ*. Thus the carboxylic acid solution obtained above was treated with (RR)-(-)-1,2-diphenyldiaminoethane (76), and the ^1H nmr spectrum compared with standard spectra of solutions containing the chiral diamine and (RS)- and (S)-2-methylbutyric acids respectively (Fig. 4.102).

The 400MHz ^1H nmr spectrum of (RS)-2-methylbutyric acid complexed with (RR)-(-)-1,2-diphenyldiaminoethane (76) in $CDCl_3$ (Fig. 4.102) showed a doubling of each proton resonance. The signals were most clearly resolved for the *C-5 methyl* protons, each enantiomer showing resolved doublets, however double resonances were also observed for other protons. The addition of (S)-(+)-2-methylbutyric acid increased the intensity of the upfield doublet. Thus the upfield doublet belongs to the *5-methyl* resonance of S-(+)-2-methylbutyric acid, whilst the downfield doublet belongs to (R)-(-)-2-methylbutyric acid.

The 2-methylbutyric acid obtained after the oxidation of tenellin (1) was similarly complexed with (RR)-(-)-1,2-diphenyldiaminoethane (76) and its 400MHz ^1H nmr spectrum obtained (Fig. 4.103). The *5-methyl* resonance was a single clean doublet, consistent with a single enantiomer (clearly no racemisation occurred during the oxidation process). The addition of a small amount of commercial racemic (RS)-2-methylbutyric acid resulted in the appearance of a new doublet upfield of that originally observed. The upfield doublet was previously assigned to (S)-(+)-2-methylbutyric acid and so the 2-methylbutyric acid obtained by the oxidation of tenellin (1) showed exclusively the R-configuration. Tenellin (1) obtained from *B. bassiana* is therefore. (R)-(-)-3-(*E,E* -4,6-dimethylocta-2,4-dienoyl)-1,4,-dihydroxy-5-(*p* -hydroxyphenyl)-2(1H)-pyridone (1a).

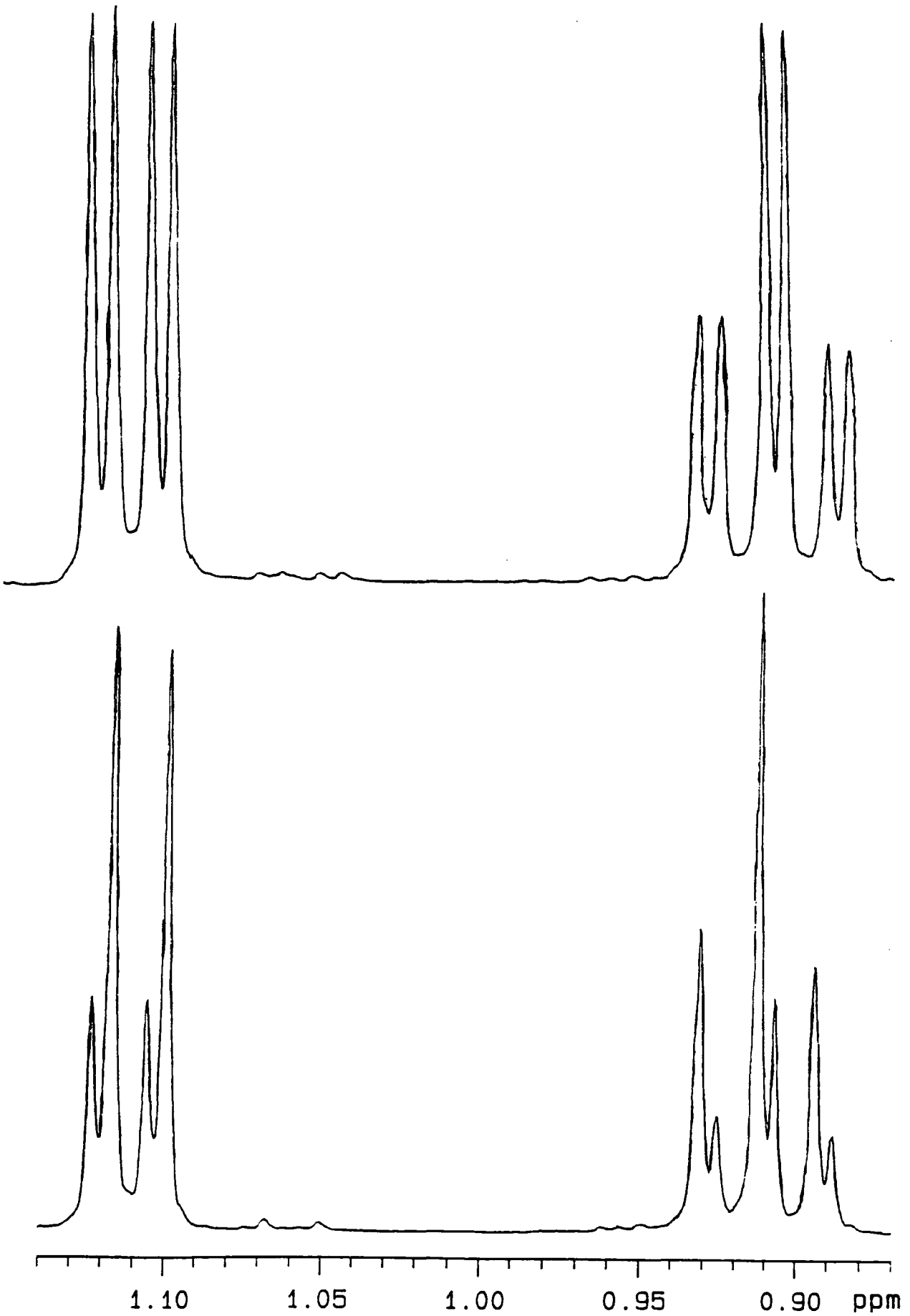


Fig. 4.102. Methyl region of 400MHz ^{1}H nmr spectra of 2-methylbutyrates complexed with (76). (a) (RS)-2-methylbutyric acid + (76), (b) 75% (S) + 25% (R) 2-methylbutyric acid + (76)

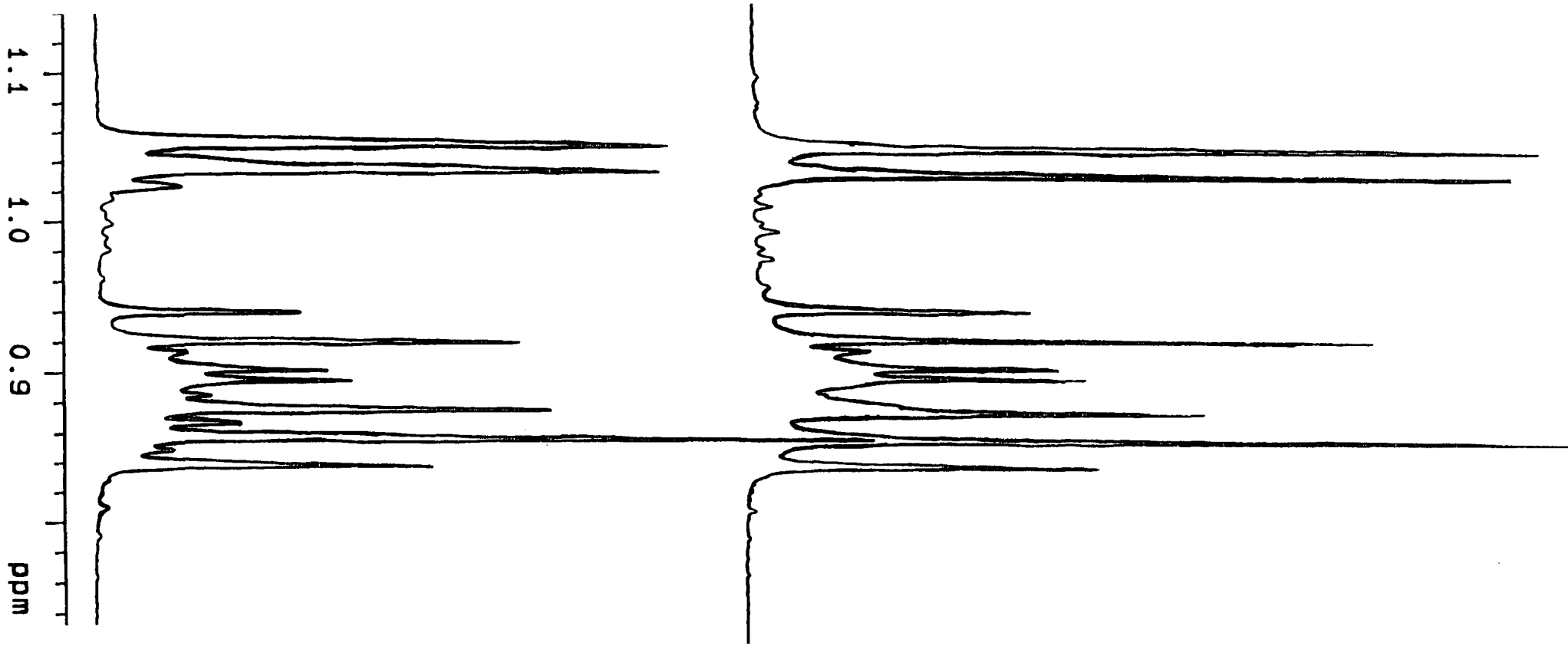


Fig. 4.103. Methyl region of 400MHz ^{1}H nmr spectra of 2-methylbutyrate, isolated from tenellin oxidation, complexed with (76).
(a) 2-methylbutyric acid from tenellin + (76) (contaminated with ether),
(b) as (a) + (RS)-2-methylbutyric acid.

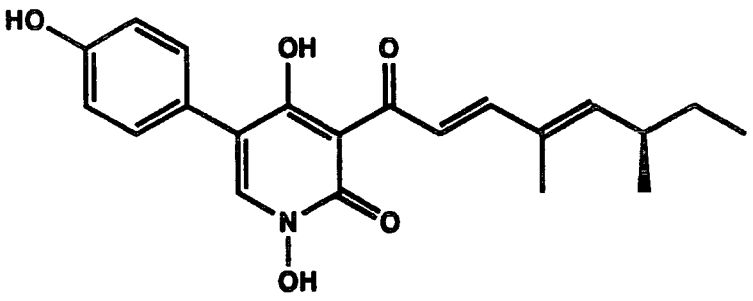
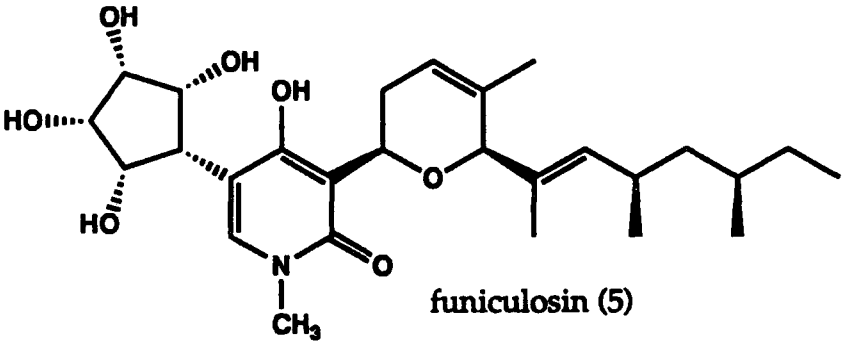


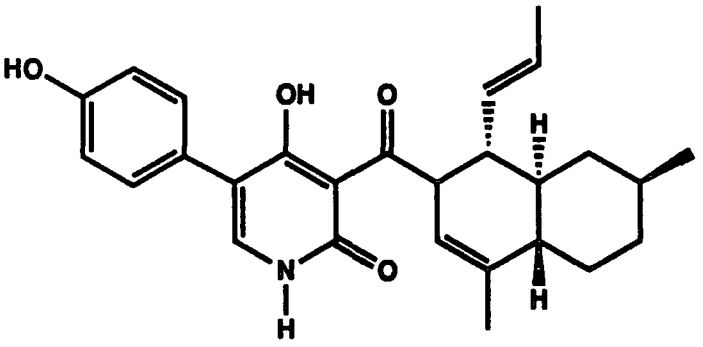
Fig. 4.104. The absolute configuration of tenellin (1a).

4.2 Discussion.

The R-configuration of tenellin (1) matches similar configurations of methylated positions in the other 2-pyridones funiculosin (5) and ilicicolin-H (6).



funiculosin (5)

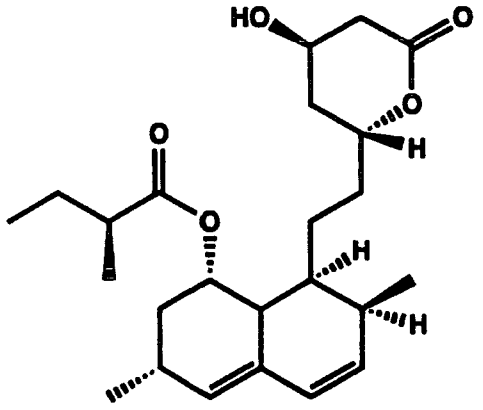


ilicicolin-H (6)

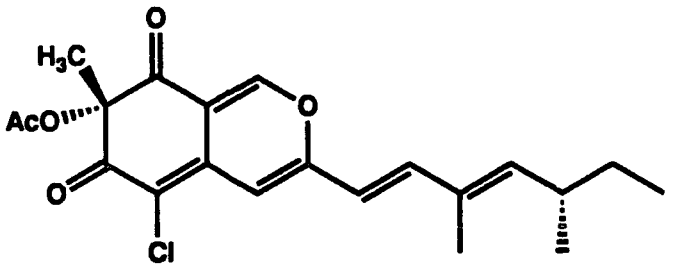
Fig. 4.201. Metabolites containing R-methylated centres.

The generation of R-configuration at methylated centres is not a general feature of fungal PKS systems, many metabolites are known to posess the (S) configuration. Notably, the *Penicillium sclerotiorum* metabolites[7], (+) and (-)-sclerotiorin (70a,b) possess a structurally identical triketide segment, differing from tenellin (1) only by virtue of the (S) configuration at the chiral centre[8]. The polyketide derived 2-methylbutyryl residue of mevinolin (69)[9] (*Aspergillus terreus*) also possesses the (S)-configuration, as does alternaric acid (71)[10], a

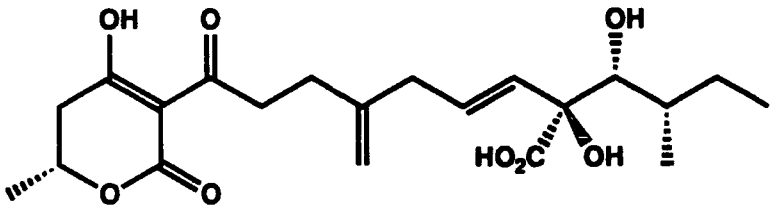
metabolite of the potato blight causing fungus *Alternaria solani,* and the acyl moiety of lunatoic acid-A (72)[11] (*Cochliobolus lunata*).



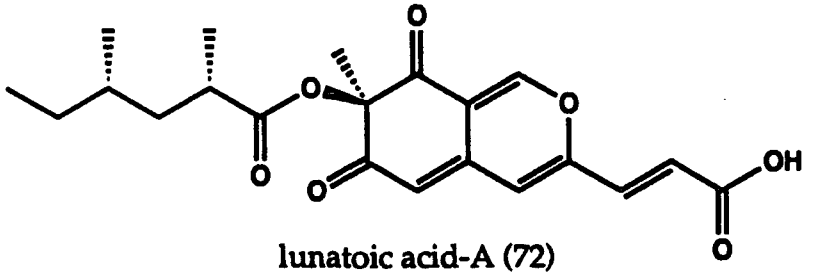
mevinolin (69)



(-) sclerotiorin (70a)



alternaric acid (71)



lunatoic acid-A (72)

Fig. 4.202. Metabolites containing S-methylated centres.

Making the assumption that methylations of the fungal polyketides occur at the β-keto stage, as seems likely (Chapter 2, section 2.52), then the chirality will be generated at the methylated centre by the action of an enoyl reductase (ER) activity of the PKS. The formation of an (R)-stereocentre is consistent with presentation of hydrogen to the *Si* -face of the α-carbon of the methyl-enoyl

intermediate (Fig. 4.203), whilst presentation of hydrogen to the *Re* face of the α-carbon will generate a methylated centre with the opposite configuration. The lack of stereochemical consistency in different fungal metabolites is evidence of variation of the various fungal enoyl reductases and reflects the situation for FAS where the selectivity of the enoyl reductases also varies between organisms. In fact all four possible stereochemical combinations of hydrogen addition to the enoyl double bond have been identified in different systems (chapter 2, section 2.2).

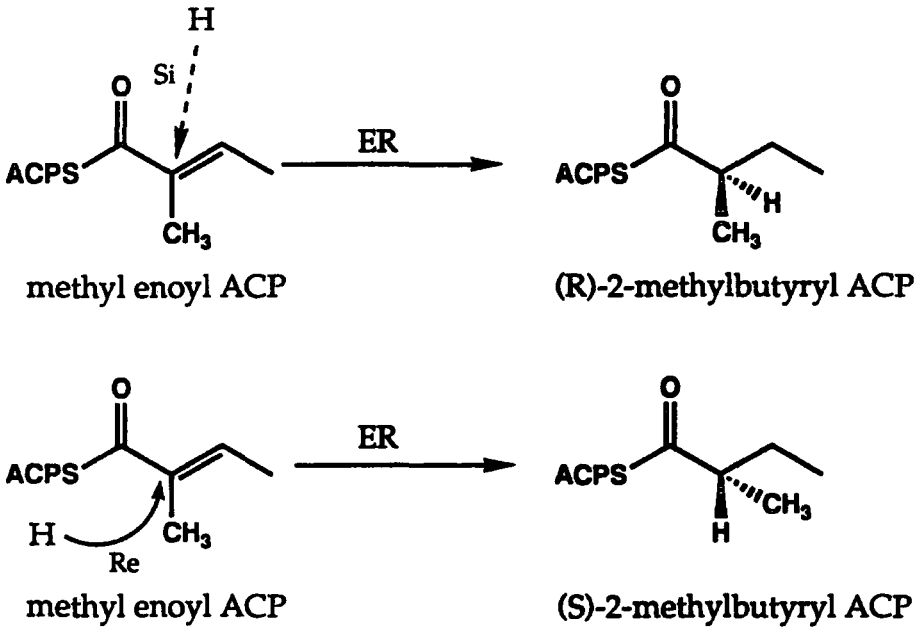


Fig. 4.203. Presentation of hydrogen to opposite faces of the α–methyl-enoyl ACP generates opposite absolute configurations at methylated centres.

Several investigations of fungal PKS enoyl reductases have been carried out, utilising the non-methylated fungal polyketides cladosporin[12] (*Cladosporium cladosporoides*), dehydrocurvularin[13] (*Alternaria cinerariae*), antibiotic A-26771B (*Penicillium turbatum*) and brefeldin-A[14] (*Penicillium brefeldianum*). These studies have only probed the stereospecificity at the α–carbon by determining the final location of deuterium from [2-^{13}C, 2-$^{2}H_3$]-acetate. In the four cases examined to-date the enoyl system is reduced by presentation of hydrogen to the Re face at the α–carbon as evinced by deuterium occupying the 2-*pro*-S position of the growing chain in all cases. Interestingly FAS from the same organisms processed the enoyl systems with the *opposite* absolute configuration, generating fatty acids where deuterium was incorporated into the 2-*pro* -R positions of the growing chain[12,13,14].

Clearly the stereochemical course of the enoyl reductases generating the chiral centre of tenellin (1) and the other R-methylated metabolites, is opposite

to that observed for these non-methylated systems. In contrast the enoyl reductase activities which generate the S-methylated positions of alternaric acid (71) and sclerotiorin (70), for example, have the same stereochemical course to those of the non methylated systems. In the case of FAS all four possible modes of hydrogen addition (Re-Re, Re-Si, Si-Si and Si-Re) to the double bond have been discovered (chapter 2, section 2.2). For the fungal secondary metabolites, however, the lack of information concerning the stereospecificity of the various enoyl reductases at the β--position precludes any deeper comparisons. It will be interesting to compare the modes of reduction of methylated and unmethylated enoyl systems by enoyl reductases within the same PKS, this could be done for instance in the cases of leporin-A (7) and ilicicolin-H (6) (chapter1, section 1.1), where methylated centres are present together with acetate C-2 derived methylene groups.

4.3 References.

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Chapter 5.

The synthesis of isotopically labelled compounds.

5.01 3-Amino-2-phenylpropionic acid (104 a, b, c).

5.02 Functionalised 2-methylbutyrates (109-112a, b, c, d).

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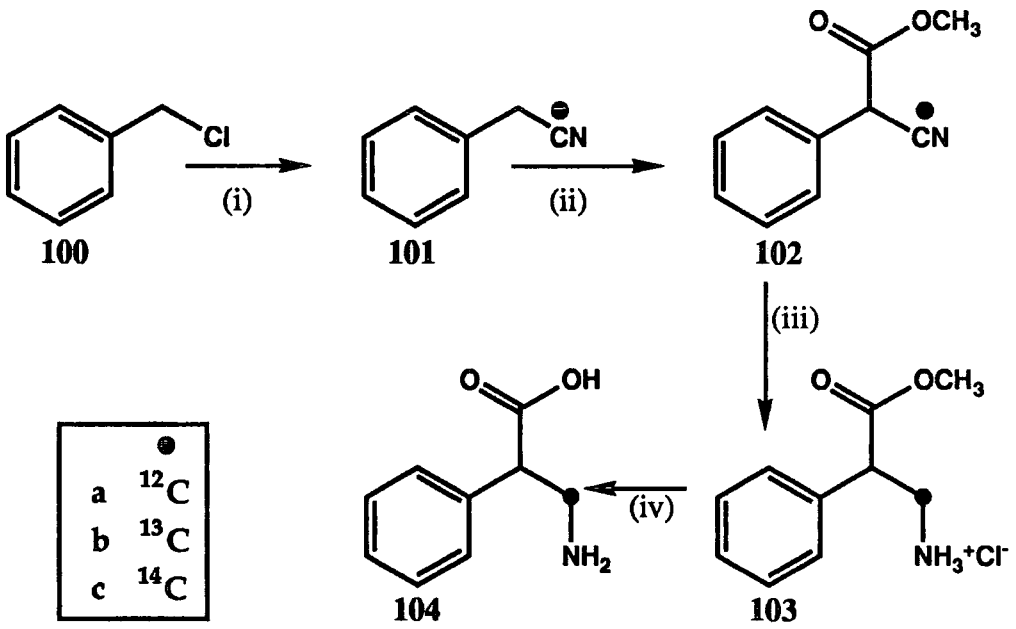
5.2 References.

5.01 The synthesis of 3-amino-2-phenylpropionic acids (104a, b, c).



(i) KCN, 18-Crown-6, 18h, RT. (ii) ^{t}BuLi, CO_2, CH_2N_2. (iii)H_2, PtO_2, EtOH/$CHCl_3$. (iv) KOH, DOWEX.

Scheme 5.01. The synthesis of 3-amino-2-phenylpropionic acid (104).

Potassium cyanide, labelled as either [1-^{13}C] or [1-^{14}C], was chosen as a convenient source of a carbon labelled C-N unit. The reaction of benzyl chloride (100) with one equivalent of potassium [1-^{13}C] or [1-^{14}C] cyanide proceeded smoothly in acetonitrile at room temperature, when a catalytic amount of the phase transfer agent 18-crown-6 was used, to produce [1-^{13}C] or [1-^{14}C] benzyl cyanide (101a,b) in quantitative yield. The reaction was significantly slower, and in fact did not run to completion even under prolonged reflux conditions, when benzyl bromide was utilised as the alkylating agent. These findings are in agreement with Liotta *et al.* who showed a reversal of the normally accepted order of reactivities for primary alkyl halides to nucleophilic displacement under similar conditions[1]. It was also observed that the specific activity of ^{14}C labelled 3-amino-2-phenylpropionic acid (104c) obtained *via* this route was 16% less than that of the [^{14}C]-KCN used as a starting material, this fact can be explained if a low level of cyanide exchange occurs between the solvent and the labelled reagent.

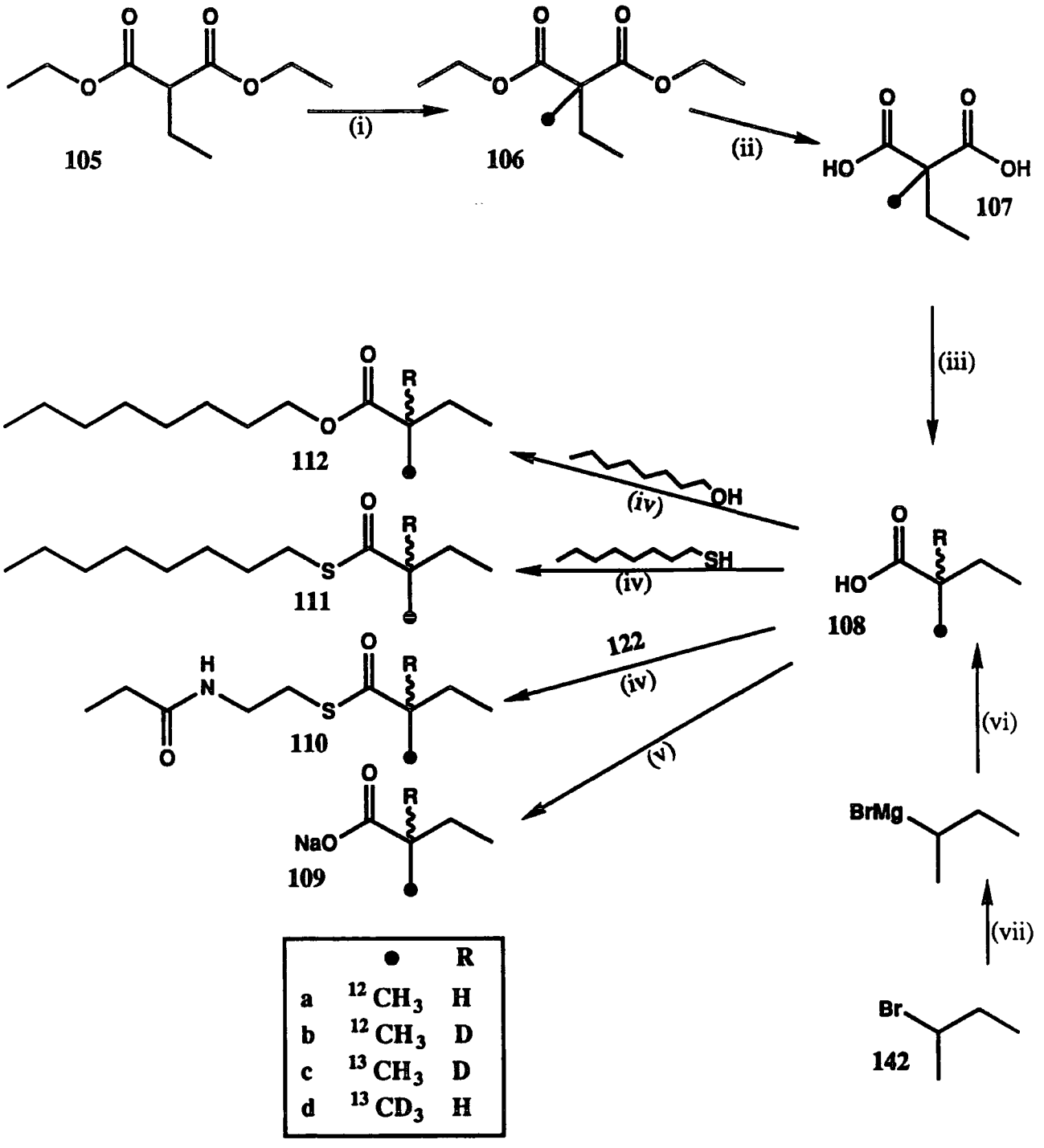
Benzyl cyanide (101a-c) was carboxylated in moderate yield by treatment with one equivalent of ^{t}butyl lithium, followed by quenching with a suspension of solid carbon dioxide in diethyl ether. The carboxylate formed could be isolated as either the lithium salt or the free carboxylic acid, but was usually acidified and treated with an excess of an ethereal solution of diazomethane[2], *in*

situ, to afford methyl cyanophenylacetate (102a-c) directly. Methylation was performed for two reasons, firstly to prevent the slow decomposition of phenylcyanoacetic acid back to carbon dioxide and benzyl cyanide, and also to protect the carboxylate group in order to prevent side reactions and salt formation in the next synthetic step.

Catalytic hydrogenation[3] was found to be the preferred method for converting the nitrile to an amine functionality, it being superior to the use of borane reagents (*i.e.* diborane[4] and trifluoroacetoxy borohydride[5]) in respect of convenience and yield. Methyl phenylcyanoacetate (102a-c) was hydrogenated under an atmosphere of hydrogen (3atm) using Adam's catalyst (PtO_2) in ethanol. The addition of 10% chloroform to the reaction solvent conveniently ensured the *in situ* formation of the amine hydrochloride salt (103), and prevented side reactions between the formed amine and reaction intermediates. After the catalyst was removed by filtration, methyl 3-amino-2-phenylpropionate hydrochloride (103a-c) was furnished, by evaporation of solvent, as a pure white crystalline solid, in excellent yield. Recovered catalyst was found to be equally active in further hydrogenations. The use of concentrated aqueous HCl as an *in situ* amine trap was also investigated, but catalysis of trans-esterification between the substrate and the reaction solvent, and generally poor yields of mixed ester products meant that chloroform was preferred in this rôle.

Basic ester hydrolysis of the amine hydrochloride salt, followed by ion exchange chromatography, utilising Dowex 50X-8 resin, afforded the labelled amino acids (104a-c) in good yield as amorphous cream solids. One crystallisation from ethanol/water yielded [3-^{13}C] and [3-^{14}C] 3-amino-2-phenylpropionic acids (104b,c) as pure white crystals.

5.02 The synthesis of functionalised 2-methylbutyrates (109-112a, b, c, d).



	●	R
a	$^{12}CH_3$	H
b	$^{12}CH_3$	D
c	$^{13}CH_3$	D
d	$^{13}CD_3$	H

(i) NaH, MeI. (ii) KOH. (iii) Δ, H_2O or D_2O. (iv) DCC, DMAP. (v) NaOH. (vi) CO_2, H_3O^+. (vii) Mg.

Scheme 5.02. The synthesis of functionalised 2-methylbutyrates (109-112).

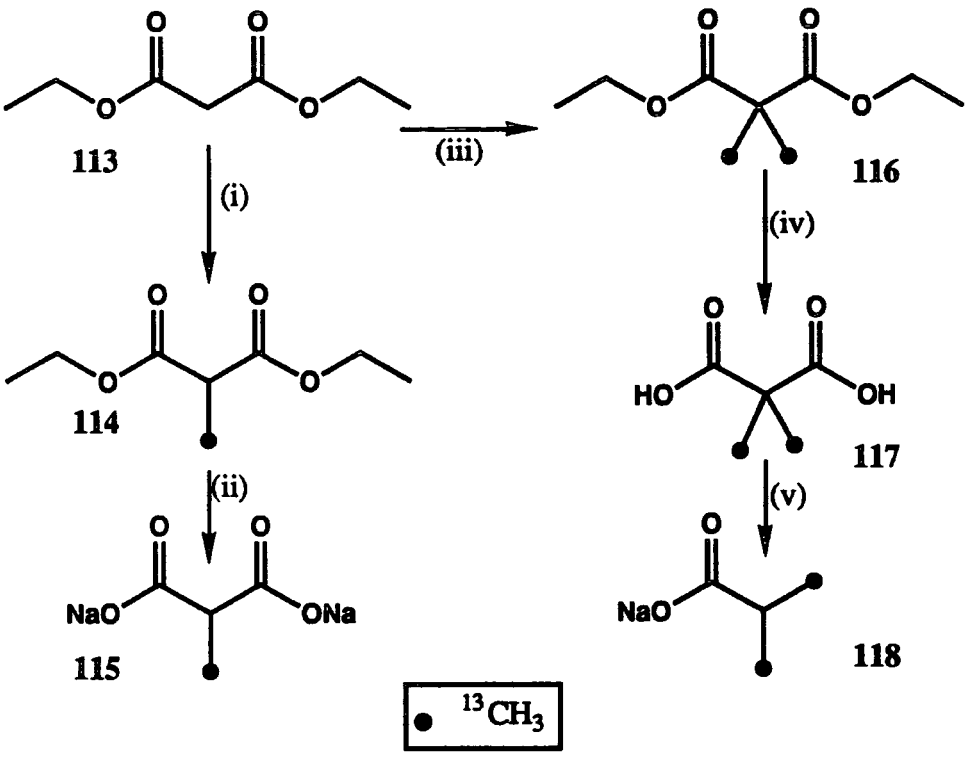
At the outset Grignard methodology was used to access labelled 2-methylbutyrates (108a,b). It was envisaged that [^{13}C]-carbon dioxide, generated by the action of concentrated acid on [^{13}C]-barium carbonate, would provide the source of label. 2-Butyl magnesium bromide was generated from 2-bromobutane (142) and reacted with an ethereal suspension of solid carbon dioxide. Acidification afforded 2-methylbutyric acid (108a) in low yield. The route was rejected due to the large excess of carbon dioxide, the source of isotopic label, required. Additionally it was found that the introduction of deuterium at the 2-position into 2-methylbutyric acid (108a) was not straightforward using this route. Esterification followed by base treatment, D_2O quench, and ester hydrolysis was not an efficient process, and the method of Atkinson *et al.* [6] involving five 24hr cycles of high pressure treatments in D_2O at 180°C was also potentially low yielding.

In an alternative, and ultimately more successful, strategy the required acids were generated by the decarboxylation of functionalised malonates[7]. Diethyl ethylmalonate (105) was alkylated by treatment with one equivalent of sodium hydride and one equivalent of appropriately labelled methyl iodide to afford labelled diethyl 2-ethyl-2-methylmalonates (106a,c,d) in high yield. Basic ester hydrolysis gave 2-ethyl-2-methylmalonic acids (107a,c,d) in good yield. Decarboxylation of the malonic acids was achieved, again in near quantitative yield, by heating aqueous solutions of the labelled acids to 180°C for 3hr in an evacuated Carius tube. High (>95%, as evinced by observation of α and β shifts in ^{13}C nmr spectra) deuterium incorporations at the α–position of the formed 2-methylbutyric acids (108b,c) could be achieved by carrying out the decarboxylation reactions in D_2O.

Thiolesterification and esterification of the 2-methylbutyric acids was achieved using DCC coupling methodology[8,9]. N-propionylcysteamine thiolesters were formed by the reaction of one equivalent of acid (108a-d), two equivalents of thiol (122), and one equivalent each of DCC (dicyclohexylcarbodiimide) and DMAP (4-dimethylaminopyridine) in diethyl ether at room temperature overnight. The thiolesters were purified and obtained in good yield by flash chromatography eluting with ether. The materials were stable when stored at 0°C under N_2, but decomposed somewhat in air. The octyl ester (112d) and thiolester (111d) were prepared in a similar fashion. The octyl-thiolester (111d) was seperable from 1-octane thiol by flash chromatography eluting with methylene dichloride:hexane/25:75, but the octyl-ester (112d) was not separable from octanol, and a mixture of the two compounds was isolated. Sodium [5-*methyl* -$^{13}C^2H_3$]-2-methylbutyrate (109d) was prepared by the combination and base hydrolysis of various [5-*methyl* -$^{13}C^2H_3$]-2-methylbutyrates (110d, 111d, 112d) obtained from fermentation

broths of *B. bassiana* at the end of unsuccessful feeding experiments.

5.03 The synthesis of methylmalonate (115) and isobutyrate (118).



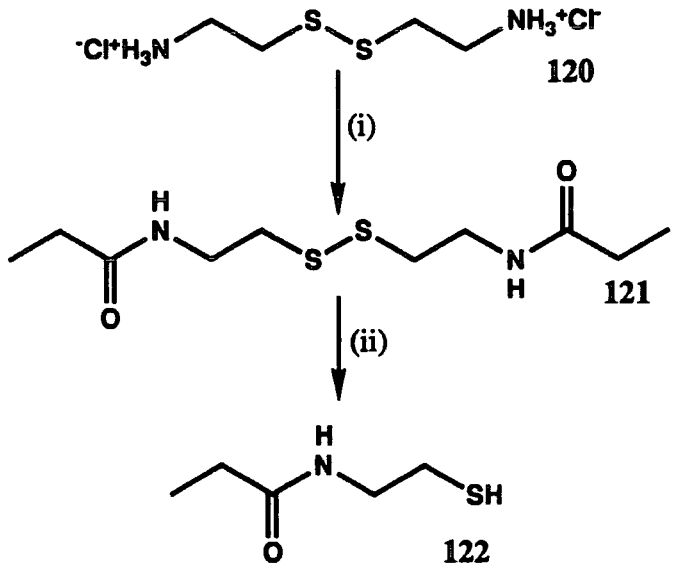
(i) NaH, MeI. (ii) NaOH. (iii) 2NaH, 2MeI. (iv) NaOH, H_3O^+. (v) Δ, NaOH.

Scheme 5.03. The synthesis of sodium methylmalonate (115) and sodium isobutyrate (118).

The alkylation of diethyl malonate was exploited in the synthesis of both disodium [*methyl*-^{13}C]-methylmalonate (115) and sodium [3,3'-$^{13}C_2$]-isobutyrate (118). Diethyl [*methyl* -^{13}C]-methylmalonate was synthesised by adding a small excess of diethylmalonate to one equivalent of sodium hydride, before the addition of one equivalent of [^{13}C]-methyliodide. Heating under reflux afforded the singly methylated diethyl [3-*methyl* -^{13}C]-methylmalonate (114) in high yield, which was hydrolysed in aqueous base to furnish sodium [3-^{13}C]-methylmalonate (115).

Similar methodology was employed in the synthesis of sodium [3,3'-$^{13}C_2$]-isobutyrate. One equivalent of diethylmalonate (113) was treated with three eqivalents of sodium hydride, and two eqivalents of [^{13}C]-methyl iodide, and heated under reflux to afford diethyl [3,3'-$^{13}C_2$]-2,2-dimethylmalonate (116) in good yield. Aqueous base hydrolysis afforded [3,3'-$^{13}C_2$]-2,2-dimethylmalonic acid (117) in near quantitative yield, and decarboxylation by heat treatment at elevated pressure for 3 hours gave [3,3'-$^{13}C_2$]-isobutyric acid, which was purified by lyophillisation and isolated in high yield as its sodium salt (118), after neutralisation.

5.04 The synthesis of N-propionylcysteamine (122).



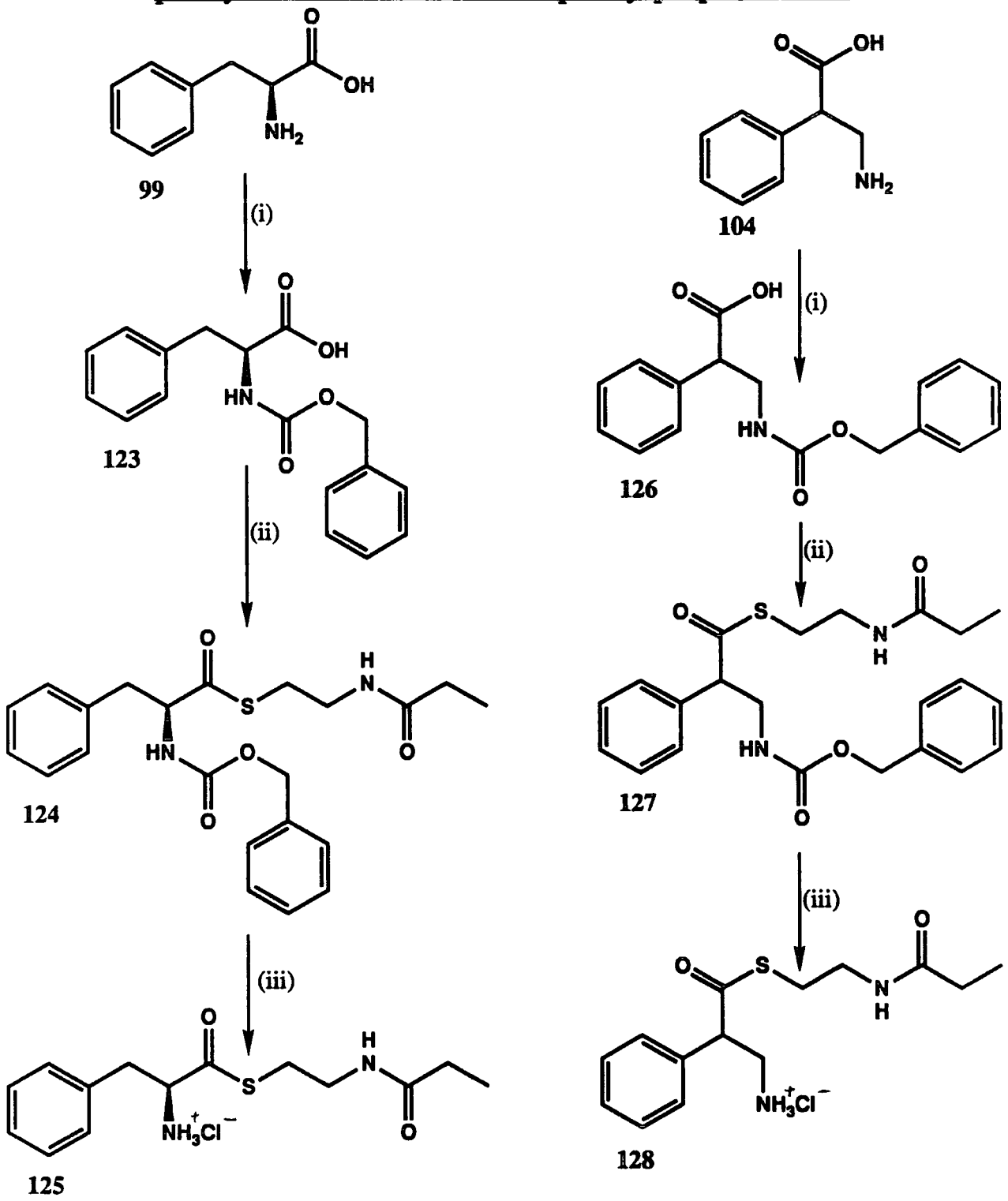
(i) CH_3CH_2COCl, KOH. (ii) Na/Hg 3%, RT 90min, H^+.

Scheme 5.04, The synthesis of N-propionylcysteamine (122).

Acylation of cystamine dihydrochloride (120) was achieved in high yield by the treatment of the free amine in aqueous basic solution with two equivalents of propionyl chloride to afford N,N'-dipropionylcystamine (121). The dipropionyl compound was a much more tractable material than the diacetyl analogue, which was insoluble in most solvents and only poorly soluble in methanol.

Reduction of the disulfide bond of (121) could be performed by two methods. Reaction with finely divided zinc in refluxing methanolic tetrahydofuran afforded N-propionylcysteamine (122) after 18 hours in good yield, but reaction with 3% sodium amalgam (119)[10], in methanol at room temperature, afforded the same compound in high yield after 90min, and was the preferred method. The free thiol (122) was prepared immediately prior to use, from the more stable disulfide (121), as significant degradation of the thiol (122) was observed over the period of several hours under ordinary conditions.

5.05 The synthesis of N-propionylcysteamyl thiolesters of L-phenylalanine and 3-amino-2-phenylpropionic acid.



a	unlabelled.
b	[UL-^{14}C] - phenylalanine.

(i) CBZCl, NaOH. (ii) (122), DCC, DMAP. (iii) BBr_3, H_3O^+.

Scheme 5.05. The synthesis of N-propionylcysteamyl thiolesters of L-phenylalanine (125) and 3-amino-2-phenylpropionic acid (128).

The synthesis of the N-propionylcysteamine thiolester of 3-amino-2-phenylpropionic acid (128) was initially attempted by a modification of the synthetic route leading to the amino acid (104) itself. Thus benzyl cyanide (101), generated from benzyl chloride (100), was carboxylated to afford phenylcyanoacetic acid. Attempted coupling of this acid with N-propionylcysteamine (122) using DCC coupling methodology was unsuccessful. The addition of DCC to an ethereal solution of phenylcyanoacetic acid led to rapid decarboxylation, as evinced by effervescence, and the formation of benzyl cyanide (101) in quantitative yield (see fig 5.051. for proposed mechanism).

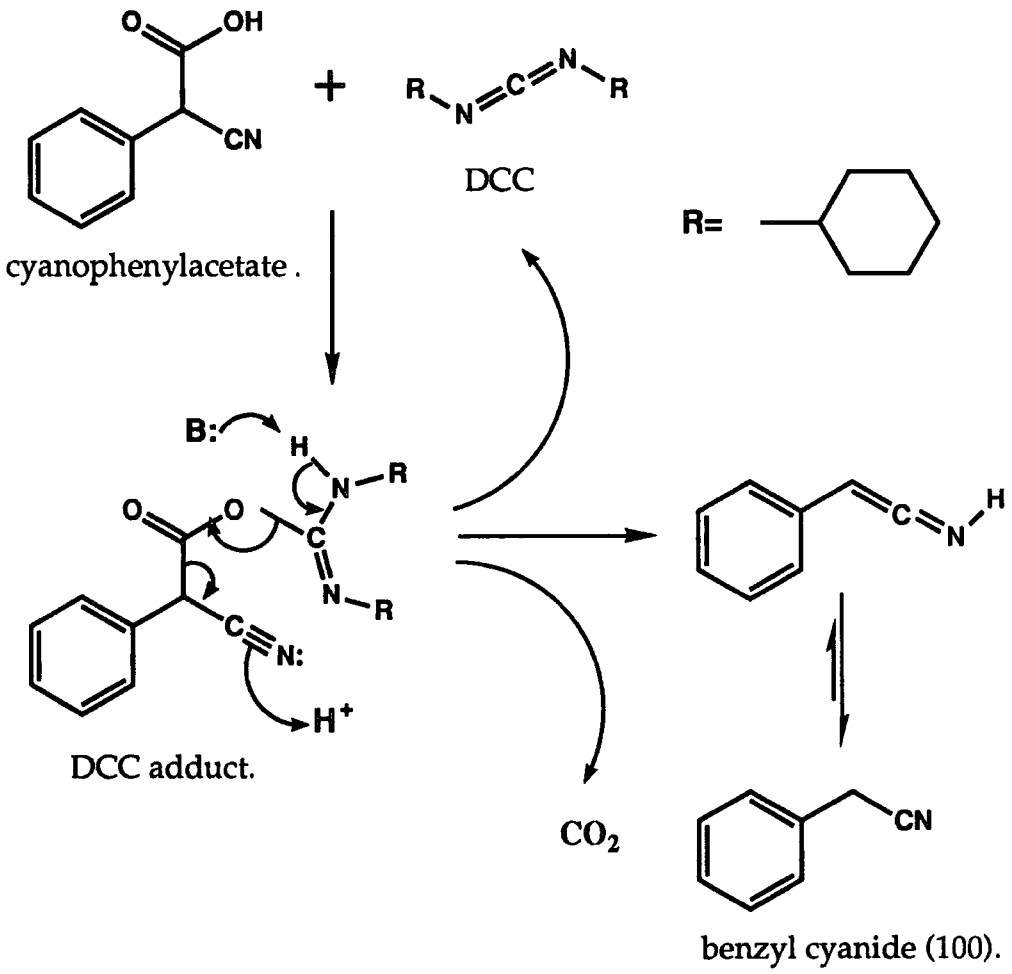


Fig. 5.051. Proposed mechanism for the decarboxylation of cyanophenylacetate by DCC.

The inability to synthesise this key intermediate led to the consideration of a second strategy based on the formation of the thiolester by reaction with the amino acid itself. Because of the insolubility of the amino acid in ether, and the possibility of oligormerisations of the amino acid under coupling conditions, an N-protection/deprotection protocol was devised. In order to circumvent the

synthesis of 3-amino-2-phenylpropionic acid (104) in bulk from scratch, the reaction sequence was developed using commercially available L-phenylalanine and [UL-^{14}C]-L-phenylalanine, and applied to generate the target molecule after optimisation.

Thus L-phenylalanine (99), [UL-^{14}C]-L-phenylalanine (99) and 3-amino-2-phenylpropionic acid (104) were protected with the benzyloxycarbonyl (CBZ) group, in high yields, by reaction with a small excess of benzyloxycarbonyl chloride (CBZCl) in dilute aqueous base[11]. CBZ was chosen as the protecting group because of its ease of removal under neutral hydrogenolysis conditions. The N-protected amino acids were then successfully converted to their N-propionylcysteamine thiolesters (124a, 124b, 127), in good yields, by treatment with an excess of N-propionylcysteamine (122) and one equivalent each of DCC and DMAP in ether at room temperature overnight. The N-protected amino acid thiolesters were purified by flash chromatography over silica eluting with acetonitrile.

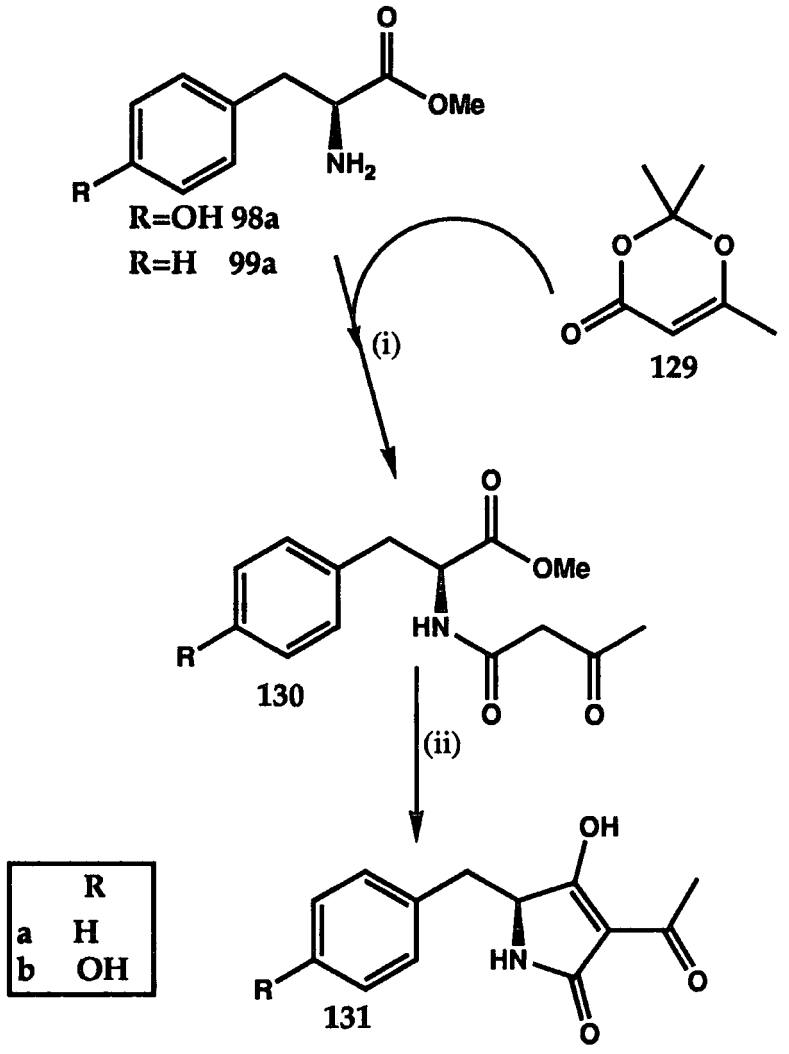
Deprotection of the amine proved to be a more formidable task than was originally hoped. Three hydrogenolysis methods were attempted. The standard method[11] of benzyloxycarbonyl cleavage under hydrogen (3atm), utilising 10% palladium on charcoal as a catalyst, failed to remove the protecting group even on prolonged reaction time and increased catalyst concentration.

Two methods of transfer hydrogenolysis were also investigated. Firstly the method of Felix[12], utilising palladium on charcoal as the catalyst and 1,4 cyclohexadiene as the hydrogen source in ethanol, was attempted at varying diene and catalyst concentrations. Secondly the use of formic acid as the hydrogen source was assessed[13]. Neither protocol resulted in even partial deprotection of the amine group. The three hydrogenolysis methods were also tried utilising platinum dioxide (Adam's catalyst) but without success. Acid promoted cleavage with concentrated aqueous HCl[14] or trifluoroacetic[15] acid in organic solvents was similarly unsuccessful.

Benzyloxycarbonyl cleavage was finally achieved by using a solution of boron tribromide in dichloromethane[16]. Thus solutions of the protected amino acid thiolesters (124a, 124b, 127) were treated with five equivalents of boron tribromide, giving rise to a yellow solid precipitate. After the addition of water to the reaction mixture, breaking up this solid with effervescence, the aqueous solution contained the hydrobromide salt of the aminoacid thiol ester, whilst the organic layer contained benzyl bromide. The free amine was isolated after basification of the aqueous solution and extraction into organic solvent. Prolonged standing of a chloroform solution containing the free amine thiolester resulted in the precipitation of a white insoluble material, presumably an oligomer or polymer of the amino acid. The amino acid thiolesters were

therefore isolated as their hydrochloride salts (125a, 125b, 128), materials which were stable to storage and which had the added advantage of being water soluble.

5.06 The synthesis of short-chain acyl tetramic acids (131a, b).



(i) Acetone diketene adduct (129), toluene, Δ. (ii) NaOMe/MeOH.
Scheme 5.06. The synthesis of acyl tetramic acids (131a,b).

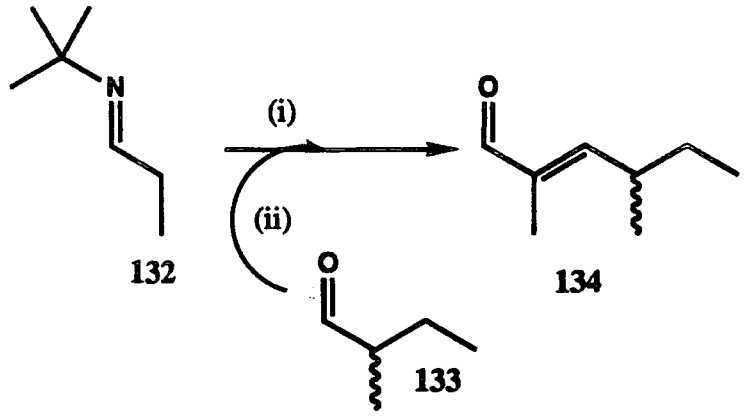
L-Phenylalanine (99a) and L-tyrosine (98a) methyl esters were conveniently acetoacetylated by heating a toluene solution of the amino ester with one equivalent of the masked diketene (129) at reflux temperature. Heat treatment above 100°C decomposes the acetone diketene adduct (129) to afford diketene *in situ*, which rapidly reacts with the amine nucleophile[17]. Acetone was observed as a gaseous by-product. This procedure conveniantly obviates the need to use the toxic diketene itself which has been used[18] in the past to generate similar compounds. A high yield of the phenylalanine methyl ester β–ketoamide (130a) was obtained after purification by flash chromatography. The tyrosine methyl ester analogue (130b) was not so easily purified and was used in crude form.

Dieckmann cyclisations were effected by heating a methanolic solution of

the amino ester amides (130a,b) with a small excess of sodium methoxide for two hours under reflux. A moderate yield of 5(S)-3-acetyl-5-benzyl-2,4-dioxopyrrolidine (131a) was obtained after recrystallisation. Much poorer yields of 3-acetyl-5-(*p*-hydroxybenzyl)-2,4-dioxopyrrolidine (131b) were obtained after recrystallisation, this was probably due to competing O-acetoacetylation in the first step of the synthetic sequence.

The benzyl substituted acyl tetramic acid (131a) was observed to retain stereochemical integrity at C-5, whereas the *p* -hydroxybenzyl analogue (131b) was obtained in racemic form. This centre is evidently more susceptible to epimerisation under the basic ring closing conditions. Epimerisation at this position has been previously reported and is a general feature of base promoted ring closure in these systems[19].

5.07 The synthesis of 2,4-dimethylhex-2-enal (134).



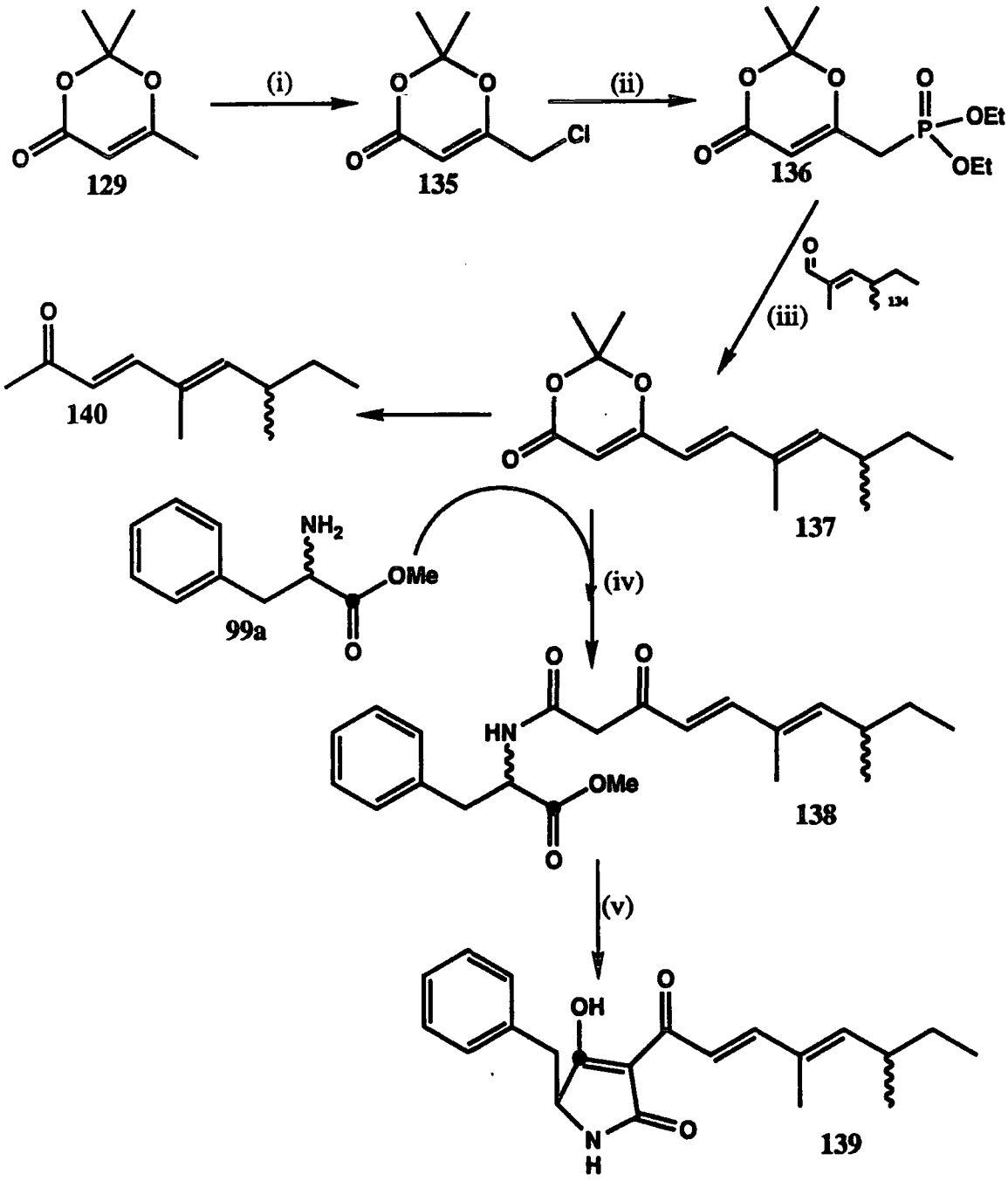
(i) BuLi, -78°C. (ii) (133), -78° C to 0°C, H_3O^+.

Scheme 5.07. The synthesis of E-2,4-dimethylhex-2-enal (134).

The directed aldol condensation method of Wittig[20] was used to synthesise the E-unsaturated aldehyde (134). The condensation between ^{t}butylamine and propionaldehyde afforded (1,1-dimethyl-N-propylidene)ethylamine (132)[21] after separation of the reaction mixture by standing over potassium hydroxide pellets at 0°C overnight. The crude material had to be distilled three times from potassium hydroxide before pure, dry imine could be obtained. The compound was unstable over a matter of weeks when stored at 0°C under dry nitrogen, and was routinely distilled from potassium hydroxide under dry nitrogen prior to use.

Treatment of the imine (132) with butyllithium at -78°C afforded the lithiated species which was condensed with one equivalent of freshly distilled 2-methylbutyraldehyde (133). The lithiated imino alcohol thus formed was decomposed to E-2,4-dimethyl-hex-2-enal (134)[22] by treatment with an excess of an aqueous slurrey of oxalic acid. The aldehyde was unstable, and did not store for more than a few days at -20°C, it was therefore prepared and purified, by flash chromatography, immediately prior to usage.

5.08 The synthesis of an acyl tetramic acid analogue of tenellin (1).



(i) LDA, -78°C, C_2Cl_6, -50°C (ii) $(EtO)_2P(O)^-$, H_3O^+. (iii) LHMDS, (134).
(iv) (98), toluene, Δ. (v) $^tBuO^-$, ^tBuOH.

Scheme 5.08. The synthesis of tenellin tetramic acid analogue (139).

The protocol developed for the synthesis of the short-chain acyl tetramic acids discussed above was modified in order to be able to synthesise the putative tetramic acid (139) intermediate in tenellin (1) biosynthesis.

Treatment of freshly distilled acetone-diketene adduct (129) with one equivalent of LDA at -78°C yielded the lithiated species as a yellow suspension

in THF. This material could be chlorinated[23] in high yield if the suspension, cooled to -78°C, was added dropwise to a solution of hexachloroethane in tetrahydrofuran, maintained at -50°C. When addition was complete the hexachloroethane solution was allowed to warm to -20°C. Quenching of the reaction mixture with ice-cold dilute hydrochloric acid and extraction into the organic phase led to the isolation of 2,2-dimethyl-6-chloromethyl-1,3-dioxin-4-one (135). Poorer yields were obtained if the hexachloroethane solution was added to the solution of the lithiated 2,2,6-trimethyl-1,3-dioxin-4-one. The chlorinated acetone-diketene adduct (135) could be purified by flash chromatography, but was routinely adequately purified by trituration with hexane, to remove excess hexachloroethane prior to the formation of phosphonate (136).

Preparation of the Wadsworth-Emmons reagent (136) proceeded smoothly when the chlorinated acetone-diketene adduct (135) was treated with a solution of potassium diethylphosphite in dimethylformamide. Acidification of the reaction solution and removal of excess solvent, and diethylphosphite, by distillation under diminished pressure afforded 2,2-dimethyl-6-(diethylphosphonomethyl)-1,3-dioxin-4-one (136) in good yield. The crude material was conveniently stored at 0°C and purified by flash chromatography immediately prior to use.

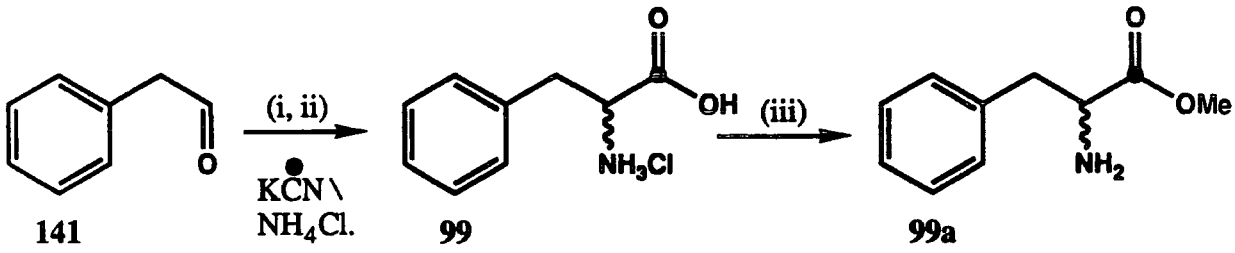
Wittig olefination of the purified phosphonate to form the all E triene (137) proceeded in moderate yield[24]. The phosphonate (136) was treated with 1 equivalent of lithium hexamethyldisilazide (LHMDS), forming the anion as a deep blood red solution in tetrahydrofuran at 0°C. The addition of one equivalent of freshly prepared and purified 2,4-dimethylhex-2-enal (134), at -78°C, afforded the key intermediate, triene (137), after purification by flash chromatography. LHMDS[25] was found to be a more effective base for this reaction than either LDA or butyllithium. Sodium hydride gave very poor yields of triene (137) as the reflux temperatures required for full deprotonation of the phosphonate presumably caused significant decomposition of the protected diketene moiety.

In one experiment where the triene (137), was purified using activated silica the decarboxylation product (140) was isolated. Apart from this one instance, the triene (137) was found to be stable to storage under nitrogen at 0°C.

Treatment of unlabelled L-phenylalanine or [1-^{13}C]-DL-phenylalanine methyl esters (99a) with one equivalent of the protected ketene (137) in refluxing toluene afforded the corresponding β-keto amides (138a,b) after purification by flash chromatography in high yield. Treatment of these amides with sodium methoxide in methanol resulted in large-scale decomposition,

affording the methyl ester of phenylalanine as the only identifiable by-product. Deickmann cyclisation was achieved by utilising freshly sublimed potassium ^{t}butoxide as the base in ^{t}butanol as the solvent[26]. The highly polar acyl tetramic acid was an orange waxy oil which could not be purified by chromatography over silica or by recrystallisation. The recovered material was not fully characterised due to this difficulty in purification (similarly poor characterisations of acy tetramic acids have been reported in the literature, however[25,26]). The recovered material was shown to be free from amide starting material, and more importantly free from phenylalanine, by ^{13}C nmr and mass spectroscopy. The material was thus confidently used in a feeding experiment with *B. bassiana*.

5.09 The synthesis of phenylalanine methyl ester (99a).



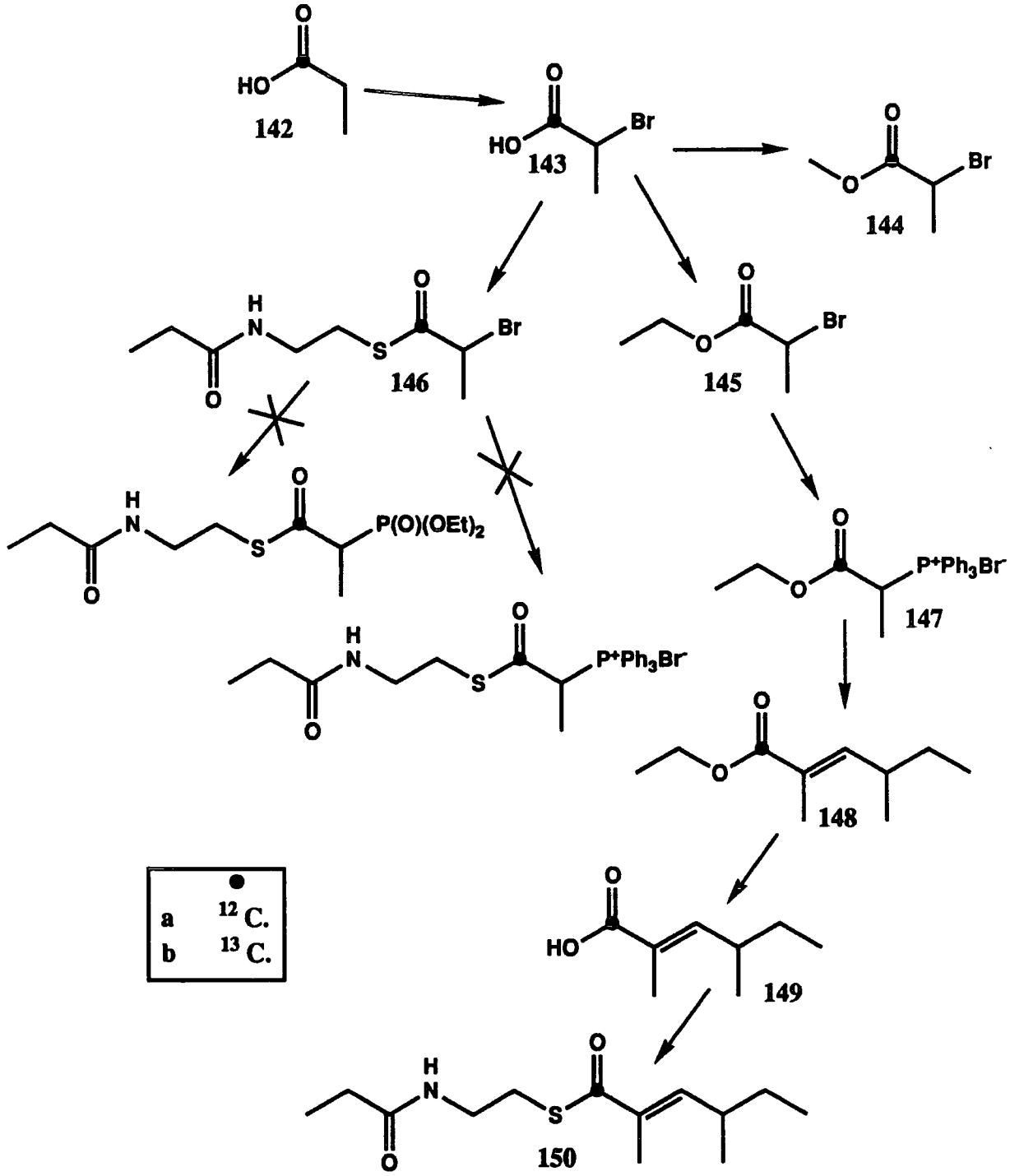
141 99 99a

(i)KCN, NH_4Cl. (ii) H_3O^+, Δ. (iii) MeOH, $SOCl_2$.

Scheme 5.09. The synthesis of 1-^{13}C labelled DL-phenylalanine (97).

The synthesis of [1-^{13}C]-DL-phenylalanine was achieved by Strecker methodology[27]. Phenylacetaldehyde was treated with [^{12}C] or [^{13}C] potassium cyanide and ammonium chloride in aqueous methanol to afford [1-^{12}C] or [1-^{13}C] 2-amino-3-phenylpropionitrile hydrochlorides. The nitriles were hydrolysed in refluxing concentrated aqueous hydrochloric acid to afford [1-^{12}C] and [1-^{13}C]-DL-phenylalanine hydrochlorides (99) in good yield after 48hr. The amino acids were methylated, again in high yields, by treatment with a methanolic solution of an excess of thionyl chloride[28], to form [1-^{12}C]- and [1-^{13}C]-DL-phenylalanine methyl esters (99a) after basification with sodium bicarbonate solution. The free amines polymerised slowly on standing, and were usually prepared immediately prior to use.

5.10 The synthesis of N-propionylcysteamine thiolester of 2,4 dimethylhex-2-enoate (150).



Scheme 5.10. The synthesis of NAC thiolester of E-2,4-dimethylhex-2-enoate.

[1-^{12}C]- And [1-^{13}C] propionic acids (142a,b) were α–brominated by a modification of the method of Ropp[29], in quantitative yield by the treatment of the acids, activated to the trifluoroacetic mixed anhydrides, with bromine in the presence of red phosphorus. Methylation of the α-bromoacids by either

ethereal diazomethane[2] or by reflux in acidic methanol afforded the methyl esters (144a,b) in low yield. The ethyl esters of [1-^{12}C] and [1-^{13}C] α-bromopropionic acids (145a,b) were prepared by reflux in acidic ethanol in moderate yields. The N-propionylcysteamine thiolester (146a) was prepared in high yield by the treatment of α-bromopropionic acid (143a) with one equivalent of N-propionylcysteamine (122) and one equivalent each of DCC and DMAP in ether.

The N-propionylcysteamine thiolester (146) could not be converted to the triphenylphosphonium bromide by treatment with one equivalent of triphenylphosphine in either refluxing toluene or acetonitrile. Further attempts using Arbusov conditions[30], with triethylphosphite under reflux, also failed to provide the Wadsworth- Emmons reagent.

The triphenylphosphonium bromide salts (147a,b) of ethyl 2-bromopropionates (145a,b) were formed in good yields by the treatment of either ethyl [1-^{12}C] or [1-^{13}C]-2-bromopropionate (145a,b) with one equivalent of triphenylphosphine in refluxing acetonitrile[31].

The triphenylphosphonium bromide salts were converted to the phosphorus ylides[32] by treatment with one equivalent of triethylamine in refluxing methylene dichloride, and Wittig olefination[33] was achieved *in situ* by the reaction of the ylides with two equivalents of 2-methylbutyraldehyde (133). The formed ethyl [1-^{13}C]-2,4-dimethylhex-2-enoate was shown to possess the E configuration exclusively, by measurement of the ^{13}C-^{13}C coupling constant (5.6Hz) between C-1 and the methine carbon position[34]. In Z olefins this coupling is of the order 1-2Hz, in E olefins it is approximately 7Hz. This conclusion was further bolstered by the results from two NOE experiments which showed that the alkenic methyl group was closer to the methine attached methyl group than to the alkenic hydrogen, and that the methine hydrogen was judged to be relatively close to the alkenic methyl group (fig 5.11).

The exclusive formation of the E-configuration contrasts with the results of Handa[35] *et al.* who obtained a mixture of E (30%) and Z (70%) isomers of ethyl 2,4-dimethylhex-2-enoate (148) when the Wadsworth-Emmons reagent ethyl α−diethylphosphonopropionate was condensed with 2-methylbutyraldehyde (133) during the synthesis of the ant pheremone Manicone.

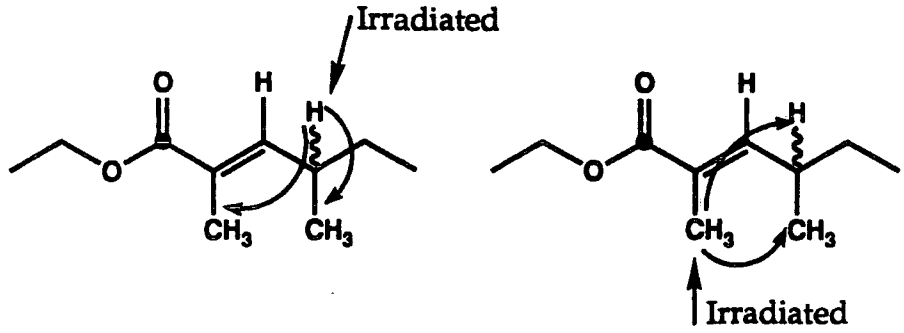


Fig 5.11. Results of two NOE experiments showing peak enhancements observed when the indicated resonances were irradiated.

[1-^{12}C] And [1-^{13}C] ethyl 2,4-dimethylhex-2-enoates were hydrolysed to their corresponding acids by treatment with refluxing dilute aqueous base. The acids were then converted in good yields to their N-propionylcysteamyl thiolesters by DCC coupling with N-propionylcysteamine (122), with DMAP in ether. The compounds were purified by flash chromatography over silica eluting with ether.

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Chapter 6

Experimental.

6.1 General.

6.2 Experimental.

6.3 References.

6.1 General.

^1H n.m.r. spectra were recorded on either a Varian Gemini 200 instrument operating at 199.975MHz, a Brucker AC 250 instrument operating at 250.13 MHz or a Varian 400S instrument operating at 399.952 MHz. ^{13}C n.m.r. spectra were recorded on a Varian Gemini 200 instrument operating at 50.3 MHz or a Varian VXR 400S instrument operating at 100.577 MHz. Chemical shifts are quoted in ppm relative to TMS in $CDCl_3$ and H_2O in D_2O, to two decimal places for ^1H and one decimal place for ^{13}C, all coupling constants are in Hz (J_{gem}, J_{vic} and J_{av} refer to geminal, vicinal and averaged coupling where resolution is poor, respectively). IR spectra were recorded on Perkin Elmer 577 and 377 grating spectrophotometers, samples being embedded in a KBr disc for solids or neat between KBr plates for liquids. Mass spectra were obtained using a VG Analytical 7070E mass spectrometer operating at 70 eV in the indicated mode. Melting points were determined using a Gallenkamp melting point aparatus and are uncorrected. Specific optical rotations were measured using an Optical Activity AA-10 automatic polarimeter. A Beckman J2-21M/E centrifuge was used for all centrifugations.

All solvents were distilled and dried before use[1]. Reagents and starting materials were used without prior purification unless otherwise indicated. Silica gel 60 (particle size 0.035-0.070mm, Brockmann activity 2-3) was used for all chomatographic separations under 'flash' chromatography conditions[2]. Commercial silica gel-60 coated plates, coated to a thickness of 0.2mm and impregnated with fluorescent indicator (F-254), were used for t.l.c. analyses, using the solvent systems described, and samples were visualised by irradiation with ultra-violet light (254 nm) unless otherwise indicated. Specific activities for ^{14}C compounds were obtained from samples dissolved in 'Ecoscint A' or Packard 'Ultima Gold' scintillation solutions using a Packard 2000CA liquid scintillation analyser. Physical and spectrometric properties of ^{14}C-labelled compounds were identical to the unlabelled materials. Properties of ^{13}C and ^2H labelled compounds differed from the unlabelled compounds where indicated.

6.2 Experimental.

Benzyl cyanide (101a).

To a solution of benzyl chloride (100) (distilled, b.p. 22-24°C, 0.005 mbar, 1.94g, 15.3mmol) and 18-crown-6 (0.3g, 1.1 mmol) in acetonitrile (6ml) was added potassium cyanide (1.0g, 15.4mmol). After stirring for 18h at room temperature, methylene dichloride (50ml) was added, and the suspension filtered. Organic washings were washed with water (2x50ml), dried ($MgSO_4$), evaporated *in vacuo*, and distilled (b.p. 46°C, 0.1 mbar) to yield benzyl cyanide as a clear colourless oil (1.68g, 14.4mmol, 93.8%). $\delta_H(CDCl_3)$ 7.30 (5H, m), 3.66 (2H, s). $\delta_C(CDCl_3)$ 23.0 (CH_2), 117.7 (Ph), 127.5 (Ph), 126.7 (Ph), 129.7 (Ph). ν_{max}(liq. film)/cm^{-1} 3100, 3078, 3042, 2975, 2925, 2250, 1605, 1500, 1456 and 1418.

[1-^{13}C]-Benzyl cyanide (101b).

$K^{13}CN$ (1.0g, 15.2mmol, 99 atom% ^{13}C) was used to synthesize [1-^{13}C]-benzyl cyanide by the method described above for (101a). (1.62g, 13.7mmol, 90.3%). $\delta_H(CDCl_3)$ 7.30 (5H, m), 3.66 (2H, d, J10.0).

[1-^{14}C]-Benzyl cyanide (101c).

^{14}C-Labelled KCN (1.0g, 15.4 mmol) of specific activity 9.47μCi/mmol was used to make [1-^{14}C]-benzyl cyanide (1.48g, 12.6 mmol, 83.7%) by the method described above for (101a).

Methyl phenylcyanoacetate (102a).

A solution of benzyl cyanide (101a) (1.68g, 14.4mmol) in tetrahydrofuran (20ml) was stirred under dry N_2 and cooled to -78°C . ^tBuLi in pentane (1.7M, 10ml, 17mmol) was added via dry N_2 flushed syringe and the yellow solution was stirred for 5min, then added to an excess of solid CO_2 in tetrahydrofuran (50ml). The white suspension thus formed was acidified with dilute aqueous H_2SO_4 and extracted into methylene dichloride (3x50ml). The organic fractions were dried ($MgSO_4$) and treated with an excess of an ethereal solution of diazomethane. The solvent was removed by evaporation *in vacuo* and the oil distilled (bp 78-80°C, 0.08 mbar) to afford (102a) as a clear yellow oil (1.07g, 6.11mmol, 42.5%). $\delta_H(CDCl_3)$ 7.37 (5H, m), 4.78 (1H, s), 3.71 (3H, s). ν_{max}(liq. film)/cm^{-1} 3080-3020, 2962, 2260,

1755, 1600, 1500, 1458 and 1438.

[3-^{13}C]-Methyl phenylcyanoacetate (102b).

[1-^{13}C]-Benzyl cyanide (101b) (1.62g, 13.7mmol,) was used without further purification to prepare [3-^{13}C]-methyl phenylcyanoacetate (102b) (0.92g, 5.2mmol, 38.2%) by the method described above for (102a).

[3-^{14}C]-Methyl phenylcyanoacetate (102c).

[1-^{14}C]-Benzyl cyanide (101c) (1.48g, 12.6 mmol) was used without further purification to give [3-^{14}C]-methyl phenylcyanoacetate (102c) (0.84g, 4.8mmol, 38.1%) by the method described above for (102a).

Methyl 3-amino-2-phenylpropionate hydrochloride (103a).

A solution of methyl phenylcyanoacetate (102a) (1.07g, 6.11mmol), in ethanol (50ml) and chloroform (5ml) was shaken under 2.5 atm H_2 with PtO_2 (Adam's catalyst, 200mg) for 18 h. The catalyst was removed by filtration and the solvent evaporated *in vacuo* to afford a cream solid, which was washed with ethyl acetate, collected by filtration and air dried to afford methyl 3-amino-2-phenylpropionate hydrochloride (103a) (1.12g, 5.20 mmol, 85.1%) as a white crystalline solid . $\delta_H(D_2O)$ 7.34-7.22 (5H, m), 4.02 (1H, t, J_{av}7.6), 3.59 (3H, s), 3.53 (1H, dd, J_{vic}7.2, J_{gem}13.2), 3.29 (1H, dd, J_{vic}7.2, J_{gem}13.2). ν_{max}(KBr disc)/cm^{-1} 3450(br), 3300-2500 (br), 1734, 1605, 1590, 1495, 1460, 1440 and 1410. m/z(CI) 180.18 (35.29%).

Methyl [3-^{13}C]-3-amino-2-phenylpropionate hydrochloride (103b).

[3-^{13}C]-Methyl phenylcyanoacetate (102b) (0.92g, 5.2mmol) prepared as above was used to afford methyl [3-^{13}C]-3-amino-2-phenylpropionate hydrochloride (103b) (450mg, 2.08 mmol, 40.0%) by the method described for (103a).

Methyl [3-^{14}C]-3-amino-2-phenylpropionate hydrochloride (103c).

Methyl [3-^{14}C]-phenylcyanoacetate (102c) (0.84g, 4.8mmol) prepared as above was used to afford the methylated amino acid hydrochloride (103c) (900mg, 4.18 mmol, 87.0%) by the method described for (103a).

3-Amino-2-phenylpropionic acid (104a)

A solution of methyl 3-amino-2-phenylpropionate hydrochloride (103a) (1.12g, 5.2 mmol) in aqueous potassium hydroxide (1.2M, 50ml) was stirred for 12h at RT. The solution was acidified to pH 7 and purified by ion exchange chromatography (DOWEX 50X8-200 resin). Aqueous washings containing the amino acid (ninhydrin test) were evaporated *in vacuo* to afford 3-amino-2-phenylpropionic acid (104a) as white crystals after recrystallisation from ethanol/water. (500mg, 3.03mmol, 58.3%). m.p. 223.7-223.9°C (ethanol/water, lit[3]., 222-224°C). $\delta_H(D_2O)$ 2.99 (1H, dd, J_{vic1}7.6, J_{gem}12.8), 3.19 (1H, dd, J_{vic2}7.6, J_{gem}12.4), 3.56 (1H, t, J_{av}7.6), 7.26 (5H, m). $\delta_C(D_20)$ 40.9 (CH_2), 48.4 (CH), 128.2 (Ph), 128.8 (2Ph), 129.5 (2Ph), 134.2 (Ph), 174.5 (CO). ν_{max}(KBr disc)/cm^{-1} 3420, 3200-2400 (br), 2200, 1660-1490 (br), 1452 and 1400. m/z(CI) 166.23 (74.65%) ($C_9H_{11}NO_2$ requires C65.44%, N8.48%, H6.71%, found: C65.05%, N8.33%, H7.02%).

[3-^{13}C]-3-Amino-2-phenylpropionic acid (104b).

Methyl [3-^{13}C]-3-amino-2-phenylpropionate hydrochloride (103b) (450mg, 2.08 mmol) prepared as above was used to generate [3-^{13}C]-3-amino-2-phenylpropionic acid (104b) (200mg, 1.2mmol, 57.7%). $\delta_H(D_2O)$ 7.25 (5H, m), 3.65 (1H, dt, J_{av}7.3, $J_H{}^{13}C$6.4), 3.32 (1H, ddd, J_{vic1}7.3, J_{gem}12.8, $J_H{}^{13}C$145.7), 3.14 (1H, ddd, J_{vic2}7.3, J_{gem}12.8, $J_H{}^{13}C$145.7), $\delta_C(D_2O)$ 42.3 (CH_2, 99% enriched), 51.4 (CH, d, J_{CC}37Hz), 127.9 (Ph), 128.1 (2Ph), 129.2 (2Ph), 137.2 (Ph), 178.3 (CO). m/z(CI) 166.86 (46.33%).

[3-^{14}C]-3-amino-2-phenylpropionic acid (104c).

[3-^{14}C]-Methyl 3-amino-2-phenylpropionate hydrochloride (103c) (900mg, 4.18 mmol) prepared as above was used to generate [3-^{14}C]-3-amino-2-phenylpropionic acid (104c) (475mg, 2.88 mmol, 68.9%), s.a. 7.97μCi/mmol.

Diethyl 2-ethyl-2-methylmalonate (106a).

To a suspension of sodium hydride (940mg, 36 mmol) in tetrahydrofuran (20ml) was added diethyl ethylmalonate (105) (3.76g, 20mmol) and methyliodide (2.84g, 20mmol) at 0°C. The solution was heated under reflux for two hours, cooled to RT and quenched with ethanol (10ml). The clear brown solution was added to water (20ml) and extracted into methylene dichloride (2x25ml). The organic extracts were combined, dried ($MgSO_4$) and evaporated *in vacuo* to yield an orange oil. Diethyl 2-ethyl-2-methylmalonate (106a) was distilled under vacuum as a clear pale orange oil (b.p. 26°C, 0.02 mbar, 2.21g, 10.9mmol, 54.7%). $\delta_H(CDCl_3)$ 0.83 (3H, t, J6.0), 1.25 (6H, t, J7.0), 1.37 (3H, s), 1.90 (2H, q ,J6), 4.12 (4H, q, J7.0). $\delta_C(CDCL_3)$ 8.6 (CH_3), 14.0 (2xCH_3), 19.2 (CH_3), 28.6 (CH_2), 53.9 (C-2), 60.9 (2xCH_2), 172.0 (CO). ν_{max}(liq. film)/cm^{-1} 2980, 2935, 1738, 1465, 1380, and 1310. m/z (CI) 220 (M+18, 26.4%), 203 (M+1, 100%).

Diethyl [2-*methyl* -^{13}C]-2-ethyl-2-methylmalonate (106c).

Sodium hydride (1.44g, 60 mmol), diethyl ethylmalonate (105) (6.6g, 35mmol) and [^{13}C]-methyliodide (5.0g, 35mmol) were used to prepare diethyl [2-*methyl* -^{13}C]-2-ethyl-2-methylmalonate (106c) by the method described above. The title compound was distilled under vacuum as a clear pale orange oil (b.p. 26°C, 0.02 mbar, 6.27g, 30.9mmol, 88.3%).

Diethyl [2-*methyl* -$^{13}C^2H_3$]-2-ethyl-2-methylmalonate (106d).

To sodium hydride (0.34g, 7mmol) in tetrahydrofuran (25ml) stirred at 0°C under dry N_2 was added diethyl ethylmalonate (105) (1.32g, 7mmol) and [$^{13}C^2H_3$]-methyliodide (1g, 6.85mmol). The suspension was heated to reflux for 2h and then quenched with ethanol (10ml) and water (50ml). The clear solution was extracted into methylene dichloride (4x50ml) and the organic layers combined, dried ($MgSO_4$) and evaporated *in vacuo* to yield the title compound as a pale yellow oil which was used without further purification (1.15g, 5.58mmol, 81.5%). $\delta_H(CDCl_3)$ 0.87 (3H, t, J7.52), 1.25 (6H, t, J7.14), 1.90 (2H, m), 4.17 (4H, q, J7.16), 4.18 (2H, q, J7.12). $\delta_C(CDCL_3)$ 8.7 (CH_3), 14.1 (2CH_3), 18.6 (sep, J_{CD}19.67, $^{13}CD_3$), 28.6 (CH_2), 53.9 (d, J34.5, C-2), 61.0 (2CH_2), 172.2 (CO). ν_{max}(liq. film)/cm^{-1} 2980, 2950, 2230, 1740, 1465, 1380, 1365 and 1290. m/z (CI) 207.07 (100%).

2-Ethyl-2-methylmalonic acid (107a).

A solution of diethyl 2-ethyl-2-methylmalonate (106a) (2.12g, 10.9mmol) in aqueous potassium hydroxide (12.5M, 10ml) was heated under reflux for 24h. The cooled solution was acidified with dilute aqueous sulphuric acid and the white suspension formed was added to water (50ml) and extracted into diethyl ether (4x50ml). The ethereal solution was dried ($MgSO_4$) and evaporated *in vacuo* to afford brown crystals of (107a) which were washed with pentane and obtained by filtration as a beige solid (1.04g, 7.13 mmol, 65.4%). $\delta_H(D_2O)$ 0.73 (3H, t, J6.0), 1.23 (3H, s), 2.73 (2H, q, J6.0).

[2-*methyl* -^{13}C]-2-Ethyl-2-methylmalonic acid (107c).

Diethyl [2-*methyl* -^{13}C]-2-ethyl-2-methylmalonate (106c) (6.27g, 30.9 mmol) was used to make (107c) by the method described for (107a) above, as brown crystals which were washed with pentane and obtained by filtration as a beige solid (3.15g, 21.4 mmol, 69.3%). $\delta_H(D_2O)$ 0.64 (3H, t, J7.8), 1.15 (3H, d, J132.5), 1.64 (2H, m).

[2-*methyl* -$^{13}C^2H_3$]-2-Ethyl-2-methylmalonic acid (107d).

A solution of diethyl [2-*methyl*-$^{13}C^2H_3$]-2-ethyl-2-methylmalonate (106d) (1.15g, 5.58mmol) in aqueous KOH (2M, 20ml), was heated under reflux for 24h. The solution was acidified with dilute aqueous HCl and extracted into diethyl ether (5x50ml). The organic washings were combined, dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as a white solid (600mg, 4.0mmol, 71.7%). $\delta_H(D_2O)$ 0.72 (3H, t, J7.56), 1.72 (2H, dq, J_{vic}7.54, $J_H{}^{13}C$4.3). $\delta_C(D_2O)$ 10.9 (CH_3), 21.1 (sep, J19.41, $^{13}CD_3$), 57.0 (d, J34.4, C-2), 179.9 (CO). m/z (CI) 168 (M+18, 75%), 106 (25%).

[*dimethyl* -$^{13}C_2$]-2,2-Dimethylmalonic acid (117).

To sodium hydride (432mg, 18mmol) in diethyl ether (50ml) stirred under dry N_2 and cooled in an icebath was added a solution of diethyl malonate (113) (960mg, 6mmol) in diethyl ether (10ml) dropwise over 10 minutes. [^{13}C]-Methyliodide (1.72g, 753µl, 12mmol) was added and the supension heated under reflux for 1h. Ethanol (10ml) was added, followed by water (50ml). The solution was extracted into diethyl ether (5x50ml) and the ethereal solution was dried ($MgSO_4$) and evaporated *in vacuo* to yield diethyl [*dimethyl* -$^{13}C_2$]-2,2dimethylmalonate (116) as a colourless oil (0.75g,

3.95mmol, 65.8%).

A solution of diethyl [*dimethyl* -$^{13}C_2$]-2,2-dimethylmalonate (116) (0.75g, 3.95mmol) in aqueous sodium hydroxide (1M, 20ml) was heated under reflux for 24h. The solution was then acidified with dilute aqueous hydrochloric acid and extracted with diethyl ether (5x50ml). The ethereal solution was dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as white crystals (500mg, 3.73mmol, 94.5%). $\delta_H(D_2O)$ 1.26 (6H, dd, J130.5, J4.58). ν_{max}(KBr disc)/cm^{-1} 3410 (br.) 2985, 2900, 1705, 1455, 1395, 1365, 1285 and 1260.

Sodium [*methyl* -^{13}C]-2-methylmalonate (115).

To sodium hydride (120mg, 5mmol) in tetrahydrofuran (30ml) was added a solution of diethyl malonate (113) (900mg, 5.6mmol) in tetrahydrofuran (10ml) dropwise while stirring under dry N_2 at RT. [^{13}C]-Methyliodide (314µl, 0.715g, 5mmol) was then added and the solution heated under reflux for 2h and then quenched with ethanol (10ml) and water (50ml). The clear solution was extracted into methylene dichloride (4x50ml) and the organic layers combined, dried ($MgSO_4$) and evaporated *in vacuo* to yield diethyl [3-*methyl* -^{13}C]-2-methylmalonate (114) as a pale yellow oil, which was used without further purification.

A solution of diethyl [*methyl* -^{13}C]-2-methylmalonate (114) in aqueous potassium hydroxide (2M, 20ml) was heated under reflux for 24h. The solution was acidified and extracted into diethyl ether (4x50ml). The ethereal extracts were dried ($MgSO_4$), and evaporated *in vacuo* to afford [*methyl* -^{13}C]-2-methylmalonic acid. The acid was neutralised with dilute aqueous sodium hydroxide and the sodium salt obtained by evaporation of solvent *in vacuo* (390mg, 2.4mmol, 35.0%). $\delta_H(D_2O)$ 1.30 (3H, dd, J_{vic}5.48, $J_H{}^{13}{}_C$131.0), 3.51 (1H, m). $\delta_C(D_2O)$ 16.2 ($^{13}CH_3$ enriched), 48.87 (d, J33.80, C-2), 177.268 (CO).

2-Methylbutyric acid (108a).

A solution of 2-ethyl-2-methylmalonic acid (107a) (1.04g, 7.13 mmol) in water (20ml) was sealed in an evacuated Carius tube and heated to 180°C for 3h. The tube was cooled to -78°C and then opened and the contents acidified with dilute aqueous HCl and extracted into diethyl ether (3x25ml) after warming to RT. The ethereal extracts were combined, dried ($MgSO_4$), and evaporated *in vacuo* to afford crude (108a) as a clear pale brown oil (670mg, 6.57mmol, 92.1%).

An alternative synthesis was achieved as follows. To magnesium turnings (5.4g, 222mmol) stirred under dry N_2 in diethyl ether (100ml) was added 2-bromobutane (142) (22.3ml, 27.4g, 200mmol) dropwise until reaction was initiated. 2-Bromobutane was added at such a rate so as to preserve gentle reflux of the diethyl ether. The solution was heated in the final stages of reaction under reflux for 35min. The black solution was cooled to 0°C and added to a suspension of solid CO_2 (approx. 100g) in diethyl ether (100ml). The suspension was allowed to warm to RT then acidified with dilute aqueous HCl (100ml). The ethereal layer was separated, and the aqueous layer extracted further into diethyl ether (2x100ml). The combined ethereal layers were dried ($MgSO_4$), and evaporated *in vacuo* to yield the crude title compound as a pale brown oil (12.12g) which furnished pure (108a) as a clear colourless oil upon distillation. (5.6g, 55mmol, 27.5%, b.p. 22°C, 0.005mbar). $\delta_H(CDCl_3)$ 0.95 (3H, t, J7.50), 1.18 (3H, d, J6.89), 1.50 (1H, m, J6.94), 1.72 (1H, m, J8.47), 2.40 (1H, m, J6.85). δ_C(DMSO D_6) 11.4 (CH_3), 16.5 (CH_3), 26.2 (CH_2), 40.2 (CH), 177.3 (CO).

[2-^{2}H]-2-Methylbutyric acid (108b).

A solution of 2-ethyl-2-methylmalonic acid (107a) (1.04g, 7.13 mmol) in D_2O (15ml) was sealed in an evacuated Carius tube and heated to 180°C for 3h. The tube was cooled to -78°C before opening and the contents acidified with dilute aqueous HCl and extracted into diethyl ether (3x25ml) after warming to RT. The ethereal extracts were combined, dried ($MgSO_4$), and evaporated *in vacuo* to afford crude (108b) as a clear pale brown oil (0.51g, 4.90mmol, 68.7%). $\delta_H(CDCl_3)$ 0.95 (3H, dt, J2.0, J7.6), 1.17 (3H, s), 1.50 (1H, dq, J7.2, J13.8), 1.71 (1H, dq, J14.0, J6.8), 11.65 (0.5H, s(br)). $\delta_C(CDCl_3)$ 11.6 (CH_3), 16.351 (CH_3, ß shifted from 16.435, 92%D), 26.607 (CH_2, ß shifted from 26.687, 92%D), 40.7 (t, C-D, α shifted from 41.081), 183.5 (CO).

[5-*methyl* -^{13}C, 2-^{2}H]-2-Methylbutyric acid (108c).

A solution of [2-*methyl* -^{13}C]-2-ethyl-2-methylmalonic acid (107c) (3.15g, 21.4 mmol) in D_2O (35ml) was treated as above for (108a) to yield (108c) as a clear pale brown oil (1.7g, 16.19 mmol, 75.6%). $\delta_H(CDCl_3)$ 0.95 (3H, t, J7.2), 1.17 (3H, d, J_{CH}128.0), 1.50 (1H, m), 1.70 (1H, m). $\delta_C(CDCl_3)$ 11.29 (CH_3), 16.071 ($^{13}CH_3$, 99% enriched), 26.335 (CH_2, ß shifted from 26.414, 98%D from peak area), 40.8 (d, J_{C-CH}34.1 due to undeuterated C-2), 40.41 (dt, J_{C-C}34.2, J_{C-D}19.7 due to deuterated material α shift of 0.384ppm), 183.299 (CO).

[5-*methyl* -$^{13}C^2H_3$]-2-Methylbutyric acid (108d).

A solution of [2-*methyl* -$^{13}C^2H_3$]-2-ethyl-2-methylmalonic acid (107d) (600mg, 4.0mmol) in water (10ml) was heated to 175°C for 4h in a sealed evacuated Carius tube. The tube was cooled to -78°C before opening and the contents acidified with dilute aqueous HCl and extracted into diethyl ether (5x50ml) after warming to RT. The ethereal extracts were combined, dried ($MgSO_4$) and evaporated *in vacuo* (cold water bath) to afford the title acid as a pale brown aromatic oil (420mg, 3.96mmol, 99.1%). $\delta_H(CDCl_3)$ 0.95 (3H, t, J7.56), 1.75 (1H, m), 1.69 (1H, m), 2.37 (1H, m). 12.05 (1H, s(Br)). $\delta_C(CDCl_3)$ 11.8 (CH_3), 15.8 (sep, J19.5, $^{13}CD_3$), 26.8 (CH_2), 41.1 (d, J33.95), 184.1 (CO). ν_{max}(liq. film)/cm^{-1} 2960, 2940, 2220, 1710 and 1420. m/z (EI) 107 (31%), 89 (16.68%), 78 (100%), 61 (72%).

Sodium [5-*methyl* -$^{13}C^2H_3$]-2-methylbutyrate (109d).

This material was generated by the base hydrolysis of appropriately labelled esters and thiolesters (110-112) (recovered from feeding experiments) of [5-*methyl* -$^{13}C^2H_3$]-2-methylbutyric acid (108d), followed by acidification, lyophillisation and neutralisation. $\delta_C(D_2O)$ 14.5 (CH_3), 19.5 (sep, J19.14, $^{13}CD_3$), 30.1 (CH_2), 47.6 (d, J40.59, CH), 174.037 (CO).

Sodium [dimethyl-$^{13}C_2$]-isobutyrate (118).

A solution of [*dimethyl* -$^{13}C_2$]-2,2-dimethylmalonic acid (117) (500mg, 3.73mmol) in water (10ml) was heated to 180°C in a sealed evacuated Carius tube for 3h. Upon cooling, the contents of the tube were acidified with dilute aqueous hydrochloric acid and lyophilised. The lyophilisate was carefully neutralised with dilute aqueous sodium hydroxide and the title compound obtained as an amorphous white solid by freeze drying. (330mg, 2.95mmol, 79.0%). δ_H (D_2O) 0.86 (6H, ddd, J126.5, J5.54, J6.82), 2.32 (1H, m), $\delta_C(D_2O)$ 22.4 (2x$^{13}CH_3$ 99% enriched), 39.8 (t, J33.5, C-2), 204.5 (CO). ν_{max}(KBr disc)/cm^{-1} 3425 (br.), 2965, 1560, 1475, 1420, 1370 and 1260.

(N-propionyl)Cysteamyl 2-methylbutyrate (110a).

To 2-methylbutyric acid (108a) (1.02g, 10mmol) was added a solution of DCC (2.06g, 10mmol), DMAP (1.08g, 10mmol) and N-propionylcysteamine (122) (2.66g, 20mmol) in diethyl ether (20ml). The suspension was stirred overnight at RT. Methylene dichloride (50ml) was added and the suspension filtered. The organic washings were combined and washed with dilute aqueous HCl (50ml). The acidic aqueous layer was washed with methylene dichloride (2x50ml) and the combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo* to yield crude (110a) which was purified by chromatography (R_f 0.36, diethyl ether) on silica, eluting with diethyl ether, to afford the title compound as a clear pale yellow oil after evaporation of product containing fractions (1.26g, 5.1mmol, 51.0%). $\delta_H(CDCl_3)$ 0.89 (3H, t, J7.08), 0.95 (3H, t, J8.06), 1.20 (3H, d, J6.44), 1.51 (1H, m), 1.77 (1H, m), 2.25 (2H, q, J7.67), 2.60 (1H, m), 3.07 (2H, t, J6.47), 3.46 (2H, dt, J6.12), 6.75 (1H, s(br)). ν_{max}(liq. film)/cm^{-1} 3310 (br), 3090, 2980, 2940, 2885, 1695, 1655, 1550, 1465, 1408, 1378 and 1230.

(N-propionyl)Cysteamyl [2-^{2}H]-2-methylbutyrate (110b).

To [2-^{2}H]-2-methylbutyric acid (108b) (700mg, 6.8mmol) was added a solution of DCC (1.44g, 7.0mmol), DMAP (0.85g, 7.0mmol) and N-propionylcysteamine (122) (950mg, 7.14mmol) in diethyl ether (20ml). The suspension was stirred overnight at RT. Methylene dichloride (50ml) was added and the suspension filtered. The organic washings were combined and washed with dilute aqueous HCl (50ml). The acidic aqueous layer was re-extracted into methylene dichloride (2x50ml) and the combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo* to yield crude (110b) which was purified by chromatography (R_f 0.36, diethyl ether) over silica eluting with diethyl ether to afford the title compound as a pale yellow oil (1.1g, 5.05mmol, 74.2%). $\delta_H(CDCl_3)$ 0.91 (3H, t, J7.2), 1.14 (3H, t, J7.6), 1.16 (3H, s), 1.47 (1H, m), 1.72 (1H, m), 2.21 (2H, q, J7.6), 3.03 (2H, t, J6.4), 3.43 (2H, dt, J6.4, J6.0), 6.41 (1H, s(br)). $\delta_C(CDCl_3)$ 9.8 (CH_3), 11.6 (CH_3), 17.091 (CH_3, ß-shifted from 17.190), 27.074 (CH_2, ß shifted from 27.173), 28.1 (CH_2), 29.6 (CH_2), 39.7 (CH_2), 49.709 (CH, t, J19.8, α-shifted from 50.157), 174.1 (CON), 204.3 (COS).

(N-propionyl)Cysteamyl [5-*methyl* -^{13}C, 2-^{2}H]-2-methylbutyrate (110c)

To a solution of [5-*methyl* -^{13}C, 2-^{2}H]-2-methylbutyric acid (108c) (525mg, 5.0 mmol) in diethyl ether (15ml) was added DCC (1.03g, 5 mmol), DMAP (0.61g, 5.0 mmol) and N-propionylcysteamine (122) (665mg, 5.0 mmol). The solution was stirred for 12h at RT. A fine white precipitate appeared within 30 min. The suspension was filtered and evaporated to yield a pale yellow oil containing white crystals. The oil was purified by chromatography (R_f 0.36, diethyl ether) over silica eluted with diethyl ether (400ml). Product containing fractions were combined and evaporated to afford the thiolester as a clear colourless oil (0.69g, 3.15 mmol, 63%). δ_H($CDCl_3$) 0.91 (3H, t, J7.2), 1.14 (3H, t, J7.6), 1.15 (3H, d, J128), 1.48 (1H, m), 1.71 (1H, m), 2.21 (2H, q, J7.6), 3.04 (2H, t, J6.4), 3.43 (2H, dt, J6.4, J6.0), 6.50 (1H, s(br)). δ_C($CDCl_3$) 9.8 (CH_3), 11.6 (CH_3), 17.1 ($^{13}CH_3$, 99% enriched), 27.1 (CH_2), 28.1 (CH_2), 29.6 (CH_2), 39.6 (CH_2), 49.715 (C-D, dt, J_{C-C}33.8, J_{C-D}19.7, alpha shift of 0.444ppm), 50.2 (d, J_{C-CH}33.8 due to non deuterated material), 174.2 (CON), 204.3 (COS). m/z (CI) 220 (100%), 134 (37%).

(N-propionyl)Cysteamyl [5-*methyl* -^{13}C^{2}H$_3$]-2-methylbutyrate (110d).

To [5-*methyl* -^{13}C^{2}H$_3$]-2-methylbutyric acid (108d) (210mg, 2.0mmol) was added a solution of DCC (410mg, 2.0mmol), DMAP (245mg, 2.0mmol) and N-propionylcysteamine (122) (270mg, 2.0mmol) in diethyl ether (20ml). The suspension was stirred overnight at RT. Methylene dichloride (50ml) was added and the suspension filtered. The organic washings were combined and washed with dilute aqueous HCl (50ml). The acidic aqueous layer was washed with methylene dichloride (2x50ml) and the combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo* to yield crude (110d) which was purified by chromatography (R_f 0.36, diethyl ether) over silica eluting with diethyl ether. (155mg, 0.70mmol, 70.0%). δ_H($CDCl_3$) 0.90 (3H, t, J7.52), 1.14 (3H, t, J7.76), 1.45 (1H, m), 1.70 (1H, m), 2.24 (2H, q, J7.68), 2.55 (1H, m), 3.03 (2H, t, J7.74), 3.40 (2H, dt, J7.74), 7.45 (1H, t(Br)). δ_C($CDCl_3$) 9.2 (CH_3), 10.7 (CH_3), 15.5 ($^{13}CD_3$, sep. J19.44), 26.3 (CH_2), 27.3 (CH_2), 28.6 (CH_2), 38.6 (CH_2), 49.1 (CH, d, J33.9), 173.7 (CON), 202.7 (COS). m/z (CI) 222 (14.5%), 134 (4.8%).

Octyl [5-*methyl* -$^{13}C^2H_3$]-2-methylbutyrate (112d).

To [5-*methyl* -$^{13}C^2H_3$]-2-methylbutyric acid (108d) (210mg, 1.98mmol) was added a solution of DCC (410mg, 2.0mmol), DMAP (244mg, 2.0mmol) and 1-octanol (520mg, 4mmol) in diethyl ether (20ml). The suspension was stirred overnight at RT. Methylene dichloride (50ml) was added and the suspension filtered. The organic washings were combined and washed with dilute aqueous HCl (50ml). The acidic aqueous layer was re-extracted into methylene dichloride (2x50ml) and the combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo* to yield a mixture of 1-octanol and the title compound which was used without further purification. $\delta_H(CDCl_3)$ 0.90 (2xCH_3), 1.30 (6xCH_2), 1.75 (CH), 1.90 (CH), 3.20 (CH), 4.05 (OCH_2). $\delta_C(CDCl_3)$ 11.5 (CH_3), 12.2 (CH_3), 15.7 ($^{13}CD_3$, sep. J19.36), 27.5 (CH_2), 28.2 (CH_2), 30.0 (CH_2), 30.4 (CH_2), 31.1 (CH_2), 32.0 (CH_2), 33.2 (CH_2), 41.5 (CH, d, J37.4), 63.2 (OCH_2), 154.6 (CO).

Thiooctyl [5-*methyl* -$^{13}C^2H_3$]-2-methylbutyrate (111d).

To [5-*methyl* -$^{13}C^2H_3$]-2-methylbutyric acid (108d) (210mg, 1.98mmol) was added a solution of DCC (410mg, 2.0mmol), DMAP (245mg, 2mmol) and 1-octanethiol (580mg, 4mmol) in diethyl ether (20ml). The suspension was stirred overnight at RT. Methylene dichloride (50ml) was added and the suspension filtered. The organic washings were combined and washed with dilute aqueous HCl (50ml). The acidic aqueous layer was re-extracted into methylene dichloride (2x50ml) and the combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo* to yield a mixture of 1-octanethiol and the title compound which were separated by flash chromatography (R_f 0.46, methylene dichloride/hexane 25/75) on silica eluting with methylene dichloride/hexane 25/75. The title compound was obtained as a colourless oil (80mg, 0.34mmol, 17.2%). $\delta_H(CDCl_3)$ 0.85 (3H, t, J7.2), 0.89 (3H, t, J7.6), 1.24 (12H, m(Br)), 1.45 (1H, m), 1.60 (1H, m), 2.50 (1H, m), 2.83 (2H, t, J8.3). $\delta_C(CDCl_3)$ 11.6 (CH_3), 14.08 (CH_3), 16.40 ($^{13}CD_3$, sep, J19.41), 22.6 (CH_2), 27.1 (CH_2), 28.5 (CH_2), 28.8 (CH_2), 29.1 (CH_2), 29.14 (CH_2), 29.6 (CH_2), 31.8 (CH_2), 50.0 (CH, d, J33.6), 204.1 (COS).

Sodium amalgam (~3%) (119).

Clean, dry sodium pellets (13g) were placed in a 250ml 3 necked round bottomed flask though which dry N_2 was passed. From 430g of distilled mercury, an aliquot (approx. 50g) was run into the flask. Reaction was initiated by gentle heating with a Bunsen flame. Reaction was maintained by the addition of further mercury, and in the later stages of reaction by strongly heating with a Bunsen. When visible signs of reaction had ceased (approx. 20min), the molten compound was poured into a glass crystallising dish to cool, and cut up before fully solid. The solidified material was roughly ground with a mortar and pestle and stored in a stoppered bottle.

N,N'-Dipropionylcystamine (121).

To a solution of cystamine dihydrochloride (120) (22.5g, 100mmol) in water (20ml) was added aqueous sodium hydroxide solution (12.5M) until the pH was 8.2. Propionyl chloride (20g, 216 mmol) was added dropwise and the pH was maintained above 8.0 by the addition of further base as required. When about half of the propionyl chloride had been added a white precipitate started to appear. The precipitate was collected by Büchner filtration. Further material was precipitated on standing at 4°C overnight. Combined solids were washed with cold water and dried under reduced pressure in a dessicator to afford N,N'-dipropionylcystamine (121) as a cream solid (18.16g, 68.8mmol, 68.8%). mp 93.9-95.3°C. $\delta_H(CDCl_3)$ 1.16 (6H, t, J8.0), 2.26 (4H, q, J7.8), 2.84 (4H, t, J6.8), 3.56 (4H, dt, J6.4, J6.0), 6.91 (2H, t, J6.0). $\delta_C(CDCl_3)$ 10.3 (CH_3), 30.0 (CH_2), 38.3 (CH_2), 38.9 (CH_2), 175.1 (CON). ν_{max}(KBr disc)/cm^{-1} 3285, 3070, 2980, 2950, 2885, 1660, 1640, 1630, 1550, 1470, 1430 and 1410. m/z (CI) 265.19 (6.79%).

N-Propionylcysteamine (122).

To a solution of N,N'-dipropionylcystamine (121) (2.64g 10mmol) in methanol (50ml) under dry nitrogen was added 3% sodium amalgam (119) (26g). The suspension was stirred for 90min at RT, then filtered to remove mercury. The methanolic solution was poured into a separatory funnel containing dilute aqueous HCl (50ml) and methylene dichloride (50ml). The acidified solution was immediately extracted with further methylene dichloride (2x50ml). Organic washings were dried and evaporated *in vacuo* to afford N-propionylcysteamine (122) as a pale yellow oil (2.27g, 17.1mmol, 85.3%).

An alternative method of reduction is as follows. A solution of N,N'-dipropionylcystamine (121) (1.55g, 5.87mmol) in tetrahydrofuran (50ml) containing methanol (10ml) was stirred under reflux with powdered zinc (1.5g, 22.9mmol) overnight. After this time excess zinc was removed by filtration and the solution acidified with concentrated aqueous HCl (10ml). The solution was concentrated *in vacuo* to a volume of approx. 10ml and then extracted into methylene dichloride (3x25ml). Organic washings were combined, dried ($MgSO_4$) and evaporated *in vacuo* to afford (122) as a clear pale yellow oil (950mg, 7.14mmol, 60.8%). $\delta_H(CDCl_3)$ 1.16 (3H, t, J7.6), 1.49 (1H, t, J8.4), 2.26 (2H, q, J7.6), 2.67 (2H, dt, J6.8, J8.4), 3.42 (2H, dt, J6.0, J6.8), 7.06 (1H, s(br)). $\delta_C(CDCl_3)$ 9.2 (CH_3), 23.3 (CH_2), 28.5 (CH_2), 41.8 (CH_2), 174.0 (CON). ν_{max}(liq. film)/cm^{-1} 3290, 3075, 2975, 2938, 2880, 2540, 1710, 1650 and 1545. m/z (EI) 134 (56.4%), 74 (22.5%), 57 (21.1%).

(L)-N-Benzyloxycarbonyl phenylalanine (123a).

To a solution of L-phenylalanine (99) (6.6g, 40 mmol) in aqueous sodium hydroxide (2M, 20ml), cooled and stirred at -5°C, was added benzyloxycarbonyl chloride (6ml, 7.17g, 42.0mmol) and aqueous sodium hydroxide (2M, 24ml) in 6x1ml and 6x4ml portions respectively, over a period of 30 min. The solution was acidified with concentrated aqueous HCl (10ml) and extracted into methylene dichloride (3x50ml). Organic washings were combined, dried ($MgSO_4$), and evaporated *in vacuo* to give (123a) as a clear glassy solid. (9.35g, 31.27 mmol, 78%). m.p. 84.4-85.7°C. $\delta_H(CDCl_3)$ 3.14 (2H, m), 4.65 (1H, m), 4.82 (2H, s), 5.28 (1H, d, J8.08, NH) 7.25 (m, 10H), 10.40 (s, 1H). $\delta_C(CDCL_3)$ 37.7 (CH_2), 54.6 (CH), 67.2 (OCH_2), 127.2 (Ph), 128.1 (Ph), 128.2 (Ph), 128.5 (Ph), 128.6 (Ph), 129.3 (Ph), 135.5 (Ph), 136.0 (Ph), 156.0 (CO), 176.3 (CO). $[\alpha]_D^{22}$ +2.46° (c 8.1, EtOH). ν_{max} (KBr disc)/cm^{-1} 3340, 3075, 3040, 2940, 1715, 1695, 1610, 1590, 1540, 1530, 1500, 1460, 1420 and 1300.

(L)-[UL-^{14}C]-N-Benzyloxycarbonyl phenylalanine (123b).

To a solution of L-phenylalanine (99) (3.3g, 20mmol) in aqueous sodium hydroxide (10ml, 2M) was added L-[UL-^{14}C]-phenylalanine (20μCi). The solution was treated with benzyloxycarbonyl chloride (3.5ml, 4.18g, 24.5mmol) as described above to give a clear pale yellow oil. The oil crystallised under pentane after two days to form (123b) as a white solid (5.90g, 19.7mmol, 98.5%), s.a. 1.0μCi$mmol^{-1}$.

N-(benzyloxycarbonyl)-3-Amino-2-phenylpropionic acid (126).

A solution of 3-amino-2-phenylpropionic acid (104) (0.5g, 3.03mmol) in aqueous sodium hydroxide (2M, 10ml) was treated with benzyloxycarbonyl chloride (1ml, 1.2g, 7.0mmol) and aqueous sodium hydroxide solution (2M, 5ml) as described above to afford the title compound as a white solid after washing with pentane. (710mg, 2.37mmol, 78.4%). m.p. 109.4-114.0°C. $\delta_H(CDCl_3)$ 3.59 (2H, m), 3.90 (1H, m), 5.07 (2H, s), 5.23 (1H, t(br), NH), 7.32 (10H, m) 9.75 (1H, s, OH). $\delta_C(CDCl_3)$ 43.94 (CH_2), 51.99 (CH), 67.46 (OCH_2), 128.48 (Ph), 128.56 (Ph), 128.65 (Ph), 128.83 (Ph), 129.05 (Ph), 129.48 (Ph), 136.16 (Ph), 137.0 (Ph), 156.89 (CO), 178.0 (CO). ν_{max}(KBr disc)/cm^{-1} 3375, 3075, 3045, 2940, 1705, 1690, 1605, 1555, 1500, 1455, 1425 and 1365. m/z(CI) 300 (1.1%).

(L)-N-Benzyloxycarbonyl phenylalanine (N-propionylcysteamine) thiolester (124a).

To a solution of (L)-N-benzyloxycarbonyl phenylalanine (123a) (2.99g, 10mmol) in diethyl ether (25ml) was added DCC (2.06g, 10mmol), DMAP (1.22g, 10mmol) and an excess of N-propionylcysteamine (122) (2.0g, 15.0mmol). The solution was stirred under dry N_2 for 12h, methylene dichloride (50ml) was added and the white suspension filtered and the organic solution was evaporated *in vacuo* to afford a thick oil containing white crystals. This was chromatographed on silica eluting with acetonitrile (700ml). Product containing fractions were combined and evaporated *in vacuo* to give the title compound as a white solid. m.p. 86.5-90.2°C, (3.63g, 8.44mmol, 84.4%). $\delta_H(CDCl_3)$ 1.07 (3H, t, J7.6), 2.12 (2H, q, J7.6) 2.96 (2H, m, CH_2S), 3.17 (2H, m, CH_2Ph), 3.32 (2H, m, CH_2N), 4.625 (1H, m, CHN), 5.01 (2H, s, CH_2O), 6.44 (1H, d, J8.4, NH), 6.64 (1H, t, J5.6, NH), 7.20 (10H, m, 2Ph). $\delta_C(CDCL_3)$ 9.6 (CH_3), 28.4 (CH_2), 29.3 (CH_2), 37.9 (CH_2), 38.8 (CH_2), 61.7 (CH), 67.0 (OCH_2), 127.0 (Ph), 127.8 (Ph), 128.0 (Ph), 128.3 (Ph), 128.5 (Ph), 129.1 (Ph), 135.5 (Ph), 135.9 (Ph), 155.7 (CO), 174.0 (CO), 200.8 (COS). $[\alpha]_D^{22}$ -9.62° (c 6.2, EtOH). ν_{max}(KBr disc)/cm^{-1} 3410, 3245, 3070, 3045, 2980, 2940, 1720, 1705, 1545, 1500, 1415, 1378 and 1250. m/z (CI) 415.17 (6.87%).

(L)-N-Benzyloxycarbonl-[UL-^{14}C]-phenylalanine (N-propionylcysteamine) thiolester (124b).

A solution of (L)-N-benzyloxycarbonyl-[UL-^{14}C]-phenylalanine (123b) (5.90g, 19.7mmol) in diethyl ether (100ml) was treated with DCC (4.12g, 20mmol), DMAP (2.44g, 20mmol) and an excess of N-propionylcysteamine (122) (3.33g, 25mmol) as described above for (124a) to afford (124b) as a white solid after evaporation. The solid was washed with pentane and collected by filtration. (5.42g, 13.1mmol, 65.5%, s.a. 0.985μCi/mmol).

N-(benzyloxycarbonyl) 3-amino-2-phenylpropionic acid (N-propionylcysteamine) thiolester (127).

A solution of N-(benzyloxycarbonyl) 3-amino-2-phenylpropionic acid (126) (650mg, 2.17mmol) in diethyl ether (50ml) was treated with N-propionylcysteamine (122) (2.46g, 20mmol), DCC (2.06g, 10mmol) and DMAP (1.22g, 10mmol) as described above for (124a) to afford (127) as a white solid after chromatography (R_f 0.82, acetonitrile) on silica eluting with acetonitrile. (750mg, 1.81mmol, 83.4%). $\delta_H(CDCl_3)$ 1.03 (3H, t, J7.6), 2.06 (2H, q, J7.6), 2.95 (2H, t, J5.6, CH_2S), 3.30 (2H, m, CH_2N), 3.50 (1H, m), 3.75

(1H, m), 4.1 (1H, m), 5.05 (2H, s), 5.65 (1H, t (br), NH), 6.40 (1H, t (br), NH), 7.27 (10H, m, 2Ph).

(L)-Phenylalanine (N-propionylcysteamine) thiolester hydrochloride (125a).

A solution of (L)-N-benzyloxycarbonyl phenylalanine (N-propionylcysteamine) thiolester (124a) (500mg, 1.16mmol) in methylene dichloride (25ml) was cooled to -10°C. To this solution under dry nitrogen was added boron tribromide (1M in CH_2Cl_2, 6ml, 6mmol). After 1h stirring at -10°C the solution was warmed to RT. After 90min at RT a hard yellow solid had formed. The addition of 20ml of water decomposed this solid with effervescence. The aqueous layer was washed with methylene dichloride (2x50ml) and evaporated to yield a cream solid. The solid was dissolved in water (50ml) and the solution basified to pH 8 with dilute aqueous NaOH then extracted into diethyl ether (3x50ml). The combined ethereal extracts containing the free amine were then extracted into dilute aqueous HCl (2x50ml). The aqueous acid washings were evaporated *in vacuo* to afford (125a) as a pale brown sticky solid (150mg, 0.47mmol, 40.8%). m.p. 108.4-119.6°C. $\delta_H(D_2O)$ 0.93 (3H, t, J6.8, CH_3), 2.07 (2H, q, J6.8, CH_2), 2.94 (2H, m, CH_2), 3.05 (2H, m, CH_2), 3.24 (2H, m, CH_2), 4.45 (1H, m, CH), 7.22 (5H, m, Ph). $\delta_C(D_2O)$ 8.3 (CH_3), 27.3 (CH_2), 27.9 (CH_2), 35.8 (CH_2), 36.8 (CH_2), 58.8 (CH), 126.9 (Ph), 128.0 (Ph), 128.3 (Ph), 132.2 (Ph), 177.0 (CON), 196.3 (COS). m/z CI) 281 (67%), 134 (100%)

(L)-[UL-^{14}C]-Phenylalanine (N-propionylcysteamine) thiolester hydrochloride (125b).

(L)-Benzyloxycarbonyl-[UL-^{14}C]-phenylalanine (N-propionylcysteamine) thiolester (124b) (500mg, 1.2mmol) was treated with boron tribromide (1M in CH_2Cl_2, 7ml, 7mmol) for 1h at -10°C under dry N_2. The solution was warmed to RT and water (10ml) added and stirred vigorously until effervescence had stopped. The aqueous layer was washed with methylene dichloride (3x15ml) and then basified with dilute aqueous KOH and extracted into methylene dichloride (3x50ml). The combined organic extracts containing the free amine were extracted into dilute aqueous HCl (5x50ml). The aqueous acid layer was evaporated *in vacuo* to afford a colourless oil which crystallised overnight, giving (125b) as white crystals (254mg, 0.803mmol, 66.4%). s.a 1.02μCi/mmol, m/z (CI) 281.11 (6.52%), 134.05 (62.23%).

3-Amino-2-phenylpropionate (N-propionylcysteamine) thiolester hydrochloride (128).

A solution of N-(benzyloxycarbonyl) 3-amino-2-phenylpropionic acid (N-propionylcysteamine) thiolester (127) (750mg, 1.81mmol) in methylene dichloride (50ml) was stirred at -10°C. Boron tribromide (1M in CH_2Cl_2, 5ml, 5mmol) was added and stirring continued for 90min. The solution became very pale yellow and a solid appeared. The solution was warmed to RT and stirred for 30min. Water (25ml) was added and stirring continued for 20min. The separated aqueous solution was washed with methylene dichloride (3x25ml) and then basified with dilute aqueous NaOH to pH 8 and extracted into methylene dichloride (3x25ml). The combined organic layers containing the free amine were extracted into dilute aqueous HCl (3x50ml). The acid aqueous washings were combined and evaporated *in vacuo* to afford (128) as a sticky thick yellow oil (200mg, 0.63mmol, 34.8%). $\delta_H(D_2O)$ 0.80 (3H, t, J7.6), 1.89 (2H, q, J7.6), 2.78 (1H, dt, J_{gem}14.0, J_{vic}16.0), 2.90 (1H, dt, J_{gem}14.0, J_{vic}16.0), 3.12 (2H, t, J_{vic}16.0), 3.26 (1H, dd, J_{gem}13.2, J_{vic}7.6), 3.521 (1H, dd, J_{gem}13.2, J_{vic}7.2), 4.169 (1H, t, J_{vic}7.6), 7.24 (5H, m). $\delta_C(D_2O)$ 12.4 (CH_3), 31.3 (CH_2), 31.9 (CH_2), 41.0 (CH_2), 43.5 (CH_2), 59.2 (CH), 131.5 (2Ph), 132.1 (Ph), 132.5 (2Ph), 136.7 (Ph), 180.8 (CON), 203.2 (COS).

(L)-N-(acetoacetyl)-Phenylalanine methyl ester (130a).

A solution of L-phenylalanine methyl ester (99a) (3.58g, 20mmol) in toluene (20ml) was heated under reflux with freshly distilled 2,2,6-trimethyl-1,3-dioxin-4-one (129, acetone diketene adduct) (3.5g, 25mmol, b.p. 36°C, 0.03 mbar) for 2h. The resultant red solution was evaporated *in vacuo* to give a viscous red oil which was pumped free of solvent residues under vacuum. The oil was purified by chromatography (R_f 0.36, diethyl ether) on silica eluting with 50/50 hexane/ethyl acetate. Product containing fractions were combined and evaporated to yield (130a) as a yellow oil (3.95g, 15.0mmol, 75.0%). $\delta_H(CDCl_3)$ 2.32 (3H, s, CH_3), 3.04 (2H, m, CH_2), 3.31 (2H, s, CH_2), 3.67 (3H, s, OCH_3), 4.90 (1H, m, CH), 7.20 (5H, m, Ph), 7.26 (1H, d(br), NH).

5(S)-3-Acetyl-5-benzyl-2,4,-dioxopyrrolidine (131a).

A solution of (L)-N-(acetoacetyl)-phenylalanine methyl ester (130a) (3.95g, 15mmol) in methanol (50ml) was heated under reflux with a solution of sodium methoxide in methanol (2M, 10ml, 20mmol) for 1h. The solution was acidified with dilute aqueous HCl and extracted into diethyl ether (3x50ml). The ethereal washings were combined, dried ($MgSO_4$) and evaporated *in vacuo* to afford a yellow solid which was collected by filtration and washed with water (20ml). The solid was recrystallised from acetone/water to give the title compound as pale cream fine crystals. (1.92g, 8.3mmol, 55.4%). m.p. 134-135°C (decomp, lit[4] 133-134°C). $\delta_H(CDCl_3)$ 2.47 (3H, s, Me), 2.65 (1H, dd, J_{gem}13.9, J_{vic2}10.4, CH), 3.31 (1H, dd, J_{vic1}3.6, J_{gem}13.9, CH), 4.02 (1H, dd, J_{vic1}3.6, J_{vic2}10.3, CH), 6.15 (1H, s(br), NH or OH), 7.26 (5H, m, Ph). $\delta_C(CDCl_3)$ 19.6 (CH_3), 38.2 (CH_2), 63.7 (CH), 101.5 (C), 127.2 (Ph), 128.9 (Ph), 129.1 (Ph), 136.5 (Ph), 174.83 (CON), 185.43 (COH), 194.33 (COMe). $[\alpha]_D^{22}$ -134.83° (c 0.445, EtOH). m/z (CI) 232 (89.7%), 142 (3.96%).

5(RS)-3-Acetyl-5-(p-hydroxybenzyl)-2,4,-dioxopyrrolidine (131b).

To a solution of tyrosine methyl ester (98a) (3.9g, 20mmol) in toluene (25ml) was added 2,2,6-trimethyl-1,3-dioxin-4-one (129, acetone diketene adduct) (4.26g, 30mmol). The red suspension was heated under reflux for 30min giving a red solution. The solution was evaporated *in vacuo* to afford a red oil which was dissolved in diethyl ether (50ml) and washed with dilute aqueous HCl (3x50ml). The ethereal layer was dried ($MgSO_4$) and evaporated *in vacuo* to afford crude (L)-N-(acetoacetyl)-tyrosine methyl

ester (130b) as a red oil which was not purified further. δ_H($CDCl_3$) 1.99 (3H, s, Me), 2.97 (1H, m, CH_2), 3.06 (1H, m, CH_2), 3.72 (3H, s, OMe), 4.83 (1H, m, CH), 5.25 (2H, s, CH_2), 6.75 (2H, d, J8.3, Ph), 6.97(2H, d, J8.3, Ph), 7.45 (1H, d, NH) (OH off scale).

To a solution of the crude (L)-N-(acetoacetyl)-tyrosine methyl ester (130b) in methanol (50ml) was added sodium methoxide (1.35g, 25mmol). The solution was heated under reflux for 1h, then acidified with dilute aqueous HCl (50ml) and extracted into diethyl ether (5x50ml). The ethereal extracts were dried ($MgSO_4$) and evaporated *in vacuo* to yield crude (131b) as a brown solid. The tetramic acid was afforded as beige crystals by recrystallisation from acetone/methylene dichloride (528.5mg, 2.14mmol, 10.7%). mp. 199.5-200.3°C (decomp., acetone/methylene dichloride). δ_H($CDCl_3$/DMSO-D_6) 2.35 (3H, s), 2.79 (1H, dd, J_{gem}14.1, J_{vic1}6.8), 2.99 (1H, dd, J_{gem}14.1, J_{vic2}4.5), 3.97 (1H, t(br), J_{av}6.0), 6.69 (2H, d, J8.4), 6.98 (2H, d, J8.5), 8.35 (1H, s), 9.30 (2H, s(br)). δ_C($CDCl_3$/DMSO-D_6) 19.4 (CH_3), 36.1 (CH_2), 62.8 (CH), 101.0 (C), 114.8 (Ph), 125.8 (Ph), 130.0 (Ph), 155.6 (PhOH), 174.8 (COH), 184.5 (CO), 194.5 (CO). $[\alpha]_D^{22}$ 0.00° (c 0.55 EtOH). ν_{max}(KBr disc)/cm^{-1} 3410, 3215, 3060, 2920, 1705, 1650, 1610, 1520, 1460 and 1450. m/z (CI) 248 (100%), 107 (15%).

(1,1-dimethyl-N-propylidine)Ethylamine (132).

To ^{t}butylamine (36.5g, 0.5mol) stirred at 0°C was added propionaldehyde (29g, 0.5mol) dropwise over the course of two hours. When addition was complete stirring was stopped and KOH pellets (20g) were added, and the solution was left to separate at 0°C overnight. The yellow upper layer was carefully decanted, triply distilled over KOH at atmospheric pressure and collected under dry N_2 to yield the title compound as a clear colourless oil (21.4g, 189mmol, 37.8%, b.p. 103-105°C 1atm). $\delta_H(CDCl_3)$ 1.07 (3H, t, J7.6), 1.17 (9H, s), 2.24 (2H, dq, J4.98, J7.6), 7.60 1H, t, J4.98). $\delta_C(CDCl_3)$ 10.37 (CH_3), 29.38 ($3CH_3$), 56.0 (CH_2), 102.1 (C), 159.6 (CHN). ν_{max}(liq. film)/cm^{-1} 2970, 2940, 2870, 1675, 1460, 1365, 1230 and 1220.

E-2,4-Dimethyl-2-hexenal (134).

To a solution of *freshly distilled* (1,1-dimethyl-N-propylidine)ethylamine (132) (5.65g, 50mmol) in tetrahydrofuran (30ml) stirred under dry N_2 and cooled to -78°C was added butyllithium (1.8M in hexanes, 30.5ml, 55.0mmol), via dry N_2 flushed syringe dropwise over ten minutes. The solution was warmed to 0°C and stirred for 20 min. then cooled to -78°C. 2-Methylbutyraldehyde (133) (3.87g, 45mmol, distilled b.p. 92°C, 1atm) was added dropwise via dry N_2 flushed syringe and then the solution was allowed to warm to 0°C and stirred for 4h at this temperature. An aqueous slurry of oxalic acid (≈30ml) was added and the biphasic mixture was vigorously stirred for 72h. The mixture was added to water (50ml) and extracted into methylene dichloride (3x50ml). The combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo* to yield the crude aldehyde as a pale yellow powerfully aromatic oil (5.3g, 42.1mmol, 93.6%) which was stored at -20°C under dry N_2. The aldehyde was purified when required by flash chromatography (R_f 0.75, methylene dichloride) over silica eluting with methylene dichloride. $\delta_H(CDCl_3)$ 0.88 (3H, t, J7.4), 1.07 (3H, d, J6.6), 1.50 (2H, m), 1.76 (3H, s), 2.62 (1H, m), 6.26 (1H, d, J10.1), 9.40 (1H, s). $\delta_C(CDCl_3)$ 9.2 (CH_3), 11.9 (CH_3), 19.6 (CH_3), 29.6 (CH_2), 35.3 (CH), 138.0 (C), 160.4 (CH), 195.6 (CO). ν_{max}(liq. film)/cm^{-1} 2975, 2940, 2880, 2710, 1695, 1645, 1460 and 1380. m/z (CI) 127 (46.42%).

2,2-Dimethyl-6-chloromethyl-1,3-dioxin-4-one (135).

To a solution of LDA (50mmol) stirred at -78°C in tetrahydrofuran (30ml) under dry N_2 was added freshly distilled 2,2,6-trimethyl-1,3-dioxin-4-one (129, acetone diketene adduct) (7.1g, 50 mmol, b.p. 36°C, 0.03 mbar) dropwise via dry N_2 flushed syringe, giving a bright yellow suspension which was stirred for 15min. The thick suspension was carefully transferred dropwise via dry N_2 flushed teflon tubing to a stirred solution of hexachloroethane (17.7g, 75mmol) in tetrahydrofuran (30ml) cooled to -50°C under dry N_2 over a period of 30min.

The red solution thus formed was allowed to warm to -20°C over 30 min. before being quenched by addition to ice cold dilute aqueous HCl (150ml). The acidic solution was shaken to dispel colour and extracted into diethyl ether (3x50ml). The combined ethereal layers were washed with a saturated aqueous solution of $NaHCO_3$ (2x50ml), dried ($MgSO_4$), and evaporated to afford a yellow oil containing crystalline hexachloroethane which was removed by trituration with hexane (2x50ml). The crude title compound could be purified by flash chromatography (R_f 0.28, methylene dichloride) on silica eluting with methylene dichloride, but was routinely used without further purification. $\delta_H(CDCl_3)$ 1.73 (s, 6H), 4.05 (s, 2H), 5.57 (s, 1H). $\delta_C(CDCl_3)$ 24.8 (2xCH_3), 41.1 (CH_2Cl), 95.6 (CH), 107.6 (C), 160.0 (C), 164.6 (CO). ν_{max}(liq. film)/cm^{-1} 3010, 2960, 1730, 1645, 1400, 1280, 1205 and 1020.

2,2-Dimethyl-6-(diethylphosphonomethyl)-1,3-dioxin-4-one (136).

Diethylphosphite (20.7g, 150mmol, distilled b.p. 27°C, 0.001 mbar) was added dropwise to a solution of potassium ^{t}butoxide (16.8g, 150mmol) in dimethylformamide (30ml, distilled from CaH) cooled to 0°C under dry N_2. After 30min stirring 2,2dimethyl-6-chloromethyl-1,3-dioxin-4-one (135) (as prepared above) was added dropwise generating a deep purple solution which was stirred at 0°C for a further 30min. The colour was discharged by the dropwise addition of concentrated aqueous HCl (approx 10ml) and the pale yellow solution was filtered though celite. The celite pad was washed with diethyl ether (100ml) and the combined organic washings were evaporated *in vacuo* (bath temperature below 40°C) to afford a yellow oil. Excess dimethylformamide and diethyl phosphite were removed by vacuum distillation (0.02 mbar, bath temperature below 45°C) to afford crude title compound as a thick brown oil. Purification was effected by flash chromatography (R_f 0.19, ethyl acetate) on silica eluting with ethyl acetate to afford the title compound as a pale yellow oil (6.6g, 23.7 mmol, 47.48% from

(129)). $\delta_H(CDCl_3)$ 1.35 (6H, t, J7.06), 1.72 (6H, s,), 2.83 (2H, d, J22.14), 4.16 (4H, m), 5.41 (1H, d, J3.68), $\delta_C(CDCl_3)$ 16.8 (d, J_{PC}6.1, 2xCH_3), 25.3 (2xCH_3), 32.7 (d, J_{PC}136.9, CH_2), 63.0 (d, J_{PC}6.4, 2xCH_2), 96.5 (d, J_{PC}8.00, CH), 107.5 (C), 160.9 (C), 163.4 (CO). ν_{max}(liq. film)/cm^{-1} 3400(br), 2995, 2940, 2920, 1730, 1635, 1445, 1375 and 1255(br). m/z (CI) 279 (7.02%).

2,2-Dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (137).

To a solution of LHMDS (2.75mmol) in tetrahydrofuran (30ml) cooled to 0°C and stirred under dry N_2 was added a solution of 2,2-dimethyl-6-(diethylphosphonomethyl)-1,3-dioxin-4-one (136) (760mg, 2.73mmol) in tetrahydrofuran (5ml) via dry N_2 flushed syringe. The red anion formed was stirred for 20min before cooling to -78°C and the addition of *freshly prepared and purified* E-2,4-dimethyl-2-hexenal (134) (340mg, 2.70mmol). The solution was allowed to warm to RT over a period of 4h, then stirred at this temperature for a further 8h. Solvent was removed by evaporation *in vacuo* (bath temperature below 40°C) and the resulting red solid dissolved in methylene dichloride (15ml). Colloidal inorganics were removed by vacuum filtration though glass wool and the methylene dichloride solution concentrated to 5ml *in vacuo* before purification by chromatography (R_f 0.21, methylene dichloride) on silica, eluting with methylene dichloride. The title compound was obtained as a yellow oil (290mg, 1.16mmol, 42.5%). $\delta_H(CDCl_3)$ 0.85 (3H, t, J7.6), 1.00 (3H, d, J6.4), 1.32 (1H, m), 1.41 (1H, m), 1.72 (6H, s), 1.81 (3H, s), 2.46 (1H, m), 5.31 (1H, s), 5.66 (1H, d, J9.6), 5.90 (1H, d, J15.6), 6.98 (1H, d, J15.6). $\delta_C(CDCl_3)$ 12.0 (CH_3), 12.4 (CH_3), 20.3 (CH_3), 25.1 (2xCH_3), 30.1 (CH_2), 35.0 (CH), 93.7 (CH), 106.1 (C), 117.1 (CH), 131.8 (C), 143.4 (CH), 148.1 (CH), 162.1 (C), 164.2 (CO). ν_{max}(liq. film)/cm^{-1} 2970, 2880, 1730, 1660, 1625, 1595, 1580, 1460, 1380, 1375, 1270, 1205 and 1020. m/z (CI) 251 (4.3%).

E,E-5,7-Dimethylnona-3,5-dien-2-one (140).

When crude 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3dienyl)-1,3-dioxin-4-one (137) was purified using activated silica (heat treated at 180°C for 3h) a mixture of two products was obtained which was inseparable by chromatography. After toluene reflux in the presence of phenylalanine methyl ester (98a) the title compound was separated from the formed amide by chromatography (R_f 0.52, chloroform) on silica, eluting with chloroform, as a yellow oil. $\delta_H(CDCl_3)$ 0.85 (3H,t, J7.38), 1.00 (3H, d, J6.66), 1.29 (1H, m),

1.43 (1H, m), 1.79 (3H, s), 2.29 (3H, s), 2.46 (1H, m), 5.76 (1H, d, J9.6), 6.08 (1H, d, J16.0), 7.16 (1H, d, J16.0). δ_C($CDCl_3$) 11.9 (CH_3), 12.4 (CH_3), 20.1 (CH_3), 27.1 (CH_3), 29.9 (CH_2), 35.0 (CH), 125.2 (CH), 131.9 (C), 149.1 (CH), 149.5 (CH), 199.0 (CO). ν_{max}(liq. film)/cm^{-1} 2940, 2910, 2870, 1710, 1638, 1610, 1580, 1450, 1380 and 1265. m/z (CI) 167 (100%).

(L)-N-(E,E 3-oxo-6,8-dimethyldeca-4,6-dienoyl) Phenylalanine methyl ester (138a).

A solution of 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (137) (275mg, 1.10mmol) and L-phenylalanine methyl ester (98a) (300mg, 1.65mmol) in toluene (10ml) was heated to reflux temperature for 1h. After this time excess solvent was removed by evaporation *in vacuo* and the resultant red oil purified by chromatography (R_f 0.11, chloroform/ methylene dichloride 25/75) over silica to yield the title compound as an orange oil (190mg, 0.51mmol, 46.4%). δ_H($CDCl_3$) 0.85 (3H, t, J7.2), 1.00 (3H, d, J6.4), 1.30 (1H, m), 1.41 (1H, m), 1.79 (3H, s), 2.43 (1H, m), 3.12 (2H, m), 3.54 (2H, s), 3.69 (3H, s), 4.87 (1H, m), 5.8 (1H, d, J10.0), 6.10 (1H, d, J16.0), 7.25 (6H, m), 7.60 (1H, d(br), J8.5). δ_C($CDCl_3$) 11.9 (CH_3), 12.4 (CH_3), 20.1 (CH_3), 30.0 (CH_2), 35.2 (CH), 37.9 (OCH_3), 46.7 (CH_2), 52.3 (CH_2), 53.5 (CH), 123.6 (CH), 127.1 (Ph), 128.5 (Ph), 129.3 (Ph), 131.9 (C), 135.9(Ph), 150.8 (CH), 151.7 (CH), 165.9 (CON), 171.7 (COOMe), 195.2 (CO). m/z (CI) 372 (22.4%).

(DL)-[1-^{13}C]-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl) Phenylalanine methyl ester (138b).

A solution of 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (137) (290mg, 1.16mmol) and (DL)-[1-^{13}C]-phenylalanine methyl ester (99a) (220mg, 1.22mmol) in toluene (4ml) was heated to reflux temperature for 1.5h. After this time excess solvent was removed *in vacuo* and the resultant red oil was purified by flash chromatography (R_f 0.11, chloroform/ methylene dichloride 25/75) to yield the title compound as an orange oil (380mg, 1.02mmol, 87.9%). δ_H($CDCl_3$) 0.86 (3H, t, J7.58), 1.00 (3H, d, J6.6), 1.35 (2H, m), 1.79 (3H, s), 2.45 (1H, m), 3.12 (2H, m), 3.54 (2H, s), 3.70 (3H, d, J3.82), 4.85 (1H, m), 5.81 (1H, d, J9.72), 6.098 (1H, d, J15.82), 7.25 (6H, m), 7.57 (1H, d(br), J7.98). δ_C($CDCl_3$) 11.9 (CH_3), 12.4 (CH_3), 20.0 (CH_3), 29.9 (CH_2), 35.2 (CH), 37.9 (OCH_3), 46.6 (CH_2), 52.3 (CH_2), 53.5 (CH, d, J61.91), 123.6 (CH), 127.1 (Ph), 128.5 (Ph), 129.3 (Ph), 131.9 (C), 136.0 (Ph), 150.6 (CH), 151.4 (CH), 166.6 (CON), 171.6 (COOMe, 99% enriched), 195.0 (CO). m/z (CI) 373 (18.3%), 333

(12.8%), 181 (68.8%).

5(RS)-[4-^{13}C]-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-Benzyl-2,4-dioxo pyrrolidine (139b).

To a stirred solution of *freshly sublimed* potassium ^{t}butoxide (141mg, 1.26mmol) in ^{t}butanol (10ml) at RT was added a solution of (DL)-[1-^{13}C]-N-(E,E 3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (138b) (235mg, 0.63mmol) in ^{t}butanol (2ml). An immediate colour change from yellow to red occurred. After 30min dilute aqueous hydrochloric acid (10ml) and diethyl ether (25ml) were added. The orange organic layer was separated, washed with water (1x20ml), dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as a red wax. $\delta_H(CDCl_3)$ 0.85 (3H, t, J7.38), 1.00 (3H, d, J6.6), 1.27 (3H, s), 1.33 (2H, m), 2.45 (1H, m), 3.25 (2H, m), 4.75 (1H, m), 5.81 (1H, d, J10.14), 6.08 (1H, d, J15.82), 7.26 (6H, m), 7.86 (1H, d(br), J6.6), 8.76 (1H, s(br)). $\delta_C(CDCl_3)$ 11.8 (CH3), 12.3 (CH3), 19.9 (CH3), 29.8 (CH2), 35.2 (CH), 46.1(CH2, d, J2.3), 53.6 (CH, d, J59.2), 109.0 (C, d, J65), 123.3 (CH), 127.0 (Ph), 128.4 (2xPh), 129.3 (2xPh), 131.8 (C), 135.8 (Ph), 151.2 (CH), 152.0 (CH), 166.8 (CON), 174.3 (COMe, 99% enriched), 195.3 (CO). m/z (CI) 391.3 (?) (12.3%), 341.2 (0.3%).

(DL)-Phenylalanine hydrochloride (99).

To a solution of phenylacetaldehyde (141) (3g, 25mmol) in methanol (40ml) cooled to 0°C was added a solution of NH_4Cl (1.46g, 27mmol) in water (50ml). After 10min stirring, KCN (1.88g, 25mmol) was added and stirring was continued at RT for 24h. The methanol was removed by evaporation *in vacuo* and the aqueous solution extracted into methylene dichloride (3x50ml). The organic fractions were combined, dried ($MgSO_4$) and evaporated *in vacuo* to afford a brown oil which was heated under reflux with concentrated aqueous HCl (40ml) for 48h. After this time solvent was removed by evaporation *in vacuo* and the resulting solid washed with cold acetone (100ml) to afford the title compound as a white amorphous solid (1.65g, 8.2mmol, 32.8%). $\delta_H(D_2O)$ 3.07 (1H, dd, J_{vic2}7.76, J_{gem}14.55), 3.23 (1H, dd, J_{vic1}5.52, J_{gem}14.58), 4.16 (1H, dd, J_{vic1}5.62, J_{vic2}7.68), 7.26 (5H, m), $\delta_C(D_2O)$ 38.6 (CH_2), 57.4 (CH), 130.8 (Ph), 132.1 (Ph), 132.3 (Ph), 136.8 (Ph), 174.5 (CO). m/z (CI) 166 (100%).

(DL)-[1-^{13}C]-Phenylalanine hydrochloride (99).

[^{13}C]-KCN (2g, 30.25mmol) was used to make the title compound by the above method (3.95g, 19.5 mmol, 64.5%). $\delta_H(D_2O)$ 3.08 (1H, ddd, J_{gem}14.6, J_{vic}7.8, $J_H{}^{13}{}_C$2.8), 3.22 (1H, dt, J_{gem}14.8, J_{av}5.0), 4.18 (1H, dt, J_{vic}5.6, $J_H{}^{13}{}_C$7.6), 7.26 (5H, m). $\delta_C(D_2O)$ 38.5 (CH_2), 57.2 (CH, d, J_{CC}58.77), 130.9 (Ph), 132.1 (Ph), 132.3 (Ph), 137.0 (Ph), 174.6 (CO, 99% enriched). m/z (CI) 167 (42.5%), 120 (10.5%).

(DL)-phenylalanine methyl ester (99a).

To a solution of (DL)-phenylalanine hydrochloride (99) (1.65g, 8.2mmol) in methanol (10ml) cooled to 0°C was added thionyl chloride (1.0g, 8.2mmol). The suspension was stirred at RT until product formation was complete (approx. 48h) when observed by t.l.c. (silica plates eluted with nPrOH/NH_3(aq con£) 7/3, developed with ninhydrin). Solvent was removed by evaporation *in vacuo* to afford a pale yellow solid which was dissolved in saturated aqueous $NaHCO_3$ (20ml). The aqueous solution was extracted into diethyl ether (5x50ml) and the organic extracts combined, dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as a pale yellow oil (810mg, 4.5mmol, 54.8%). $\delta_H(CDCl_3)$ 1.71 (2H, s), 2.85 (1H, dd, J_{vic2}7.74, J_{gem}13.47), 3.08 (1H, dd, J_{vic1}5.16, J_{gem}13.5), 3.69 (3H, s), 3.72 (1H, dd, J_{vic1}5.2, J_{vic2}7.74), 7.24(5H, m). $\delta_C(CDCl_3)$ 41.0 (CH_3), 51.9 (CH_2), 55.8

(CH), 128.8 (Ph), 128.5 (Ph), 129.3 (Ph), 137.2 (Ph), 175.3 (CO). ν_{max}(liq. film)/cm^{-1} 3385, 3310, 3090, 3065, 3030, 3000, 2950, 2855, 1740, 1608, 1500, 1458, 1440 and 1200. m/z (CI) 180 (45.3%), 120 (6.6%).

(DL)-[1-^{13}C]-Phenylalanine methyl ester (99a).

(DL)-[1-^{13}C]-Phenylalanine hydrochloride (99) (600mg, 3mmol) was used to make the title compound by the above method. (310mg, 1.72mmol, 57.4%). δ_H($CDCl_3$) 1.50 (2H, s (br)), 2.82 (1H, ddd, J_{gem}14.0, J_{vic}8.25, $J_H{}^{13}C$3.25), 3.05 (1H, dt, J_{gem}14.0, J_{av}5.O), 3.66 (3H, d, J3.7), 3.68 (1H, m), 7.2 (5H, m). δ_C($CDCl_3$) 40.8 (CH_3), 51.6 (CH_2), 55.5 (CH, d, J58.74), 126.5 (Ph), 128.2 (Ph), 129.0 (Ph), 137.0 (Ph), 175.1 (99% enriched).

2-Bromopropionic acid (143a).

To propionic acid (142a) (distilled, b.p. 140-142°C, 1atm (lit[5] 141°C), 1.0g, 13.5mmol) stirred at 0°C was added triflouroacetic anhydride (3.0g, 14.3mmol). Stirring was continued for 5min and the temperature was raised to 60°C. Red phosphorus (20mg, 0.65mmol) was added and after 5min stirring bromine (2.24g, 14mmol) was added dropwise, each drop added as the colour of the previous one was consumed. When all of the bromine had been added the solution was stirred at 60°C for 18h. Volatiles were removed directly by evaporation *in vacuo* (bath temperature 80-90°C) to afford the crude α-bromoacid as a pale brown oil (1.92g, 12.8mmol, 95.4%). $\delta_H(CDCl_3)$ 1.84 (3H, d, J6.88), 4.40 (1H, q, J6.88), 11.98 (1H, s(br)). $\delta_C(CDCl_3)$ 21.9 (CH_3), 39.9 (CH), 177.0 (CO).

[1-^{13}C]-2-Bromopropionic acid (143b).

To [1-^{13}C]-propionic acid (142b) (1.0g, 13.3mmol) stirred at 0°C was added triflouroacetic anhydride (3.0g, 14.3mmol). Stirring was continued for 5min and the temperature was raised to 60°C. Red phosphorus (20mg, 0.65mmol) was added and, after 5min stirring, bromine (2.24g, 14mmol) was added dropwise, each drop added as the colour of the previous one was consumed. When all of the bromine had been added the solution was stirred at 60°C for 18h. Volatiles were removed directly by evaporation *in vacuo* (bath temperature 80-90°C) to afford the crude α-bromoacid as a pale brown oil (2.0g, 13.0mmol, 97.7%). $\delta_H(CDCl_3)$ 1.857 (3H, dd, J^{13}_{CH}3.0, J6.96), 4.43 (1H, dq, J^{13}_{CH}4.26, J6.92), 12.20 (1H, s). $\delta_C(CDCl_3)$ 21.2 (CH_3), 39.2 (CH, d, J_{CC}61.9), 176.5 (CO, 99% enriched).

Methyl 2-bromopropionate (144a).

The crude 2-bromopropionic acid (143a) (1.0g, 6.7mmol), obtained as above, was treated with an ethereal solution of diazomethane (approx 0.84g, 20mmol). After 30min, excess diazomethane was removed by bubbling with nitrogen, and the ethereal solution was evaporated *in vacuo* to afford the crude title compound as a brown oil. (243mg, 1.49mmol, 22.2%).

Alternatively the title compound could be synthesised by the following method. A methanolic solution of the crude 2-bromopropionate (143a) (0.92g, 6.17mmol) was heated under reflux in the prescence of *p* -toluenesulfonic acid (100mg, 0.6mmol) for 24h. The addition of saturated aqueous sodium bicarbonate solution (50ml) yielded a basic solution which

was extracted into methylene dichloride (3x50ml). The combined organic extracts were dried ($MgSO_4$) and evaporated *in vacuo* to yield the title compound as a pale brown oil. (196mg, 1.2mmol, 19.4%). $\delta_H(CDCl_3)$ 1.76 (3H, d, J6.98), 3.72 (3H, s), 4.32 (1H, q, J6.88). $\delta_C(CDCl_3)$ 21.6 (CH_3), 39.6 (CH), 52.8 (OCH_3), 170.6 (CO).

Ethyl 2-bromopropionate (145a).

To a solution of the crude 2-bromopropionic acid (143a) (1.99g, 13.35mmol), as obtained above, in ethanol (20ml) was added *p* toluenesulfonic acid (100mg, 0.6mmol). The solution was heated under reflux for 24h, then cooled and added to a saturated aqueous solution of sodium bicarbonate (50ml) which was extracted into methylene dichloride (3x50ml). The organic fractions were combined, dried ($MgSO_4$) and evaporated *in vacuo* to afford the crude title compound as a pale brown oil (1.52g, 8.39mmol, 62.8%). $\delta_H(CDCl_3)$ 1.18 (3H, t, J7.38), 1.70 (3H, d, J7.18), 4.11 (2H, q, J7.38), 4.25 (1H, q, J7.18). $\delta_C(CDCl_3)$ 13.6 (CH_3), 21.3 (CH_3), 39.9 (CH), 61.7 (OCH_2), 170.0 (CO). m/z (CI) 183 (33%), (EI) 155 (100%).

[1-^{13}C] Ethyl 2-bromopropionate (145b).

To a solution of the crude [1-^{13}C]-2-bromopropionic acid (143b) (2.0g, 13.0mmol), as obtained above, in ethanol (25ml) was added *p* - toluenesulfonic acid (100mg, 0.6mmol). The solution was heated under reflux for 48h, then cooled. A saturated aqueous solution of sodium bicarbonate (10ml) was added and the basic solution was extracted into methylene dichloride (4x25ml). The organic fractions were combined, dried ($MgSO_4$) and evaporated *in vacuo* to afford the crude title compound as a pale brown oil (1.49g, 8.18mmol, 62.9%). $\delta_H(CDCl_3)$ 1.28 (3H, t, J7.27), 1.80 (3H, dd, J^{13}_{CH}4.40, J_{HH}6.54), 4.20 (2H, dq, J2.65, J6.98), 4.33 (1H, dq, J4.39, J6.57). $\delta_C(CDCl_3)$ 13.8 (CH_3), 21.5 (CH_3), 40.1 (d, J_{CC}64.5, CHBr), 61.8 (OCH_2), 170.1 (CO, enriched).

(N-propionyl)Cysteamyl 2-bromopropionate (146a).

To a solution of the crude 2-bromopropionic acid (143a) (2.0g, 13.4mmol) in diethyl ether (50ml) was added DCC (2.75g, 13.5mmol), DMAP (1.65g, 13.5mmol) and N-propionylcysteamine (122) (1.80g, 13.5mmol). The solution was stirred for 12h, then methylene dichloride (50ml) was added and the suspension filtered and washed with dilute aqueous HCl (50ml).

The acid layer was extracted with methylene dichloride (2x50ml) and the combined organic layers dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as a pale yellow oil (2.97g, 11.1mmol, 82.8%). $\delta_H(CDCl_3)$ 1.15 (3H, t, J7.58), 1.85 (3H, d, J6.94), 2.23 (2H, q, J7.68), 3.12 (2H, t, J5.90), 3.48 (2H, dt, J6.1), 4.54 (1H, q, J6.96), 6.90 (1H, t(br)). $\delta_C(CDCl_3)$ 9.8 (CH_3), 21.9 (CH_3), 29.4 (CH_2), 29.6 (CH_2), 39.0 (CH_2), 47.8 (CH), 174.2 (CON), 196.7 (COS). ν_{max}(liq. film)/cm^{-1} 3260 (br), 3060, 2970, 2920, 2850, 1800, 1660(br), 1535(br), 1445 and 1375. m/z (CI) 270 (78.19%), 269 (7.78%), 268 (76.59%).

Ethoxycarbonylethyl(triphenyl)phosphonium bromide (147a).

To a solution of ethyl 2-bromopropionate (145a) (1.52g, 8.39mmol) in acetonitrile (25ml) was added triphenylphosphine (2.23g, 8.5mmol). The solution was heated under reflux for 12h and then the acetonitrile was removed by evaporation *in vacuo* to afford a sticky solid which was triturated with refluxing hexane (30ml). The title compound (147a) was collected as a fine white crystalline solid (1.65g, 3.72mmol, 44.4%) mp 156-157°C (lit[6] 158°C). $\delta_H(CDCl_3)$ 0.99 (3H, t, J7.04), 1.69 (3H, d, J5.0), 3.98 (2H, m), 6.70 (1H, m(br)), 7.90 (15H, m). $\delta_C(CDCl_3)$ 12.7 (CH_3), 13.4 (CH_3), 36.0 (CH, m), 62.6 (OCH_2, d, J1.87), 128.2-134.9 (14 lines, 3Φ), 167.7 (CO, enriched).

[1-^{13}C]-Ethoxycarbonylethyl(triphenyl)phosphonium bromide (147b).

To a solution of ethyl [1-^{13}C]-2-bromopropionate (145b) (1.49g, 8.18mmol) in acetonitrile (25ml) was added triphenylphosphine (2.17g, 8.3mmol). The solution was heated under reflux for 48h and then the acetonitrile was removed by evaporation *in vacuo* to afford a sticky solid which was triturated twice with refluxing hexane (30ml). The title compound (147b) was collected as a fine white amorphous solid (3.37g, 7.6mmol, 93.0%). $\delta_H(CDCl_3)$ 0.99 (3H, t, J7.04), 1.69 (3H, dd, J4.94, J18.3), 3.98 (2H, m), 6.70 (1H, m(br)), 7.90 (15H, m). $\delta_C(CDCl_3)$ 12.7 (CH_3), 13.4 (CH_3), 36.0 (CH, m), 62.6 (OCH_2, d, J1.87), 128.2-134.9 (14 lines, 3Φ), 167.7 (CO, 99% enriched).

Ethyl (E)-2,4-dimethylhex-2-enoate (148a).

To a solution of ethoxycarbonylethyl(triphenyl)phosphonium bromide (147a) (1.65g, 3.72mmol) in methylene dichloride (50ml) was added triethylamine (376mg, 3.72mmol) and 2-methylbutyraldehyde (133) (640mg, 7.44mmol). The solution was heated under reflux for 18h, and then washed with dilute aqueous HCl (2x20ml). The organic phase was dried ($MgSO_4$) and concentrated to a volume of 5ml, filtered to remove precipitated triphenylphosphine oxide, and residual solvent was removed by evaporation *in vacuo* to afford the crude title compound as a pale yellow fragrant oil. (226mg, 1.33mmol, 35.7%). $\delta_H(CDCl_3)$ 0.77 (3H, t, J7.34), 0.92 (3H, d, J6.64), 1.22 (3H, t, J7.16), 1.28 (2H, m), 1.76 (3H, d, J_{HMe}1.44), 2.35 (1H, m), 4.11 (2H, q, J7.20), 6.46 (1H, dq, J_{HMe}1.44, J_{HH}10.14). $\delta_C(CDCl_3)$ 11.8 (CH_3), 12.4 (CH_3), 14.2 (CH_3), 19.6 (CH_3), 29.6 (CH_2), 34.8 (CH), 60.3 (OCH_2), 126.4 (C), 147.8 (CH), 168.4 (CO).

[1-^{13}C] Ethyl (E)-2,4-dimethylhex-2-enoate (148b).

To a solution of [1-^{13}C]-ethoxycarbonylethyl(triphenyl)phosphonium bromide (147b) (3.73g, 7.6mmol) in methylene dichloride (40ml) was added triethylamine (808mg, 8.0mmol) and 2-methylbutyraldehyde (133) (1.38g, 16mmol). The solution was heated under reflux for 48h, and then washed with dilute aqueous HCl (2x20ml). The organic phase was dried ($MgSO_4$) and concentrated to a volume of 5ml. The addition of hexane (30ml) precipitated triphenylphosphine oxide (1.75g, 6.29mmol, 83.0%, m.p. 157-158°C, lit[5] 156-158°C), as fine white crystals, which was removed by filtration, and residual solvent was removed by evaporation *in vacuo* to afford the pure title compound as a pale yellow fragrant oil. (670mg, 3.92mmol, 51.6%). $\delta_H(CDCl_3)$ 0.86 (3H, t, J7.38), 1.00 (3H, d, J6.64), 1.30 (3H, t, J7.14), 1.38 (2H, m), 1.84 (3H, dd, $J_H{}^{13}{}_C$4.22, J_{HMe}1.44), 2.40 (1H, m), 4.19 (2H, dq, $J_H{}^{13}{}_C$2.96, J_{HH}7.16), 6.54 (1H, ddq, J_{HMe}1.44, $J_H{}^{13}{}_C$7.04, J_{HH}9.96). $\delta_C(CDCl_3)$ 11.8 (CH_3), 12.4 (CH_3. d, J3.13), 14.2 (CH_3), 19.6 (CH_3), 29.5 (CH_2), 34.8 (CH, d, J5.16), 60.3 (OCH_2), 126.4 (C, d, J37.2), 147.7 (CH, d, J1.76), 168.3 (CO, 99% enriched). m/z (CI) 172 (78%).

(E)-2,4-Dimethylhex-2-enoic acid (149a).

A solution of ethyl (E)-2,4-dimethylhex-2-enoate (148a) (226g, 1.32mmol) in aqueous potassium hydroxide (0.24M, 15ml) was heated under reflux for 18h. The solution was acidified with dilute aqueous HCl and extracted into

methylene dichloride (4x20ml). The organic layers were combined, dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as a waxy white semi-solid (160mg, 1.13mmol, 85.6%). $\delta_H(CDCl_3)$ 0.86 (3H, t, J7.38), 1.01 (3H, d, J6.66), 1.40 (2H, m), 1.85 (3H, d, J_{HMe}1.40), 2.45 (1H, m), 6.69 (1H, dq, J_{HMe}1.40, J_{HH}10.12). $\delta_C(CDCl_3)$ 11.8 (CH_3), 12.1 (CH_3), 19.5 (CH_3), 29.5 (CH_2), 35.1 (CH), 125.9 (C), 150.5 (CH), 173.8 (CO). m/z (EI) 142 (23%), (CI) 160 (M+18, 100%).

[1-^{13}C]-(E)-2,4-Dimethylhex-2-enoic acid (149b).

To an aqueous solution of potassium hydroxide (0.36M, 25ml) was added [1-^{13}C] ethyl (E)-2,4-dimethylhex-2-enoate (148b) (570mg, 3.33mmol), and the suspension was heated under reflux for 72h. The cloudy supension was acidified with dilute aqueous hydrochloric acid and extracted into methylene dichloride (4x25ml) and diethyl ether (1x25ml). The combined organic extracts were dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as a clear oily wax (443mg, 3.10mmol, 93.1%). $\delta_H(CDCl_3)$ 0.86 (3H, t, J7.38), 1.01 (3H, d, J6.64), 1.36 (2H, m), 1.85 (3H, dd, J_{H13C}4.24, J_{HMe}1.44), 2.43 (1H, m), 6.70 (1H, ddq, J_{HMe}1.44, J_{HH}10.1, J_{H13C}7.06), 12.14 (1H, s(br)). $\delta_C(CDCl_3)$ 11.9 (CH_3), 12.1 (CH_3), 19.6 (CH_3), 29.6 (CH_2), 35.2 (CH, d, J5.23), 126.0 (C, d, J68.80), 150.7 (CH, d, J2.11), 174.2 (CO, enriched). m/z (EI) 143 (31.6%), (CI) 161 (M+18, 75.8%).

N-(propionyl)-S-((E)-2,4-dimethylhex-2-enoyl)-Ethanamine-2-thiol (150a).

To a solution of 2,4-dimethylhex-2-enoic acid (149a) (160mg, 1.13mmol) in diethyl ether (15ml) was added DCC (204mg, 1.0mmol), DMAP (122mg, 1.0mmol) and N-propionylcysteamine (122) (200mg, 1.5mmol). The solution was stirred overnight and filtered to remove precipitated solids. The solution was evaporated to a volume of 1ml and then purified by column chromatography (R_f 0.64, diethyl ether) over silica eluting with diethyl ether. The title compound was afforded as a colourless oil after evaporation of product containing fractions. (188mg, 0.73mmol, 64.6%). $\delta_H(CDCl_3)$ 0.87 (3H, t, J7.56), 1.03 (3H, d, J6.64), 1.14 (3H, t, J7.38), 1.40 (2H, m), 1.89 (3H, s), 2.20 (2H, q, J7.56), 2.45 (1H, m), 3.08 (2H, t, J6.32), 3.47 (2H, dq, J6.28, J6.08), 5.97 (1H, s(br)), 6.55 (1H, d, J9.8). $\delta_C(CDCl_3)$ 10.3 (CH_3), 12.4 (CH_3), 13.2 (CH_3), 20.1 (CH_3), 28.9 (CH_2), 30.1 (CH_2), 30.2 (CH_2), 35.6 (CH), 40.2 (SCH_2), 135.1 (C), 148.0 (CH), 174.5 (CON), 194.8 (COS). m/z (CI) 258 (56%), 125 (21%).

N-(propionyl)-S-([1-^{13}C]-(E)-2,4-dimethylhex-2-enoyl)-Ethanamine-2-thiol (150b).

To a solution of [1-^{13}C]-2,4-dimethylhex-2-enoic acid (149b) (443mg, 3.10mmol) in diethyl ether (25ml) was added DCC (680mg, 3.33mmol), DMAP (406mg, 3.33mmol) and N-propionylcysteamine (122) (530mg, 4.0mmol). The solution was stirred overnight. Methylene dichloride (20ml) was added and the suspension was filtered to remove precipitated solids. The solution was evaporated to a volume of 4ml and then purified by column chromatography (R_f 0.64, diethyl ether) over silica eluting with diethyl ether. The title compound was afforded as viscous colourless oil after evaporation of product containing fractions. (493mg, 1.91mmol, 61.6%). δ_H($CDCl_3$) 0.87 (3H, t, J7.38), 1.03 (3H, d, J6.74), 1.14 (3H, t, J7.60), 1.38 (2H, m), 1.88 (3H, dd, J_{HMe}1.30, $J_{H^{13}C}$4.42), 2.22 (2H, q, J7.56), 2.45 (1H, m), 3.08 (2H, dt, J4.6, J6.46), 3.45 (2H, dt, J6.22, J5.94), 6.53 (1H, m), 6.62 (1H, t(br)). δ_C($CDCl_3$) 9.6 (CH_3), 11.6 (CH_3), 12.3 (CH_3, d, J2.99), 19.3 (CH_3), 28.1 (CH_2), 29.3 (CH_2), 29.3 (CH_2), 34.8 (CH, d, J5.46), 39.3 (CH_2), 134.3 (C), 147.0 (CH, d, J2.77), 173.9 (CON), 193.7 (COS, enriched). m/z (CI) 259 (54%) 126 (21%).

6.3 References.

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Chapter 7.

Biological materials and methods.

7.1 General.

7.2 References.

7.1 General

An authentic culture of *B. bassiana (Bals.) Vuill.*, strain number 110.25, was obtained from the CBS[1] culture collection (Oosterstraat, Delft, Netherlands.), and initiated onto agar slants. Suspension cultures were maintained in 50ml of medium, made up as indicated below, from commercially available materials, in a 250ml Erlenmeyer flask, stoppered with tightly wound cotton wool and shaken at 200rpm on a rotary shaker (3cm eccentricity) maintained at 24-26°C in the dark. Growth was initiated from agar slants into a seed medium, which was incubated for 14 days. Production cultures were initiated by inoculation (2ml) from the seed medium. After seven days these cultures were used to inoculate another production medium. Five or six generations could be raised before growth became unpredictable. Seven day old production cultures were frozen to provide a stock of cultures which could be re-initiated when required by resuspension in sterile production medium.

All sterilisations were performed using a portable gas heated autoclave, samples being heated to 121°C for 20min at 1.1 $Kgcm^{-2}$. Sterile media and agars were handled in an ethanol sterilised laminar flow hood using sterile implements and containers. Supplementation of extraneous materials to culture broths was performed using gamma sterilised 0.2μ filters (Sigma F-1387).

Cornmeal agar.

To warm distilled water (250ml) was added cornmeal agar powder (Oxoid, 4.25g). The powder was fully dissolved by vigorous stirring and warming as necessary, and the pH of the solution adjusted to 7.2 before sterilisation. The sterilised agar was poured into sterile petri dishes when hand-hot and allowed to set.

Oatmeal agar.

To warm distilled water (1l) was added oatmeal (porridge oats, 50g). After 2hr the thick suspension was filtered through muslin, and the muslin bag compressed to extract most of the moisture. Powdered agar (15g) was added to the liquor and dissolved by stirring and heating. The opaque suspension was divided into two batches, one adjusted to pH 6.0 and one to pH 7.0, and both agar solutions were sterilised before plating out into sterile petri dishes.

Seed medium.

To distilled water (990ml) was added D-glucose (20g), ammonium tartrate (4.6g), KH_2PO_4 (1.0g), $MgSO_4.7H_2O$ (0.5g), NaCl (0.1g), $CaCl_2$ (0.1g) and a mineral ion solution (10ml) containing $CuSO_4$ (39.3mgl^{-1}), $B(OH)_3$ (5.7mgl^{-1}), $(NH_4)_6Mo_7O_{24}.4H_2O$ (3.68mgl^{1}), $MnSO_4.H_2O$ (6.1mgl^{-1}), $ZnSO_4.7H_2O$ (879mgl^{-1}) and $FeSO_4.7H_2O$ (99.6mgl^{-1}). The pH was adjusted to 7.0 before sterilisation.

Production medium.

To distilled water (990ml) was added D-mannitol (50g), KNO_3 (5g), KH_2PO_4 (1g), $MgSO_4.7H_2O$ (0.5g), NaCl (0.1g), $CaCl_2$ (0.1g), $FeSO_4.7H_2O$ (20mg) and a mineral solution (10ml) containing $ZnSO_4.7H_2O$ (880mgl^{-1}), $CuSO_4.5H_2O$ (40mgl^{-1}), $MnSO_4.4H_2O$ (7.5mgl^{-1}), $B(OH)_3$ (6mgl^{-1}), and $(NH_4)_6Mo_7O_{24}.4H_20$ (4mgl^{-1}). The pH of the solution was not adjusted prior to sterilisation.

Tenellin (1) production and extraction.

B. bassiana was routinely cultured in the following manner. Cultures were initiated into 50ml of sterile production medium from a frozen sample of seven day old production medium culture by the transfer of a 2ml aliquot. The flask was shaken on an orbital shaker at 200rpm (3cm eccentricity) for seven days at 25-27°C, and the resultant culture was used to initiate a batch of production medium flasks (usually 8x250ml flasks each containing 50ml medium) by the transfer of 2ml aliquots. The cultures were incubated under the same conditions. The secondary cultures were supplemented with labelled substrates immediately prior to tenellin production, evinced by deep yellow colouration, usually on the second or third day after inoculation. Pulse fed materials were also added beginning at this time. Broths were incubated for a further seven to ten days before being harvested. Samples of unfed broth (10ml) were frozen as stock.

The mycelia was obtained either by centrifugation (14000 rpm, 4°C, 45min) or by vacuum filtration, and water washed before being extracted exhaustively with acetone in a soxhlet extractor. The acetone extract was evaporated *in vacuo* and the orange solid residue dissolved in methylene dichloride and repeatedly washed with brine to remove sugars and other water solubles. The organic phase was dried ($MgSO_4$) and evaporated *in vacuo* to afford a solid residue which was triturated with pentane to remove

lipids. Filtration afforded crude tenellin (1) as a tan to orange solid which was suitable for ^{13}C-nmr. Yields of crude tenellin were typically 600-800mg/l. Radioactively labelled material was recrystallised to constant activity from methanol and determination of label incorporation was calculated by two methods.

Absolute Incorporation (A.I.) was calculated as :

$$A.I. = \frac{\text{Total } \mu Ci \text{ recovered}}{\text{Total } \mu Ci \text{ fed}}$$

This method of calculation is subject to error caused by variable efficiency of recovery of labelled tenellin (1), and thus a better measure is Specific Incorporation (S.I.) calculated as:

$$S.I. = \frac{\text{Specific activity of tenellin } \mu Cimmol^{-1}}{\text{Specific activity of substrate } \mu Cimmol^{-1}} \times 100\%$$

Feeding experiments.

Feeding conditions with labelled substrates and the results are summarised in the relevent sections of chapters 2 and 3.

NMR experiments.

Crude tenellin (1) was dissolved in DMSO-D_6. Proton decoupled ^{13}C nmr spectra were obtained on a Varian VXR400S spectrometer operating at 100.577MHz. Dual proton and deuterium decoupling experiments were carried out by the Edinburgh University Ultra High Field NMR Centre using a Varian VXR600S instrument operating at 150.9MHz. The spectra were referenced to internal TMS. δ_C(DMSO-D_6) 11.8 (CH_3), 12.4 (CH_3), 19.9 (CH_3), 29.4 (CH_2), 34.7 (CH), 105.9 (C), 110.9 (C), 115.0 (2xPh), 122.7 (C), 123.0 (CH), 130.3 (2xPh), 132.6 (C), 140.3 (CH), 149.9 (CH), 151.1 (CH), 156.9 (COH), 157.5 (CO), 173.1 (COH), 193.8 (CO). See also table of peak assignments, Table 2.513, section 2.51)

Tenellin degradation.

Crude tenellin (1) (1.0g, 2.71mmol) was suspended in a biphasic mixture consisting of water (60ml), acetonitrile (20ml) and tetrachloromethane (20ml). To this suspension was added sodium periodate (11.76g, 55mmol) and ruthenium dioxide (30mg, 0.23mmol). Vigorous stirring was maintained for 72h. The orange suspension became green, then black, then colourless. Dilute aqueous sodium hydroxide was added until the pH was 9.0, the aqueous layer was separated, filtered through celite and the volume reduced to 30ml under reduced presure. The solution was acidified with concentrated sulphuric acid and lyophillised. The resulting clear colourless solution containing the volatile carboxylic acids was adjusted to pH 8.0 and freeze dried to afford a mixture of sodium salts as a white powder (approx. 80mg). The salts were treated with gaseous HCl under $CDCl_3$ to obtain a solution suitable for ^1H nmr examination (See Chapter 4, section 4.1).

Biotransformation.

5(S)-3-acetyl-5-benzyl-2,4,-dioxopyrrolidine (131a) was added to three day old *B. bassiana* (2x50ml) to a concentration of 1mM. After three days the culture broths were homogenised, using a Waring blender, and the supernatant, obtained by filtration, was acidified with dilute aqueous HCl and extracted into diethyl ether (5x50ml). Ethereal extracts were dried ($MgSO_4$) before being concentrated *in vacuo* to a volume of 2ml and examined by tlc using two solvent systems ($CHCl_3$/MeOH 9/1 and 8/2). A very faint spot corresponding to *para*-hydroxylated starting material (131b) (R_f 0.1 in $CHCl_3$/MeOH 9/1, R_f 0.15 $CHCl_3$/MeOH 8/2) was observed in both solvent systems. HPLC analysis was performed using a Bakerbond standard HPLC column (4.6x150mm) packed with reverse phase (octadecyl, 5µm) silica, eluting with acetonitrile/water 33/77.

Determination of amino acid uptake.

To a five day old production broth of *B.Bassiana* (1x50ml) was added equal quantities (5mg) of unlabelled L-phenylalanine (99) and 3-amino-2-phenylpropionic acid (104a). After a further 3 days the mycelia was obtained by Büchner filtration and thoroughly washed with deionised water. The cells were extracted with an aqueous solution of ethanol (70%) overnight and removed from solution by filtration. The ethanolic solution was

evaporated *in vacuo* to a volume of 2ml and examined by tlc on silica plates eluting with ^{n}propanol/con^{c}. ammonia 7/3. Spots corresponding to the supplementary materials (phenylalanine R_f 0.60, 3-amino-2-phenylpropionic acid R_f 0.55) were detected after development by spraying the tlc sheets with a 0.2% ethanolic solution of ninhydrin and then heating with a heat gun. 3-amino-2-phenylpropionic acid (104) could not be detected in non-fed cultures by chromatographic methods.

Feeding of [3-^{14}C]-3-amino-2-phenylpropionic acid (104c) to *Datura innoxia*.

[3-^{14}C]-3-Amino-2-phenylpropionic acid (104c, 80mg, 1.08x105 dpm/mg, s.a. 7.97μCi/mmol) was administered by the wick method to 10, 3 month old, *D. innoxia* plants growing in soil in a greenhouse. After four weeks the plants were harvested (fresh weight 460g) and chopped up in a mixture of chloroform (3l), diethyl ether (400ml) and concentrated aqueous ammonia (100ml). The two layers obtained after filtering were assayed for radioactivity. The aqueous ammoniacal layer contained 46%, and the organic layer only 0.26%, of the original activity. The organic solvents were evaporated, and the tropane alkaloids purified as their hydrochloride salts as previously described[2]. The level of radioactivity in the purified salts was negligible.

7.2 References.

[1] Centraalbureau voor Schimmelcultures, Oosterstraat, Delft, Netherlands.

[2] E. Leete.,*Phytochemistry*, 1972, **11**, 1713-1716.

Appendix 1.

Lectures, colloquia and seminars attended 1989-1992.

Part 1. Lectures and Seminars arranged within the Department of Chemistry.

* Lectures attended by the author are starred.

1989

* **October 17** Dr. F. Palmer, University of Notingham, "Thunder and Lightning."

October 25 Prof. C. Floriani, University of Lausanne, Switzerland, "Molecular Aggregates - A Bridge Between Homogenous and Heterogenous Systems."

November 1 Dr. J. P. S. Badyal, University of Durham, "Breakthroughs in Heterogenous Catalysis."

November 10 Prof. J. E. Bercaw, California Institute of Technology, "Synthetic and Mechanistic Approaches to Zeigler-Natta Polymerisation of Olefins."

* **November 11** Prof. N. N. Greenwood, University of Leeds, "Novel Cluster Geometries in Metalloborane Chemistry."

November 13 Dr. J. Becher, Odense University, Denmark, "Synthesis of New Macrocyclic Systems Using Heterocyclic Building Blocks."

* **November 16** Dr. D. Parker, University of Durham, "Macrocycles, Drugs and Rock 'n' Roll."

* **November 29** Prof. D.J. Cole-Hamilton, University of St. Andrews, "New Polymers from Homogenous Catalysis."

* **November 30** Dr. M. N. Hughes, King's college London, "A Bug's Eye View of the Periodic Table."

December 4 Dr. D. Graham, B.P. Research Centre, "How Proteins Absorb to Interfaces."

* **December 6** Dr. R. L. Powell, ICI, "The Development of CFC Replacements."

* **December 7** Dr. A. Butler, St. Andrew's University, "The Discovery of Penicillin, Facts and Fancies."

December 13 Dr. J. Klinowski, University of Cambridge, "Solid State NMR Studies of Zeolite Catalysts."

December 15 Prof. R. Huisgen, University of Munich, "Recent Mechanistic Studies of [2+2] Additions."

* **December 15** Dr. P.E. Brown, University of Newcastle, "Addition vs Rearrangement in Ag^+ Catalysed Solvolysis in Chromenes."

* **December 15** Dr. P. Hanson, University of York, "Investigation of Sandmeyer Hydroxylation Reaction."

* **December 15** Dr. A. Laws, Huddersfield Poyltechnic, "$\Delta 2$, $\Delta 3$ Isomerisation of Cephalosporins."

* **December 15** Dr. D. O'Hagan, University of Durham, "Structural Correlations Between Polyketide Metabolites."

* **December 15** Dr. J. Fisher, University of Leeds, "Biological Applications of NMR Spectroscopy."

<u>1990</u>

January 24 Dr. R. N. Perutz, University of York, "Plotting the Course of C-H Activations with Organometallics."

* **January 31** Dr. U. Dyer, Glaxo, "Synthesis and Conformation of C-Glycosides"

February 1 Prof. J. H. Holloway, University of Leicester, "Noble Gas Chemistry."

* **February 5** Prof. L. Crombie, University of Nottingham, "The Chemistry of Cannabis and Khat."

February 7 Dr. D. P. Thompson, University of Newcastle upon Tyne, "The Role of Nitrogen in Extending Silicate Crystal Chemistry."

* **February 8** Rev. R. Lancaster, Kimbolton Fireworks, "Fireworks Principles and Practice."

February 12 Prof. L. Lunazzi, University of Bologna, "Application of Dynamic NMR to the Study of Conformational Enantiomerism."

February 14 Prof. D. Sutton, Simon Fraser University, Vancouver B.C., "Synthesis and Applications of Dinitrogen and Diazo Compounds of Rhenium and Iridium."

February 28 Dr. R. K. Thomas, University of Oxford, "Neutron Reflectometry from Surfaces."

* **February 21** Dr. C. Bleasdale, University of Newcastle upon Tyne, "The Mode of Action of Some Anti-Tumour Agents."

February 22 Prof. D. T. Clark, I.C.I. Wilton, "Spatially Resolved Chemistry, Using Nature's Paradigm in the Advanced Materials Arena."

* **March 1** Dr. J.F. Stoddart, University of Sheffield, "Molecular Lego."

March 8 Dr. A. K. Cheetham, University of Oxford, "Chemistry of Zeolite Cages."

March 21 Dr. I. Powis, University of Nottingham, "Spinning off in a Huff: Photodissociation of Methyl Iodide."

March 23 Prof. J. M. Bowman, Emory University, "Fitting Experiment With Theory in ArOH."

* **May 7** Dr. T. J. Simpson, University of Bristol, "Polyketide Biosynthesis, Past, Present and Future."

July 9 Prof. L. S. German, U.S.S.R. Academy of Sciences, Moscow, "New Syntheses in Fluoroaliphatic Chemistry: Recent Advances in the Chemistry of Fluorinated Oxiranes."

July 9 Prof. V. E. Platonov, U.S.S.R. Academy of Sciences, Novosibirsk, "Polyfluoroindanes: Synthesis and Transformation."

July 9 Prof. I. N. Rozhkov, U.S.S.R. Academy of Sciences, Moscow, "Reactivity of Perfluoroalkyl Bromides."

October 11 Dr. W. A. MacDonald, I.C.I. Wilton, "Materials for the Space Age."

October 24 Dr. M. Bochmann, University of East Anglia, "Synthesis, Reactions and Catalytic activity of Cationic Titanium Alkyls."

October 26 Prof. R. Soulen, South Western University, Texas, "Preparation and Reaction of Bicycloalkenes."

* **October 31** Dr. R. Jackson, University of Newcastle upon Tyne, "New Synthetic Methods: α-Amino Acids and Small Rings."

* **November 1** Dr. N. Logan, University of Nottingham, "Rocket Propellants."

* **November 6** Dr. P. Kocovsky, University of Uppsala, Sweden, "Stereocontrolled Reactions Mediated by Transition and non Transition Metals."

* **November 7** Dr. D. Jones, University of Newcastle upon Tyne, "Light and Airy Chemistry."

November 7 Dr. D. Gerrard, B.P., "Raman Spectroscopy for Industrial Analysis."

November 8 Dr. S. K. Scott, University of Leeds, "Clocks, Oscillations and Chaos."

* **November 14** Prof. D. T. Bell, SUNY, Stoney Brook, U.S.A., "Functional Molecular Architecture and Molecular Recognition."

November 21 Prof. J. Pritchard, Queen Mary and Westfield College, London University, "Copper Surfaces and Catalysts."

* **November 28** Dr. B. J. Whittaker, University of Leeds, "Two Dimensional Velocity Imaging of State Selected Reaction Products."

* **November 29** Prof. D. Crout, University of Warwick, "Enzymes in Organic Synthesis."

* **December 5** Dr. P. G. Pringle, University of Bristol, "Metal Complexes with Functionalised Phosphines."

* **December 14** Prof. A. H. Cowley, University of Austin, Texas, U.S.A., "New Organometallic Routes to Electronic Materials."

<u>1991</u>

January 15 Dr. B. J. Adler, Lawrence Livermore Laboratories, California, "Hydrogen in all its Glory."

January 17 Dr. P. Sarre, University of Nottingham, "Comet Chemistry."

January 24 Dr. P. J. Sadler, Birbeck College, London, "Design of Inorganic Drugs: Pecious Metals, Hypertension and HIV."

* **January 30** Prof. E. Sinn, University of Hull, "Coupling of Little Electrons in Big Molecules. Implications for the Active Sites of (Metalloproteins and other) Macromolecules."

* **January 31** Dr. D. Lacey, University of Hull, "Liquid Crystals."

* **February 6** Dr. R. Bushby, University of Leeds, "Biradicals and Organic Magnets."

* **February 14** Dr. M. C. Petty, SEAS, University of Durham, "Molecular Electronics."

* **February 20** Prof. B. L. Shaw, University of Leeds, "Syntheses with Coordinated, Unsaturated Phosphine Ligands."

* **February 28** Dr. J. M. Brown, University of Oxford, "Can Chemists Make Better Catalysts Than Enzymes?"

March 6 Dr. C. M. Dobson, University of Oxford, "NMR Studies of Dynamics in Molecular Crystals."

* **March 7** Prof. K. Mullen, Max Plank Inst., "New Aromatic Compounds For Polymer Synthesis."

* **March 7** Dr. J. Markam, I.C.I. Pharmaceuticals, "DNA fingerprinting."

* **March 14** Prof. J. M. G. Cowie, Heriot Watt University, "Polymer Chemistry"

* **April 24** Prof. R. R. Schrock, MIT, "Metal Ligand Multiple Bonds and Metathesis Initiators."

* **April 25** Prof. T. Hudlicky, Virginia Polytechnic institute, U.S.A., "Biocatalysis and Symmetry Based Approaches to the Efficient Synthesis of Complex Natural Products."

* **May 29** Prof. H. Viehe, "Reactivity from Polar Substituent Effects."

* **June 20** Prof. M. S. Brookhart, University of North Carolina, U.S.A., "Olefin Polymerisations, Oligermerisations and Dimerisations Using Electrophillic Late Transition Metal Catalysts."

* **July 29** Dr. M. A. Brimble, Massey University, New Zealand, "Syntheteic Studies Towards the Antibiotic Griseusin-A."

* **September 12** Prof. D. J. Burton, University of Iowa, U.S.A., "Fluorinated Organometallic Reagents."

* **September 12** Prof. J. L. Adcock, University of Tenessee, Knoxville, U.S.A., "Aerosol Direct Fluorination."

* **October 17** Dr. J. A. Salthouse. University of Manchester, "Son et Lumiere."

November 6 Prof. B. F. G. Johnson, University of Edinburgh, "Cluster Surface Analogies."

November 7 Dr. A. R. Butler, St. Andrew's University, "Traditional Chinese Herbal Drugs: A Different way of Treating Disease."

* **November 13** Prof. D. Gani, St. Andrew's University, "The Chemistry of PLP-Dependent Enzymes."

* **November 19** Dr. I. Flemming, University of Cambridge, "Stereocontrol in Organic Synthesis Using Silicon."

* **November 20** Dr. R. More O'Ferrall, University college, Dublin, "Some Acid Catalysed Rearrangements in Organic Chemistry."

November 28 Prof. I. M. Ward, IRC in Polymer Science, University of Leeds, "The Science and Technology of Oriented Polymers."

* **December 4** Prof. R. Grigg, University of Leeds, "Palladium Catalysed Cyclisation and Ion Capture Processes."

December 5 Prof. A. L. Smith, ex Unilever, "Soap, Detergents and Black Puddings."

December 11 Dr. W. A. Cooper, Shell Research, "Colloid Science, Theory and Practice."

1992

January 22 Dr K. D. M. Harris, St. Andrew's University, "Understanding the Properties of Solid Inclusion Compounds."

* **January 29** Dr. A. Holmes, University of Cambridge, "Cycloaddition Reactions in the Service of the Synthesis of Piperidine and Indolizidine Natural Products."

* **January 30** Dr. M. Anderson, Sittingbourne Research Centre, Shell Research, "Recent Advances in the Safe and Selective Chemical Control of Insect Pests."

* **February 12** Prof. D. E. Fenton, University of Sheffield, "Polynuclear Complexes of Molecular Clefts as Models for Copper Biosites."

* **February 13** Dr. J. Saunders, Glaxo Group Research Limited, "Molecular Modelling in Drug Discovery."

* **February 19** Prof E. J. Thomas, University of Manchester, "Applications of Organostannanes to Organic Synthesis."

* **February 20** Prof E. Vogel, University of Cologne, "Poryphyrins: Molecules of Interdisciplinary Interest."

* **February 25** Prof. J. F. Nixon, University of Sussex, "Phosphaalkynes: New Bulding Blocks in Inorganic and Organometallic Chemistry."

February 26 Prof. M. L. Hitchman, Strathclyde University, "Chemical Vapour Deposition."

March 5 Dr. N. C. Billingham, University of Sussex, "Degradable Plastics, Myth or Magic."

* **March 11** Dr. S. E. Thomas, Imperial College, "Recent Advances in Organoiron Chemistry."

March 12 Dr. R. A. Hann, I.C.I Imagedata, "Electronic Photography - An Image of the Future."

* **March 18** Dr. H. Maskill, University of Newcastle upon Tyne, "Mechanistic studies of Organic Group Transfer Reactions."

April 7 Prof. D. M. Knight, Department of Philosophy, University of Durham, "Interpreting Experiments: the Beginning of Electrochemistry."

* **May 6** Prof. T. B. Marder, University of Waterloo, Canada, "Transition Metal Catalysed Alkene Hydroboration."

Part 2. Other lectures and colloquia attended.

All lectures in this section were attended by the author.

1990

March 7 S.C.I. Graduate symposium at the University of York.

November 11 * Prof T. J. Simpson, University of Bristol, "Biosynthesis of polyketide antibiotics"

November 11 * Dr. D. Rees, Parke Davis, "Structure activity correlation in K-opioids."

November 11 * Prof. D. Robins, University of Glasgow, "Biosynthesis of alkaloids from lysine and ornithine."

November 11 * Prof. G. Wulff, University of Dusseldorf, Germany, "Molecular recognition in synthetic polymers."

December 19 # Dr. J. Huff, M.S.D., "H.I.V. protease inhibitors."

December 19 # Dr. D. Williams, University of Cambridge, "How do molecules bind to each other?"

December 19 # Dr. G. M. Blackburn, University of Sheffield, "Phosphonates for probing biological phosphates."

December 19 # Prof. M. Valtier, University of Rennes, France, "Dichloroboranes and unctionalised boronates in organic synthesis."

December 19 # Prof. L. Paquette, Ohio state University, U.S.A., "Unleashing the synthetic potential of oxyanionic sigmatropy."

* Part of Irving review lectures, University of St. Andrew's.
# Part of Sheffield Stereochemistry one day symposium.

1991

April 4 * Dr. S. P. Stanforth, Newcastle Polytechnic, "Aspects of heterocyclic

chemistry"

April 4 * Dr. P. Bailey, University of York, "Asymetric routes to chiral piperidenes."

April 4 * Dr. O. J. Taylor, University of Newcastle upon Tyne, "Some chemistry of nucleosides relevent to toxicology."

April 4 * Dr. P. G. Steele, University of Durham, "Some approaches to taxol C-D ring system."

April 4 * Dr. D. Singh, Teeside Polytechnic, "Aspects of carbohydrate chemistry in the synthesis of natural products."

April 4 * Dr. C. Rayner, University of Leeds, "Novel transformations of 2-3 epoxy sulfides."

April 4 * Prof. O. Meth-Cohn, Sunderland Polytechnic, "Novel aspects of the chemistry of thiophenes at sulphur."

April 4 * Prof. G. Stork, Columbia, "Regio and stereo control of Diels-Alder reactions."

* Part of R.S.C. Northern regional meeting at Leeds.

1992

July 14 * A. R. Fersht, University of Cambridge. "Protein folding and stability."

July 14 * Prof. D. Gani, University of St. Andrews. "Amino acid metabolism: stereochemistry, mechanism and active site structure for key enzymes."

July 14 * Dr. N. Turner, University of Exeter. "Novel applications of enzymes for organic synthesis."

July 14 * Prof. J. Rebek Jr, Massachusetts Institute of Technology, U. S. A., "Recognition and Replication."

July 14 * Dr. C. Abell, University of Cambridge, "Mechanistic studies on

Shikimate Enzymes."

July 14 * Dr. P. D. Bailey, University of York, "The Synthesis of Peptidic 'Enzymes'."

July 15 * Prof. W. Steglich, University of Munich, Germany, "Biologically Active Compounds from Mushrooms and Toadstools."

July 15 * Dr. R. L. Edwards, University of Bradford, "New Metabolites of the Fungal Genus *Xylaria* ."

July 15 * Prof. R. E. Moore, University of Hawaii, U. S. A., "Cyanobacterial Toxins."

July 15 * Dr. D. Kelly, University of Wales, Cardiff, "Chemical Communication in the Natural World."

July 15 * Prof. H. G. Floss, University of Washington, U. S. A., "Biosynthesis of Shikimate-derived Antibiotics."

July 15 * Prof. G. Hofle, Gesellschaft fur Biotechnologische Forschung, Braunschweig, Germany, "The biosynthesis of the Antibiotic Ambruticin."

July 15 * Dr. R. L. Baxter, University of Edinburgh, "Biotin Biosynthesis."

July 16 * Prof. R. M. Kellogg, University of Groningen, The Netherlands, "Amino Acids and Sugars as Sources of Materials and Catalysts."

July 16 * Dr. J. K. M. Sanders, University of Cambridge, "Towards Model Enzymes based on Poryphyrins and Steroids."

July 16 * Prof. P. B. Dervan, Caltech, California, U. S. A., "Sequence Specific Recognition of DNA by Triple Helix Formation."

September 2 # Prof. S. M. Roberts, University of Exeter, "Stereocontrolled Synthesis of Naturally Occurring Carbocyclic Nucleosides and Analogues."

* Part of the 4th International Symposium on Progress In Natural Product Chemistry, held at the University of Nottingham, 14-16 July, 1992.

Part of the Royal Society of Chemistry Perkin Division and the Israel Chemical Society, International Symposium on Structure and Reactivity in Organic and Bioorganic Chemistry, held at the University of Durham, 2-4 September 1992.

Appendix 2. Abbreviations.

A.I.	Absolute incorporation.
atm	atmospheres.
av	average.
br	broad.
Bu	Butyl.
Bz	Benzyl.
C.I.	Chemical ionisation.
CBZ	Benzyloxycarbonyl.
d	doublet.
DCC	Dicyclohexylcarbodiimide.
DH	Dehydratase.
DMAP	4-dimethylaminopyridine.
DMF	Dimethylformamide.
E.I.	Electric ionisation.
ER	Enoyl reducase.
Et	Ethyl.
FAS	Fatty acid synthase.
gem	geminal.
h	Hours.
J	Coupling constant (Hz).
KR	β–Keto acyl reductase.
KS	β–Keto acyl synthase.
LDA	Lithium diisopropylamine.
LHMDS	Lithium hexamethyldisilazide.
m	multiplet.
m.p.	melting point.
Me	Methyl.
min	minutes.
nmr	Nuclear magnetic Resonance.
orf	Open reading frame.
p	pentet.
Ph	Phenyl.
PKS	Polyketide synthase.
Pr	Propyl.
q	quartet.
R_f	Retention factor.
RT	Room temperature, 18-20°C.
s	singlet.
s.a.	Specific activity.
S.I.	Specific incorporation.
sep	septet.
t	triplet.
t.l.c.	Thin layer chromatography.
^tBu	Tertiary butyl.
TE	Thiol esterase.
THF	Tetrahydrofuran.
vic	vicinal.