MICROBIAL DEGRADATION OF BENZENE DERIVATIVES

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To Ning, Gordon and my parents

CONTENTS

SUMMARY

The thesis is divided into two parts. Both deal with the microbial metabolism of benzene derivatives but in different aspects.

Part I attempts to elucidate some new mechanisms and stereochemistry involved in muconic acid pathways, which commonly occur in the microbial degradation of benzene derivatives. To investigate the conversion of 4-methyl into 3-methyl-muconolactone, three specially labelled muconolactones were tested with cell-free extracts of *Rhodococcus rhodocrous*. The results showed that the conversion of 4-methyl to 3-methylmuconolactone proceeds through two steps. Firstly, enzyme catalyses the formation of the new lactone ring by *anti* addition. Secondly the original lactone ring is opened enzymically by *anti* elimination. Dilactone was shown to be an intermediate. Studies on the inhibitors of the methylisomerase have been carried out from chemical point of view. Among the substituents studied, the larger substituents did not affect the biotransformation of pyrocatechols into corresponding muconolactones in *Pseudomonas putida* but affected those in *Aspergillus niger*. It was shown that the optical active 4-ethylmuconolactone can cyclise under mild condition giving the dilactone with opposite optical rotation, but 3,4-dimethylmuconolactone can not be cyclised under various conditions.

In part II, it was hoped to delineate some influences of fluorine substituents on the biosynthesis of cyclopenin group of benzodiazepine alkaloids. Three fluoro-phenylalanines were tested with the fungus *Penicillium cyclopium*. The qualitative results obtained showed that these substrates have been incorporated into benzodiazepine alkaloids with low yields.

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PART ONE

STUDY OF THE MECHANISMS OF MUCONIC ACID PATHWAYS

CHAPTER ONE
INTRODUCTION

Part I of the thesis attempts to elucidate some new mechanisms and stereochemistry involved in muconic acid pathways, which commonly occur in the microbial degradation of benzene derivatives. In introducing the work embodied in this part, some closely related research is reviewed in this chapter. A general review of muconic acid pathways from a historical prospect is firstly outlined. The following section summaries the enzymology of the muconic acid pathways, where we discuss each particular enzyme mainly from bacteria. The isolation, purification and biochemical properties of muconolactone 4-methylisomerase are stressed. Then the stereochemistry involved in the muconic acid pathways is discussed in Sec.1.3, in which the conversion of 4-methyl- into 3-methyl-muconolactone is of particular interest to the present research. Finally, the aims of the present project are presented.

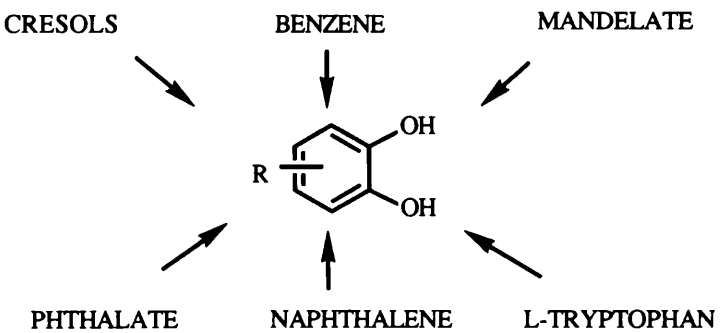
1.1 A GENERAL REVIEW OF MUCONIC ACID PATHWAYS

1.1.1 Microbial Degradation of Benzene Derivatives

Pesticides, herbicides, synthetic detergents and waste products of chemical industries constitute a formidable addition of foreign chemicals to the two major sites of

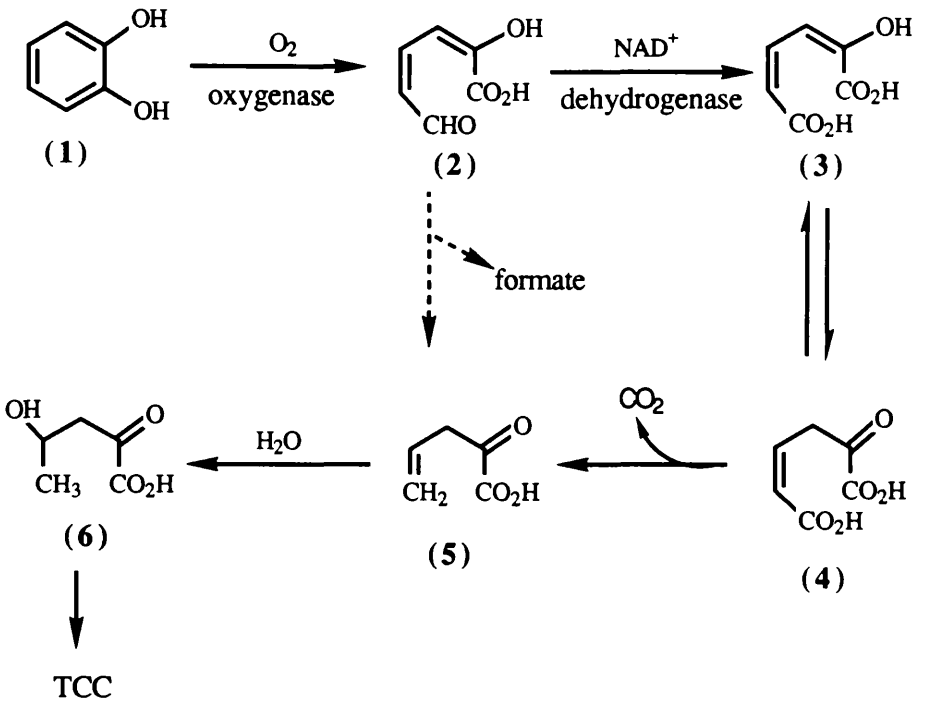
biological dissimilation: the soil and natural water. Some of these are very poisonous substances and could be hazardous to life if they accumulated in the environment. The ultimate destruction and removal of them from the biosphere is mainly dependent on microbial action and therefore is of world-wide importance. Numerous workers have described the isolation of micro-organisms from various sources and their capabilities to degrade aromatic compound (Refs. 1-7).

The oxidative attack on those chemicals carrying an aromatic ring in both bacteria and fungi leads to the formation of either pyrocatechol or its derivatives, which are the substrates for oxygenase-catalysed ring cleavage, and are therefore the last intermediates possessing an aromatic structure (Scheme 1) (Ref. 5).



Scheme 1.

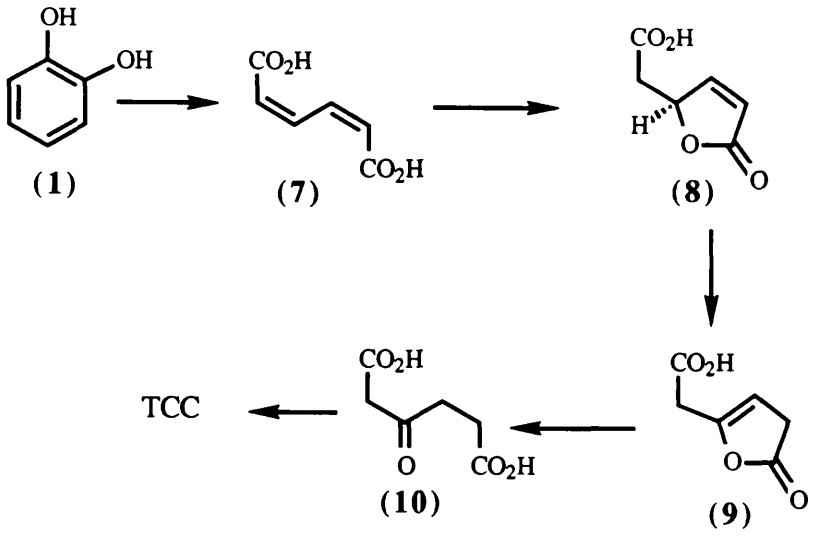
The ring cleavage of the aromatic nuclei proceeds *via* two different pathways according to the mode of ring-fission (Ref. 8). In many microorganisms, aromatic rings are metabolized by the so-called '*meta*-cleavage' illustrated in Scheme 2. The degradation of pyrocatechol (**1**) in a naphthalene-grown *Pseudomonad* gives 2-hydroxymuconic semialdehyde (**2**), which can either be enzymically hydrolysed with loss of formate, or be enzymically further oxidised and decarboxylated to 2-oxo-pent-4-enoic acid (**5**) with (**3**) and (**4**) as intermediates. After hydration of (**5**), compound (**6**) is split into acetaldehyde and pyruvate, which enters into the tricarboxylic acid cycle (TCC) (Scheme 2) (Ref.9). Alternatively, a large number of microorganisms can metabolize pyrocatechols



Scheme 2.

after *ortho*-cleavage, *i.e.* by cleavage between the two hydroxyl groups, to give muconic acids as key intermediates, which can be converted into 3-oxoadipates so that they enter into the Krebs cycle eventually. This pathway (the muconic acid pathway) (Scheme 3) provides a mechanism for the utilization of many different aromatic substrates, and has a wide taxonomic distribution, both among bacteria and fungi. In the simplest case, pyrocatechol (**1**) is converted by bacteria or fungi with uptake of oxygen into *cis,cis*-muconic acid (**7**), which is cyclized enzymically to the muconolactone (**8**) (Scheme 3). This lactone (**8**) is then converted into 3-oxoadipic acid (**10**) *via* the enol-lactone (**9**). The 3-oxoadipic acid (**10**) is further degraded to the metabolically useful acetic and succinic acids, which are constituents of the tricarboxylic acid cycle (TCC) (Ref.10).

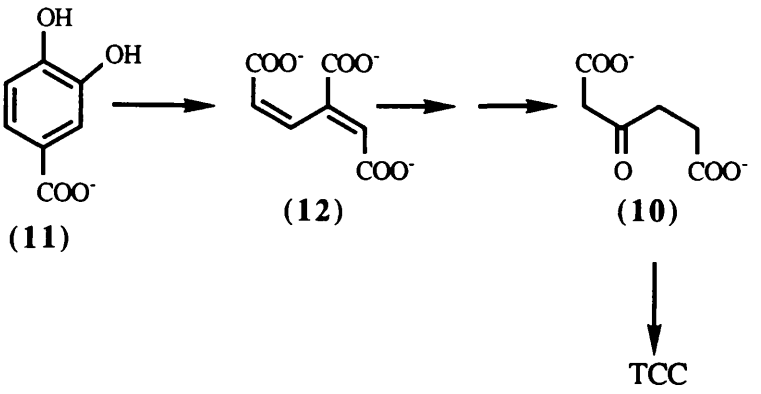
In the following text, we will only cite those which are the most closely related to the muconic acid pathway and the present project.



Scheme 3.

1.1.2 The Muconic Acid Pathways

The 'classical' muconic acid pathway shown in Scheme 3 was established gradually before any attention was given to the characterisation of the relevant enzymes and the stereochemistry of the various steps. The formation of the muconic acid (**7**) as the product of bacterial attack on pyrocatechol (**1**) was first demonstrated by Hayaishi and Hashimoto in 1950 (Ref. 11). The definitive characterization of this acid as *cis,cis*-muconic acid (**7**) followed the elucidation of the chemistry of muconic acids and their lactones by Elvidge *et al.* (Refs. 12, 13). Evans and Smith (Ref. 14) showed that only *cis,cis*-muconate (**7**), and not its stereoisomers, can be converted into 3-oxoadipate (**10**) by extracts of benzoate-induced bacteria and, in collaboration with Linstead and Elvidge (Ref. 15), they reported that such extracts can also form 3-oxoadipate (**10**) from muconolactone (**8**). The intermediary of this lactone (**8**) was confirmed by Sistrom and Stanier (Ref. 16) who separated the enzymes of *Pseudomonas fluorescens*, which convert *cis,cis*-muconate (**7**) into 3-oxoadipate, into two fractions. One fraction catalyzed

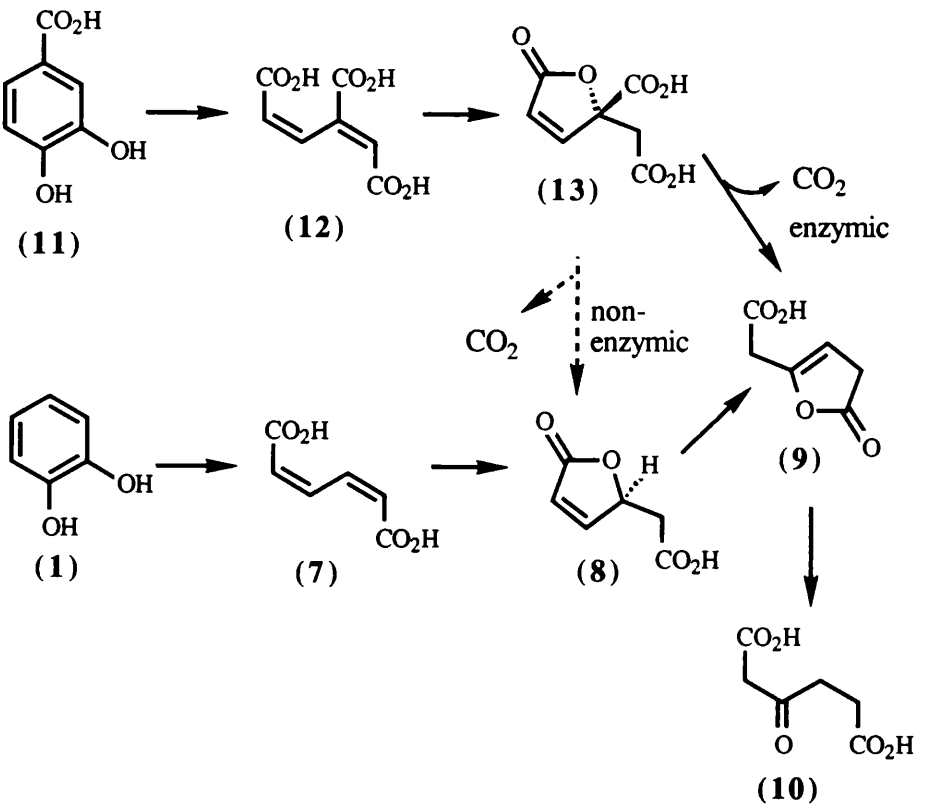


Scheme 4.

the formation of (+)-muconolactone (**8**) from *cis,cis*-muconate (**7**), the other converted (+)-muconolactone (**8**) into 3-oxoadipate (**10**).

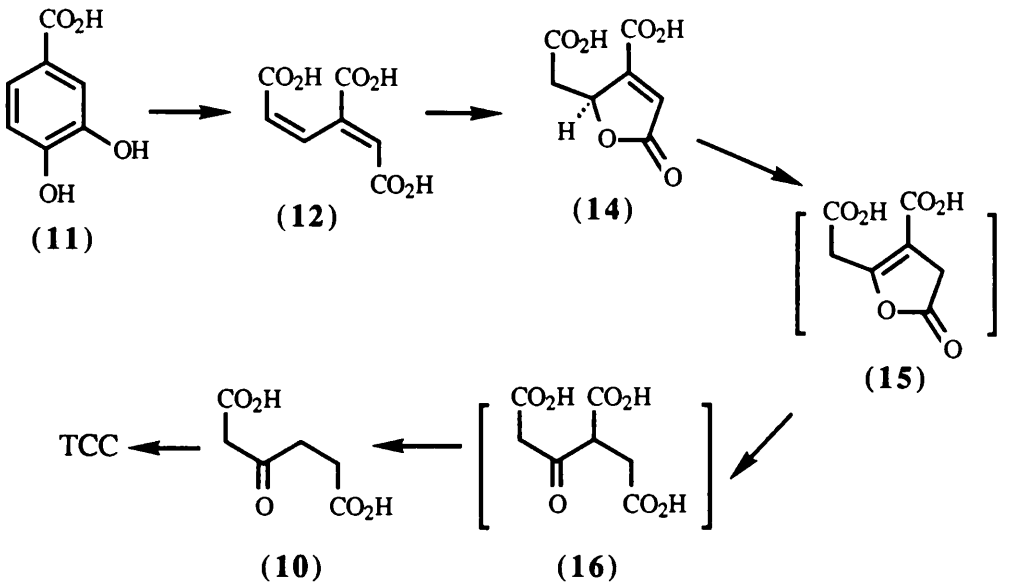
The closely related, protocatechuate (**11**) can be transformed enzymically into 3-carboxy-*cis,cis*-muconate (**12**), which was first identified by MacDonald *et al.* (Ref. 17). They also found that only the *cis,cis*-isomer of the tricarboxylic acid (**12**) was converted into 3-oxoadipate (**10**) (Scheme 4). The microbial degradation pathways provided an interesting example of evolutionary divergence. In bacteria, 3-carboxymucononate (**12**) was metabolized to 4-carboxymuconolactone (**13**), which was then decarboxylated enzymically resulting in the formation of the enol-lactone (**9**) (Scheme 5). Slow, non-enzymic decarboxylation gave muconolactone (**8**). The enol-lactone (**9**) is the first common intermediate in pyrocatechol (**1**) and protocatechuate (**11**) metabolism. The enol-lactone (**9**) is then hydrolysed enzymically to give 3-oxoadipate (**10**) (Ref. 5).

Gross *et al.* were the first to show, using *Neurospora,* that the reactions of the carboxymuconate (**12**) branch deviate in fungi from those in bacteria (Ref. 18). Lactonisation of 3-carboxymuconic acid (**12**) yielded the 3-carboxymuconolactone (**14**), which was subsequently converted into 3-oxoadipate (**10**) *via* the enol-lactone (**15**) (Scheme 6). The steps leading from the lactone (**14**) to the adipate (**10**) will be elucidated later (see Sec. 1.3.2).



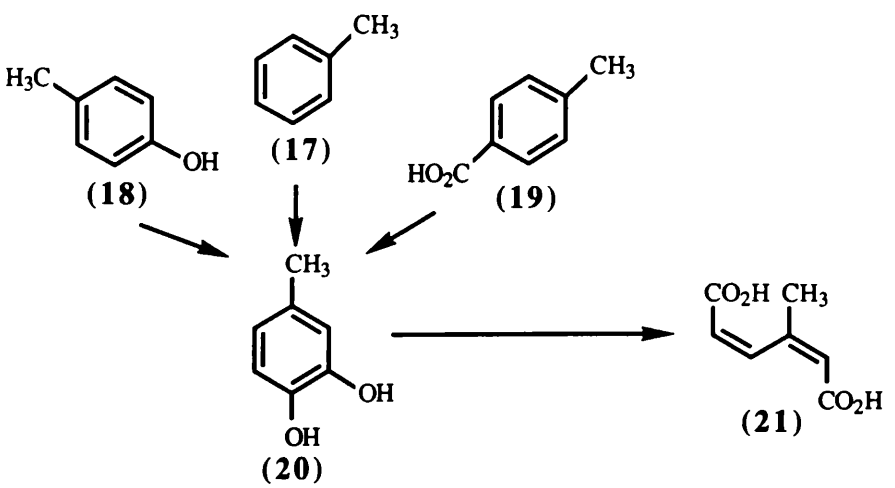
Scheme 5.

Alternatively, 4-methylpyrocatechol (**20**), derived from toluene (**17**) and its derivatives such as *p*-toluic acid (**19**) and *p*-cresol (**18**) which occur in petroleum and industrial wastes, can be transformed microbially into 3-methyl-*cis,cis*-muconic acid (**21**) (Scheme 7). Thereafter, the catabolic pathways (Scheme 8) in bacteria and fungi diverge, in the manner described before for 3-carboxymuconate (**12**). In fungi, cyclisation of 3-methylmuconate (**21**) gives 3-methylmuconolactone (**23**) (Scheme 8f), which is isomerised to 3-methyl-4-oxoadipate (**24**), a catabolic source of pyruvic and acetic acid (Ref. 19). The details of the transformation (**23**) to (**24**) have yet to be elucidated. Until recently, it was thought that the catabolism of 3-methylmuconate (**21**) in bacteria was necessarily incomplete. The cyclisation of compound (**21**) gives 4-methylmuconolactone (**22**), which is not susceptible to delactonization in a manner analogous to that by which the unsubstituted compound (**8**) and 4-carboxymuconolactone (**13**) are degraded,



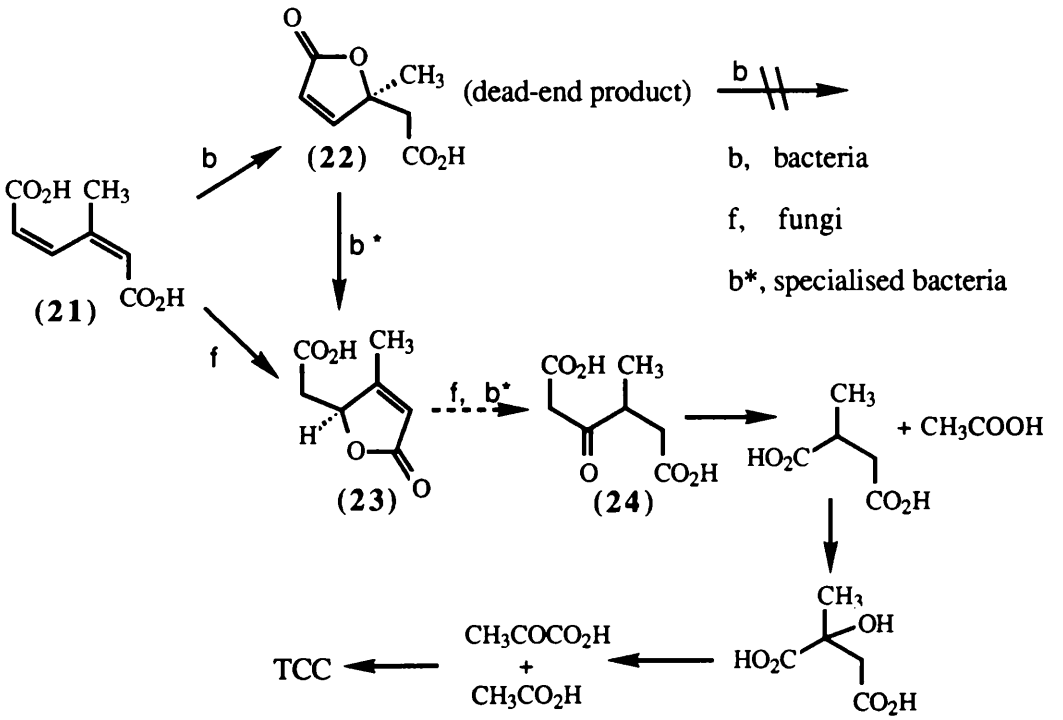
Scheme 6.

because it lacks the proton or carboxy group at C(4) to allow further transformation. Indeed, in bacteria such as *Pseudomonas putida* the 4-methylpyrocatechol (**20**) is subject to unproductive *ortho*-cleavage with accumulation of 4-methylmuconolactone (**22**) as a 'dead-end metabolite' (Refs. 20, 21) (Scheme 8b). However, the discovery (Ref. 22) that certain nocardioform actinomycetes (bacteria) could convert 4-methylmuconolactone (**22**)



Scheme 7.

into the characteristic *fungal* lactone (**23**), led to the identification of a new type of enzymic transformation. A strain of *Alcaligenes eutrophus* (Ref. 23) and several nocardioform actinomycetes (Ref. 24) including *Rhodoccus rhodocrous*, all produce enzymes able to catalyse the isomerisation of the bacterial lactone (**22**) into the fungal lactone (**23**) and thereby overcome the bacterial 'block' (Scheme 8b*). These specialised bacteria have evolved a new strategy to allow complete catabolism of toluene derivatives by a methylmuconate route.



Scheme 8.

1.2 ENZYMOLOGY OF THE MUCONIC ACID PATHWAY

1.2.1 Bacterial Enzymes

The enzymes involved in the metabolism of pyrocatechol and its derivatives have

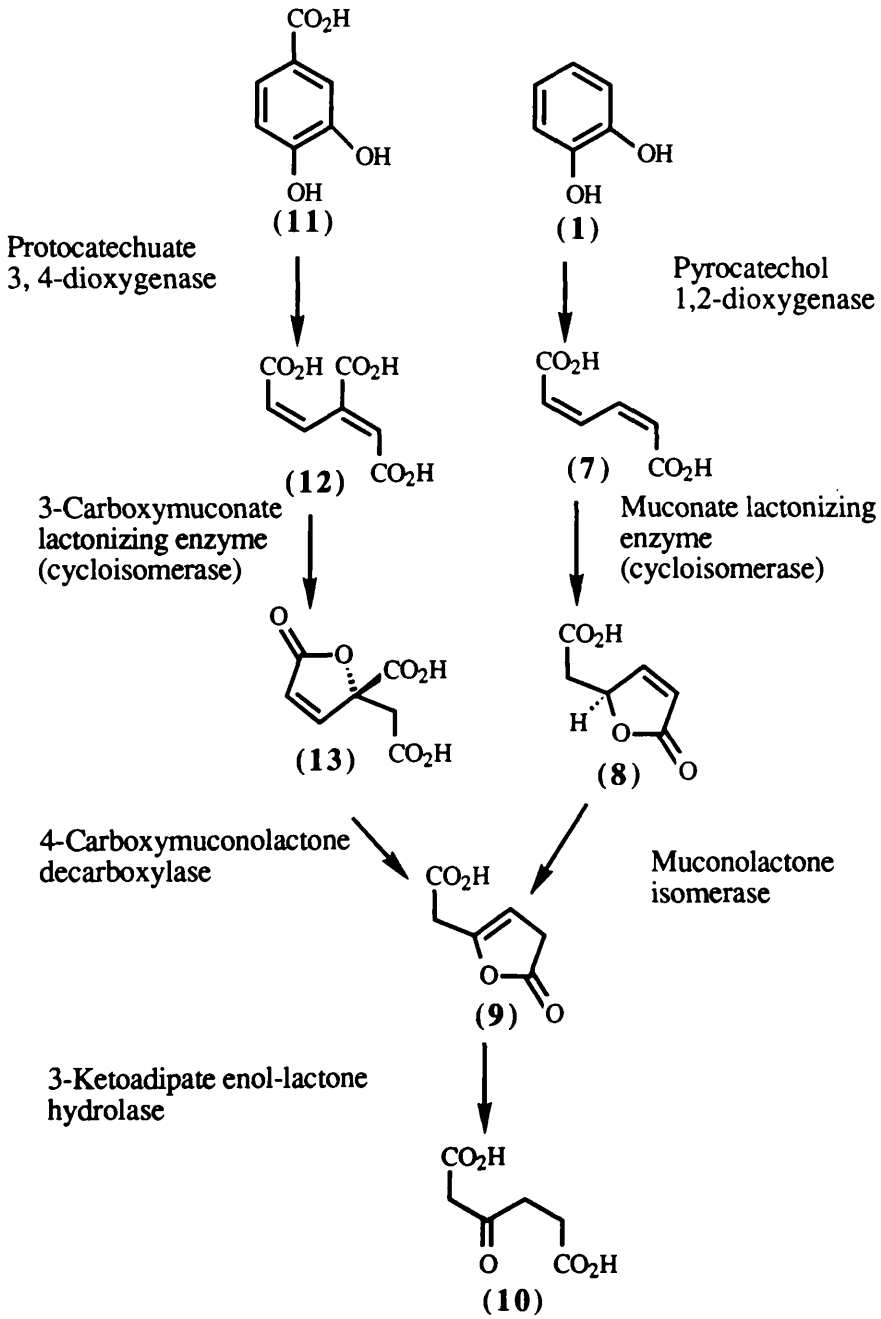
been extensively investigated. A few enzymes have been isolated from bacteria and fungi, and some have been purified and characterized. Each group of organisms has a characteristic type of enzyme. The metabolism of pyrocatechol and protocatechuate in bacteria provides good examples of the enzymology in the muconic acid pathways (Ref.8) (Scheme 9).

(1) Pyrocatechol 1,2-Dioxygenase and Protocatechuate 3,4-Dioxygenase

Pyrocatechol 1,2-dioxygenase was originally purified from a *Pseudomonas* species and it was shown to be a dioxygenase, which can introduce molecular oxygen directly into pyrocatechol (**1**), with the formation of *cis,cis*-muconic acid (**7**) (Scheme 9) (Refs. 14, 25). In 1970, Varga and Neujahr (Ref. 26) isolated pyrocatechol 1,2-dioxygenase from a strain of *Trichosporon cutaneum*. The purified enzyme was homogeneous upon polyacrylamide gel electrophoresis and red in colour as a result of the association of two ferric ions with each molecule of haematic enzyme. Its molecular weight is 109,000 daltons as determined by gel filtration. The pH optimum is 8.0. This enzyme has a broad substrate specificity; besides pyrocatechol, it is able to cleave hydroxyl- and methyl substituted pyrocatechols. This enzyme has biochemical properties resembling those of similar enzymes isolated from other microorganisms (Refs. 27, 28). The analogous enzyme, protocatechuate 3,4-dioxygenase, was purified from extracts of *Pseudomonas aeruginosa* and crystallized by Fujisawa and Hayaishi (Ref. 29). It has a molecular weight of 70,000 daltons, and like pyrocatechol 1,2-dioxygenase, is deep red, because eight ferric ions are bound to each molecule of this enzyme. The X-ray structure was reported in 1984 (Ref. 30). Both dioxygenase contained non-heme iron as a sole cofactor.

(2) *cis,cis*-Muconate-Lactonizing Enzyme and Muconolactone Isomerase

cis,cis-Muconate-lactonizing enzyme and muconolactone isomerase catalyze sequential reactions in the microbial dissimilation of *cis,cis*-muconate (**7**) *via* 3-oxoadipate pathway (Scheme 9). The enzymes are inducible in bacteria and the comparative study of the regulation of their synthesis in different bacterial genera has



Scheme 9.

been the subject of intense investigation (Refs. 8, 31, 32, 33). In 1973, Meagher and Ornston (Ref. 34) reported an improved method for direct isolation of a large quantity of pure *cis,cis*-muconate-lactonizing enzymes and muconolactone isomerase from crude extracts of *Pseudomonas putida* by DEAE-cellulose chromatography. Both enzymes were crystallized after extensive dialysis against distilled water at 5 °C. The subunit size of the *cis,cis*-muconate-lactonizing enzyme is approx. 40,000 daltons, and the amino terminus of this enzyme is threonine. The crystal structure of *cis,cis*-muconate lactonizing enzyme at high resolution (3Å) was reported by Goldman *et al.* (Ref. 35) and Neidhart *et al.* (Ref. 36). Muconolactone isomerase is composed of subunits of about 12,000 daltons. The amino terminus of the isomerase is methionine. The X-ray structure was analysed by Katz *et al.* (Ref. 37).

(3) 3-Carboxy-*cis,cis*-Muconate Lactonizing Enzyme and 4-Carboxymuconolactone Decarboxylase

In procaryotes, 3-carboxy-*cis,cis*-muconate-lactonizing enzyme and 4-carboxy-muconolactone decarboxylase are also inducible; they catalyse reactions chemically analogous to those mediated by *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase respectively (Ref. 38) (Scheme 9). The 4-carboxy-*cis,cis*-muconate lactonizing enzyme was isolated, purified and crystallised by Patel *et al* .(Ref. 39) from the same strain *Pseudomonas putida* from which *cis,cis*-muconate lactonizing enzyme was obtained. They reported that these two enzymes possess similar molecular sizes (190,000 daltons), subunit sizes (40,000 daltons), crystalline shape, and amino terminal sequences. Parke and Meagher (Ref. 40) reported that 4-carboxymuconolactone decarboxylase from *Pseudomonas putida* and muconolactone isomerase from the same strain are the products of homologous structural genes because they catalyse a similar reaction and have similar molecular sizes (93,000 daltons), subunit sizes (12,000 to 13,000 daltons) and crystalline shape (Scheme 9).

(4) 3-Oxoadipate Enol-Lactone Hydrolase

The 3-oxoadipate enol-lactone hydrolase can be isolated from many organisms, *e.g. Acinetobacter calcoaceticus* (Ref. 32) and *Alcaligenes eutrophus* (Ref. 41). Two forms of hydrolase exist, hydrolase I and hydrolase II. It has been shown that these two enzymes are subject to independent induction (Scheme 9). Its crystal structure was reported in Ref. 42.

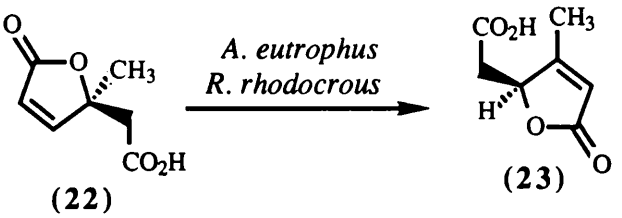
1.2.2 Fungal Enzymes

Only a few enzymes have been isolated from fungi, among them, 3-carboxy-*cis, cis*-muconate cycloisomerase from *Aspergillus niger* (Ref. 43). It has several features in common with the bacterial cycloisomerase. Both are inhibited by the tribasic anions, phosphate and citrate. Both have similar K_m values for their 3-carboxymuconic acid substrates. Both have molecular weights of about 200,000 and both are precipitated between 0.3 and 0.5 M diammonium sulphate saturation. Both are acidic proteins but the fungal enzyme is much more sensitive to thermal inactivation than that from *Pseudomonas putida.*

1.2.3 Specialised Bacterial Enzymes: 4-Methylmuconolactone Methyl-isomerases

4-Methylmuconolactone methylisomerase was observed in a strain of *Alcaligenes eutrophus* in 1985 by Pieper *et al.*(Ref. 23). Recently, they isolated and purified one 4-methylmuconolactone methylisomerase over 700-fold, to electrophoretic and gel chromatography homogeneity, from a constructed derivative of *Pseudomonas* sp. B13, named *Pseudomonas* sp. B13 FR1 (pFRC20P), which contains the gene coding for this isomerase from *Alcaligenes eutrophus* JMP 134 (Ref. 44). It has a molecular weight of 40,000 daltons and consists of a single polypeptide. This enzyme is therefore a

monomer. A similar enzyme was purified and characterized from a nocardioform actinomycetes *Rhodococcus rhodocrous* N75 by Bruce and Cain (Ref. 24). Preliminary purification of the isomerase was effected by heat treatment at 60 °C, which precipitated co-occurring proteins. Further purification was then achieved by successive ion-exchange chromatography, gel filtration, and gel electrophoresis. The enzyme was very thermo stable and had molecular weight of 75,000 daltons (Ref. 45). This enzyme consists of four identical subunits, thus in contrast with monomeric *Pseudomonas* sp. enzyme it is a tetramer. Both enzymes can be inhibited by thiol modifying agents, *e.g.* $CuSO_4$, whereas chelating agents have no effect, *e.g.* dithiothreitol. From the kinetic data obtained, they postulated that two different mechanisms might exist for these two enzymes in the conversion of 4-methyl- (**22**) into 3-methyl- (**23**) muconolactone (see Sec. 1.3.3).



(**22**) (**23**)

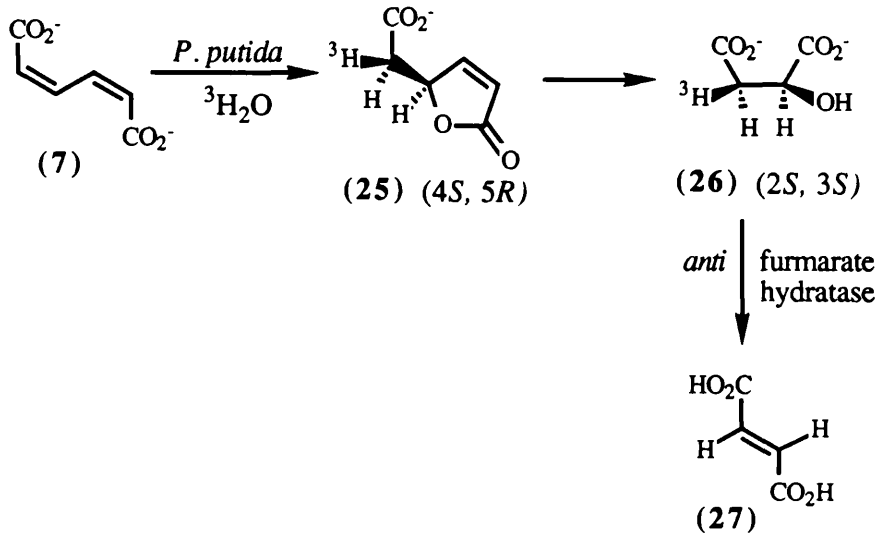
Scheme 10.

1.3 STEREOCHEMISTRY OF SOME ENZYMATIC CONVERSIONS

1.3.1 Lactonization

In 1969, Avigad and Englard first reported that the muconate lactonizing cycloisomerase from *Pseudomonas putida* catalyzed the cycloisomerization of *cis,cis*-muconic acid (**7**) to the muconolactone (**25**) by *syn* addition of carboxyl group to the double bond (Ref. 46) (Scheme 11). The absolute stereochemical course of this reaction was determined by feeding of the *cis,cis*-muconate (**7**) to *Pseudomonas* cultures in tritiated water (Scheme 11). 5-[^{3}H]-Muconolactone (**25**) was obtained enzymically with

no tritium incorporated into the residual *cis,cis*-muconate (**7**). Chemical degradation of the stereospecifically labelled (+)-muconolactone (**25**) yielded tritiated L-malate (**26**) with the same specific activity; treatment of this with fumarate hydratase resulted in release of the tritium into the medium and formation of unlabelled fumarate (**27**). Since this enzyme catalyzes *anti* elimination of (*S*)-malate, the structure of the tritiated (+)-muconolactone (**25**) is (4*S*, 5*R*) configuration, which implies *syn* addition of the carboxyl group to the double bond.

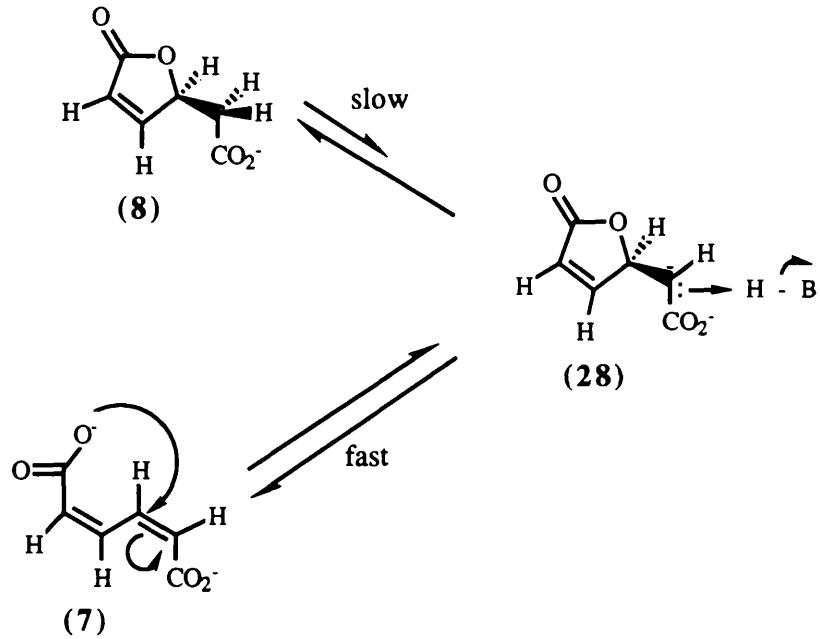


Scheme 11.

In 1983, Ngai *et al.* (Ref. 47) carried out kinetic and equilibrium isotope-effect studies with *cis,cis*-muconate cycloisomerase and found that the formation of a carbanion intermediate (**28**) from the muconolactone (**8**) is the rate determining step during the conversion of (+)-muconolactone (**8**) into *cis,cis*-muconate (**7**).

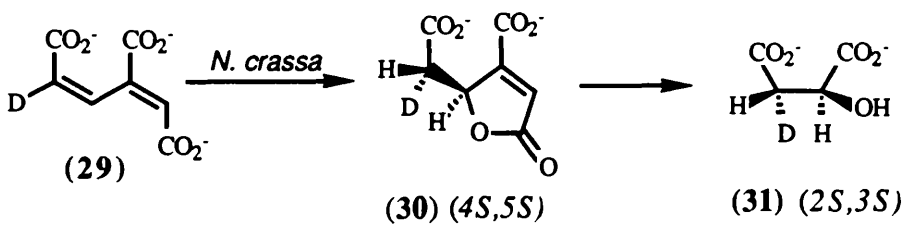
The corresponding carboxymuconate lactonization has been more widely studied. The absolute stereochemistry of the cyclization of 3-carboxy-*cis,cis*-muconate (**29**) into 3-carboxymuconolactone (**30**) in the fungus *Neurospora crassa* was investigated by Kirby *et al.* in 1975 (Ref. 48) (Scheme 13). They incubated the deuteriomuconate (**29**) with a crude preparation of the lactonizing enzyme from *N. crassa* SY4a, isolated the

resulting lactone (**30**), then decomposed this lactone (**30**) by successive treatment with ozone, manganese dioxide and formic acid-hydrogen peroxide to obtain the (2*S*, 3*S*)-[3-^{2}H]malate (**31**). The absolute stereochemistry of this degradation product followed from its optical rotation, and the relative stereochemistry by comparison of its ^{1}H NMR spectrum with that of a standard. Thus the corresponding muconolactone (**30**) had the



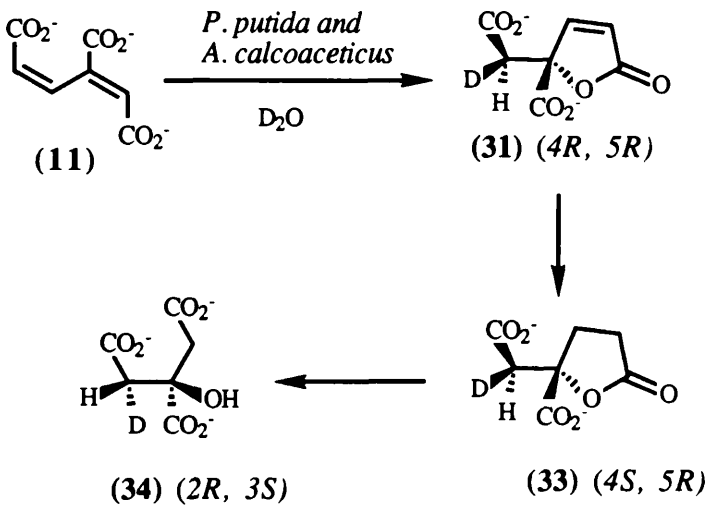
Scheme 12.

(4*S*,5*S*) configuration. This analysis, a variation of the Avigad and Englard procedure, established the reaction as a *syn*-addition with the same stereochemical course as the muconate cycloisomerase.



Scheme 13.

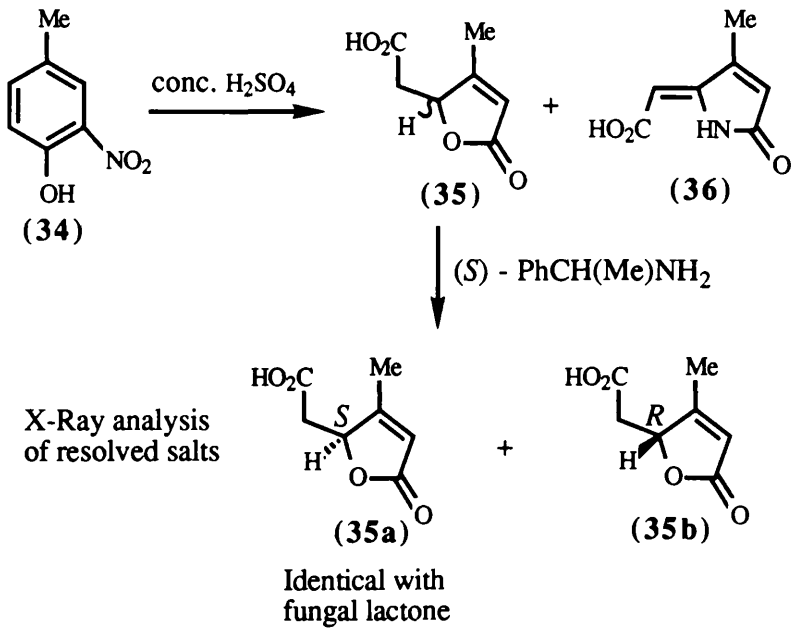
In contrast, Chari *et al.* (Ref. 49) have recently found that the enzymic cyclization of 3-carboxy-*cis,cis*-muconate (**11**), in deuterium oxide, gives the 4-carboxymuconolactone (**32**) in both bacteria *Pseudomonas putida* and *Acinetobacter calcoaceticus*, and occurs by an *anti* addition (Scheme 14). The 4-carboxymuconolactone (**32**) formed is not stable due to its spontaneous decarboxylation, so the absolute configuration was determined by reductive trapping. Catalytic hydrogenation of the lactone (**32**), and chemical degradation of the resulting 5-[^{2}H]homocitrate lactone (**33**) gave the 2-[^{2}H]citrate (**34**). The absolute configuration of 2-[^{2}H]citrate (**34**) was shown to be (*2R,3S*) from its ^{1}H NMR spectrum and hence the lactone (**32**) had the (*4R, 5R*) configuration indicating that the lactonization proceeded by an *anti* addition.



Scheme 14.

The stereochemistry of the early stage of the methylmuconate pathway has recently been studied by Cain *et al.* (Refs. 50, 51). The absolute stereochemistry of two methylmuconolactones, (**22**) and (**23**), were first investigated in 1989. (±)-3-methyl-muconolactone (**35**) was prepared in quantity from 2-nitro-4-methylphenol (**34**) (Ref.52). The muconolactone (**35**) was separated from the by-product (**36**) and resolved by repeated crystallisation of the diastereomeric salts of (*S*)-(-)-2-phenylethylamine. Both

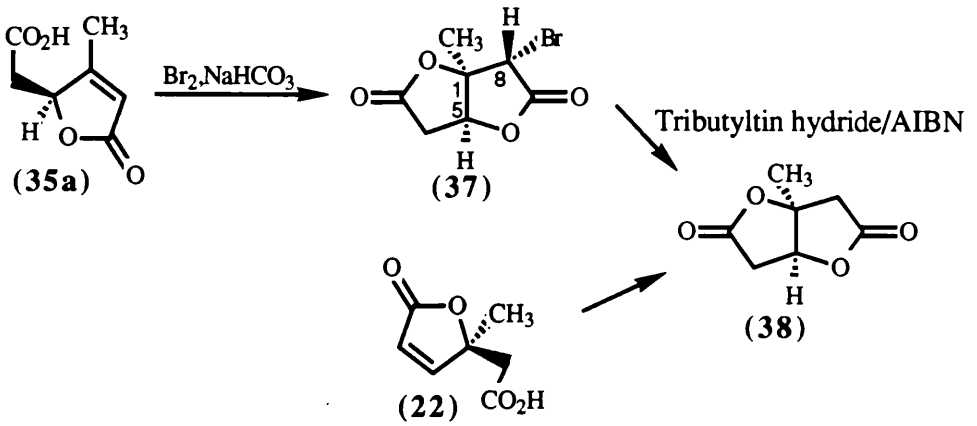
salts were subjected to X-ray crystallographic analysis, thereby determining independently the absolute configurations of the corresponding lactone enantiomers. Each lactone was liberated from its salt and the (*S*)-(-)-lactone (**35a**) was found to be identical with the lactone obtained from an *Aspergillus niger* mutant. This finding was confirmed by feeding of 4-methylpyrocatechol (**20**) to *Aspergillus niger* that accumulated (-)-3-methylmuconolactone (**23**). The muconolactone (**23**) was then purified by recrystallisation and treated with (*S*)-1-phenylethylamine. The resulting salt was shown by X-ray analysis to be identical with the synthetic salt.



Scheme 15.

Determination of the absolute configuration of the bacterial 4-methyl-muconolactone (**22**) was achieved *via* the dilactone (**38**), a possible intermediate in the enzymic, lactone isomerization (**22**) to (**23**). This was confirmed by correlation with the 3-methyl isomer (**35a**) (Scheme 16). The treatment of the (-)-lactone (**35a**) in aqueous sodium hydrogen carbonate (1 mol equiv.) with bromine in dichloromethane gave the (-)-bromodilactone (**37**). X-Ray analysis established the relative configuration and confirmed

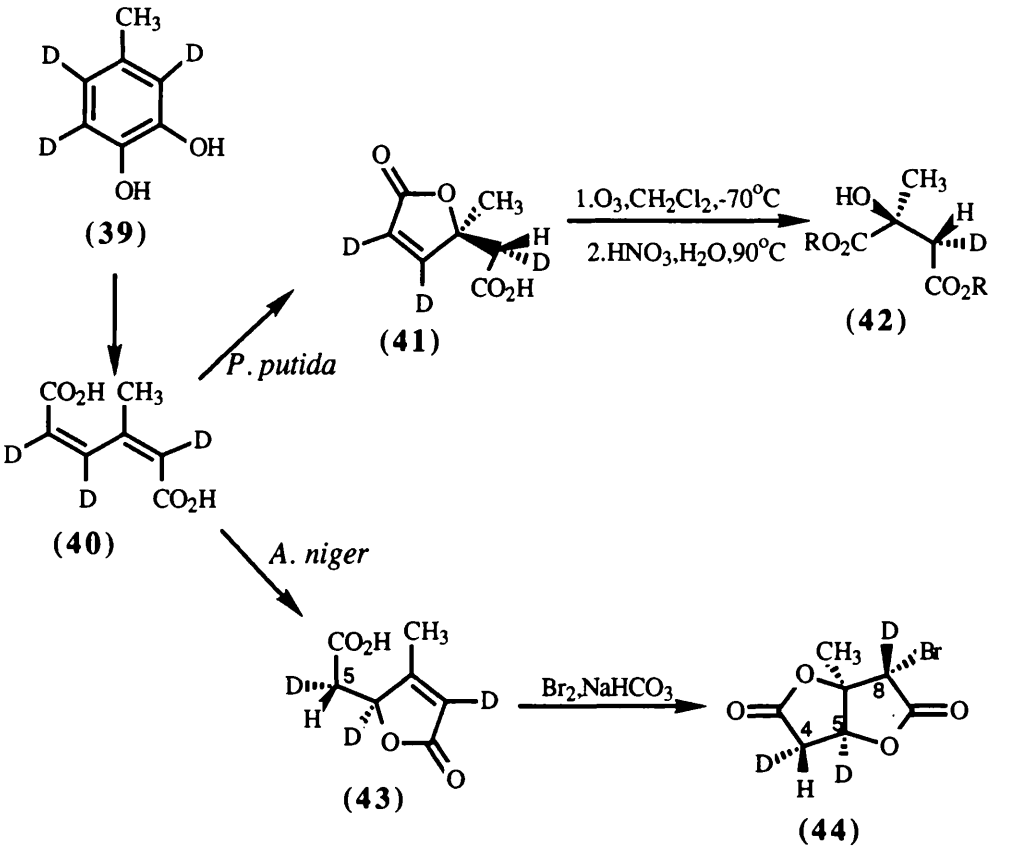
the absolute configuration at C-(5). The (-)-bromodilactone (**37**) reacted with tributyltin hydride (1.2 mol equiv.), in benzene containing azoisobutyronitrile (0.1 mol equiv.) to gave the (-)-dilactone (**38**). The ^1H NMR spectrum, m.p., and optical rotation of the synthetic (-)-dilactone (**38**) agreed well with those reported for the material formed by non-enzymic cyclization of natural (+)-4-methylmuconolactone (**22**), which must be assigned the 4*S* configuration (**22**).



Scheme 16.

With the synthesis and stereochemistry of the appropriate substrates established, attention was turned to the stereochemistry and mechanism of the enzymic reactions of the methylmuconolactone pathways (Scheme 17). Trideuteriopyrocatechol (**39**) was used as a substrate to determine the stereochemistry of the lactone formation in a typical fungus *Aspergillus niger*, and in the strain of bacterium *Pseudomonas putida* (ATCC 12633) used in the studies on the classical muconate and carboxymuconate pathways. The trideuteriopyrocatechol (**39**), prepared by exchange in D_2O-DCl, was fed in batches to a mutant strain of *Aspergillus niger* which lacked the enzyme muconolactone cyclo-isomerase and was known to accumulate 3-methylmuconolactone (**23**). The ^1H NMR spectrum of the resulting lactone (**43**) showed, as expected, that highly stereoselective cyclization had occurred. The absolute stereochemistry at C-(5) was determined using the

rigid bromodilactone (**44**). Unambiguous assignment of the C-(4) methylene signals in the ^1H NMR spectrum followed from the near-zero, vicinal coupling between the trans protons 4-H_R and 5-H [dihedral angle H(5)-C(5)-C(4)-H_R(4) 99°]. The ^1H NMR spectrum of the bromodilactone (**44**) showed a signal, δ 2.89 (t, $J_{H,D}$ 2.8 Hz), corresponding to 4-H_R, and the complementary ^2H NMR spectrum showed a signal, δ 3.34 (t, $J_{H,D}$ 2.9 Hz), corresponding to 4-D_S. Therefore enzymic cyclization in *Aspergillus niger* must have occurred by *syn* addition.



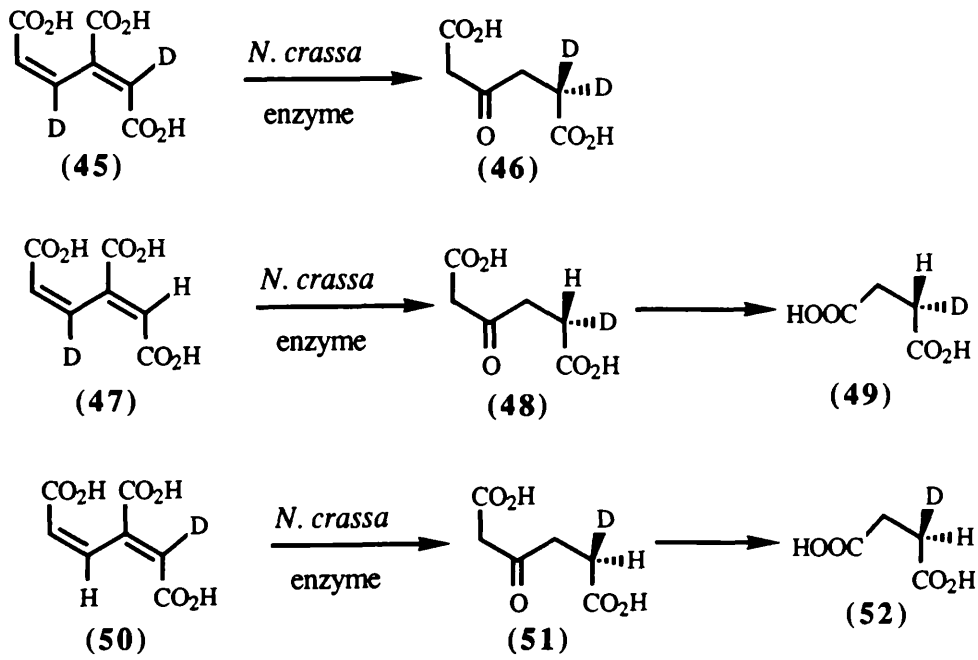
Scheme 17.

Similarly, the pyrocatechol (**39**) was fed to *Pseudomonas putida* (ATCC 12633). The absolute stereochemistry of the resulting deuteriated 4-methylmuconolactone (**41**) was established by oxidative degradation to the citramalic acid (**42**). The ^1H NMR spectrum of dimethyl monodeuteriocitramalates, having an unambiguously determined

relative configuration, has been reported by Muhll *et al.* (Ref. 53). The degradation product (**42**, R=Me) was therefore esterified with diazomethane; the proton spectrum corresponded closely with that reported in the literature. Thus, cycloisomerization of 3-methyl-*cis,cis*-muconate (**40**) in *Pseudomonas putida* proceeds by *syn*-addition to form a (4*S*)-lactone (**41**). This result contrasts unexpectedly with the *anti* cyclization of the carboxymuconic acid (**12**) in the same organism.

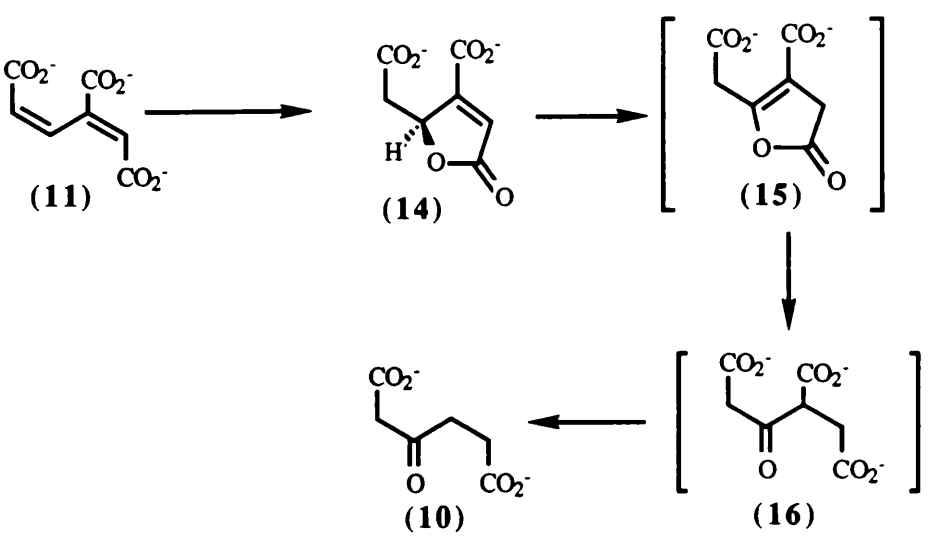
1.3.2 Conversion of 3-Carboxymuconolactone into 3-Oxoadipic Acid

In 1977, Hill *et al.* (Ref. 54) incubated the deuteriated 3-carboxymuconic acid (**45**) with a cell-free preparation from *Neurospora crassa* to afford the 3-oxoadipic acid (**46**) with, surprisingly, retention of both deuterium atoms (Scheme 18). Similarly, the monodeuteriated acids, (**47**) and (**50**), gave monodeuteriated products (**48**) and (**51**). The intermediate carboxymuconolactones were not isolated.



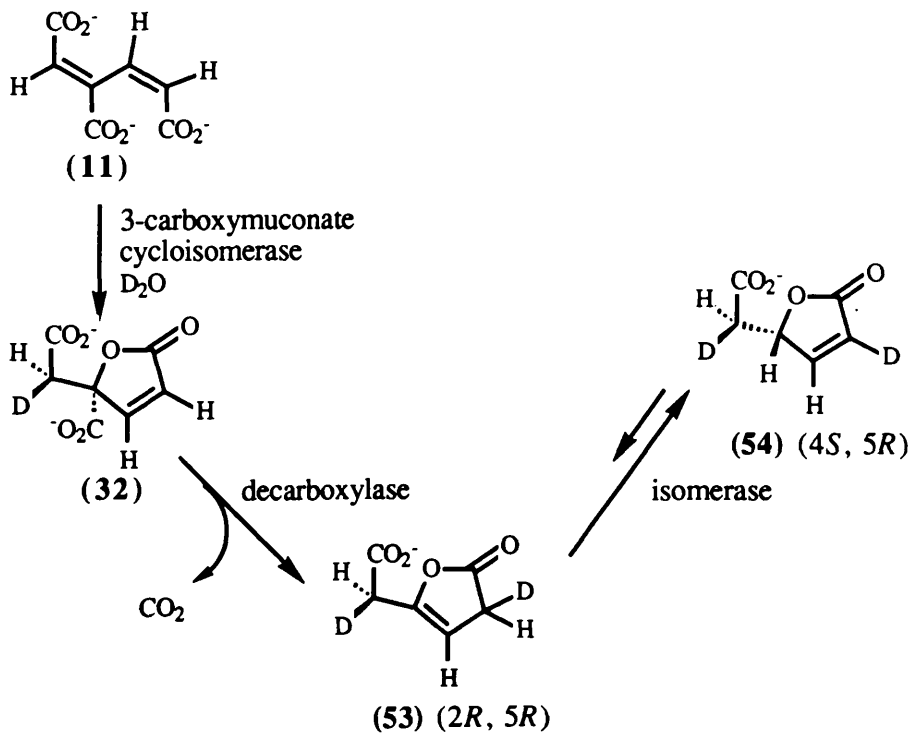
Scheme 18.

When the oxoadipic acid (**49**) enzymically obtained from the muconic acid (**47**) was degraded with aqueous alkali, the resulting 2-deuteriosuccinic acid (**49**) had the configuration shown in Scheme 18. This was established by comparison of the optical rotation at various wavelengths with that of a standard. Thus the product (**49**) was (*S*)-(+)-2-deuteriosuccinic acid. In the complementary experiment, the muconic acid (**50**) was converted *via* (**51**) into (*R*)-(-)-2-deuteriosuccinic acid (**52**). Incubation of an equal mixture of dideuterio and diprotio muconic acid gave 3-oxoadipic acid containing equal amounts of dideuteriated and diprotio species (mass spectral analysis). Thus, migration of deuterium must be intramolecular. These results strongly suggested that the first step in the enzymic degradation of the intermediate carboxymuconolactone (**14**) involves a 1,3-suprafacial migration of hydrogen (or deuterium) (Scheme 19), probably effected by a basic group on the enzyme, to form an enol-lactone (**15**). Hydrolysis of (**15**) and decarboxylation of the 3-keto-acid (**16**) would complete the process. All attempts to separate this multifunctional enzyme into components with different activities have failed and, so far, the postulated intermediates (**15**) and (**16**) have not been detected (Ref. 55). Apparently, the eukaryotes have evolved a more sophisticated catalytic system than the prokaryotes for the degradation of 3-carboxymuconate (**11**).



Scheme 19.

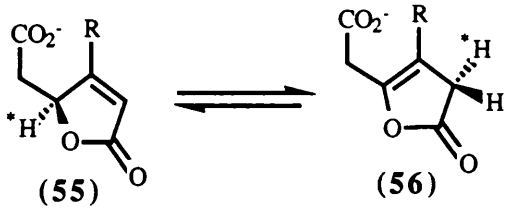
Recently, a similar 1,3-proton shift has been shown to occur in the reversible interconversion of the parent muconolactone (**54**) with the enol-lactone (**53**) (Scheme 20) (Ref. 56), since the equilibrium favours the former, it was necessary to prove that migration occurred in the direction (**53**) ⇒ (**54**). The carboxylactone (**32**) was formed enzymically in deuterium oxide from (**11**) and incubated with the decarboxylase and isomerase enzymes. The successive reactions were monitored directly by ^{1}H NMR spectroscopy. Eventually, a simple spectrum indicated near quantitative formation of the (4*S*,5*R*)-2,5-dideuteriomuconolactone (**54**). The lack of a significant resonance for 2-H (δ 6.02) established a nearly exclusive proton transfer during the transformation (**53**) ⇒ (**54**). The final spectrum also confirmed the stereochemistry at C-(5) as *R* since an identical, asymmetric collapse of the C-(5) AB quartet was observed for the reaction of *cis,cis*-muconate (**11**) with the cycloisomerase, which yielded (4*S*,5*R*)-5-deuteriomuconolactone. The stereochemistry at C-(2) in enol-lactone (**53**), formed



Scheme 20.

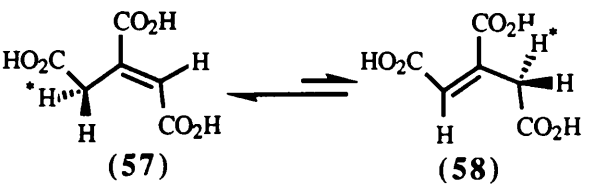
quantitatively from 4-carboxymuconolactone (**32**) by decarboxylase, was determined unambiguously by ^1H NMR spectroscopy and thus had (2*R*,5*R*) configuration. Therefore the intramolecular 1,3-shift of deuterium is almost certainly *syn* rather than *anti*.

There are therefore two examples of 1,3-proton shifts shown to occur in the muconic acid pathways, namely, (**55**) ⇔ (**56**), where R = H or CO_2^- (Scheme 21). A similar shift is involved, with the same absolute stereochemistry, in the interconversion of the aconitic acids (**57**) and (**58**), although here there is almost complete exchange of the migration proton with the medium (Ref. 57).



(H^* retained in molecule)

cf.



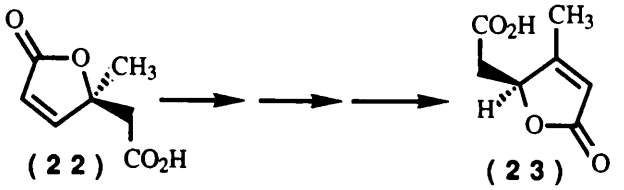
(H^* exchanges with medium)

Scheme 21.

1.3.3 Conversion of 4-Methylmuconolactone (22) into 3-Methylmuconolactone (23).

It was found that the bacterium *Alcaligenes eutrophus* and several naturally occurring nocardioform actinomycetes including *Rhodococcus ruber*, all produced an enzyme able to catalyze the transformation of the 4-methyl-lactone (**22**) into the 3-methyl-

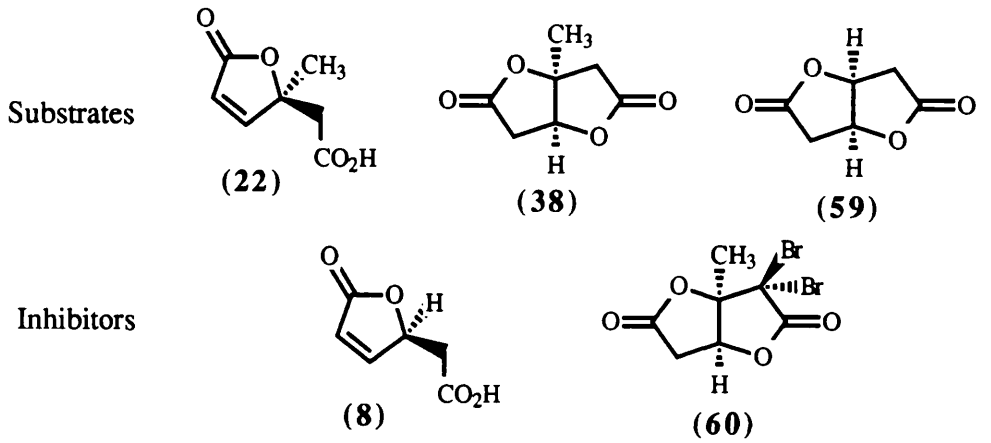
lactone (**23**) (Scheme 22). This has led to the investigation of a new type of mechanism for enzymic transformation.



Scheme 22.

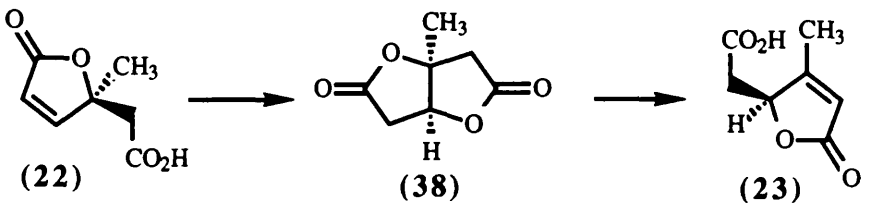
Using *Rhodococcus rhodocrous* N75, a nocardioform actinomycetes, Bruce *et al.* (Ref. 45) showed that this bacterium contains a novel enzyme which has the property of catalysing the conversion of 4-methylmuconolactone into its 3-methyl isomer. They isolated and purified this methylisomerase and showed it to be highly specific with respect to the interconversion of its substrates (Scheme 23). This enzyme only accepts (+)-(4*S*)-4-methylmuconolactone (**22**), the putative isomerization intermediate 1-methyldilactone (**38**), and simple dilactone (**59**) as substrates, and yielding (-)-(4*S*)-3-methylmuconolactone (**23**) and (*S*)-muconolactone as product. The dilactone (**59**) has a higher turnover rate (2.2 times higher) than 4-methylmuconolactone (**22**) itself. (+)-Muconolactone (**8**) is a moderately effective, competitive inhibitor while the dibromodilactone (**60**) is a strong irreversible inhibitor. The transformation (**22**) ⇒ (**23**) proceeds quantitatively, although in principle it should be reversible.

Significantly, the purified enzyme exhibited no activity towards either the *cis,cis*- or the 2-*cis*,4-*trans*- isomer of 3-methylmuconic acid; neither were they effective competitive inhibitors. This excluded the possibility that the interconversion of the 4-methyl- to 3-methylmuconolactone first involved ring opening followed by recyclization in the opposite sense either directly or after isomerization to the 3-methyl-2-*cis*-4-*trans*-muconate. From kinetic data obtained with this enzyme, Bruce *et al.* postulated that a 1-methyldilactone (**38**) was an intermediate in the overall isomerization, but perhaps enzyme-bound, linked to a thiol group at the active site (Scheme 24). This would account



Scheme 23.

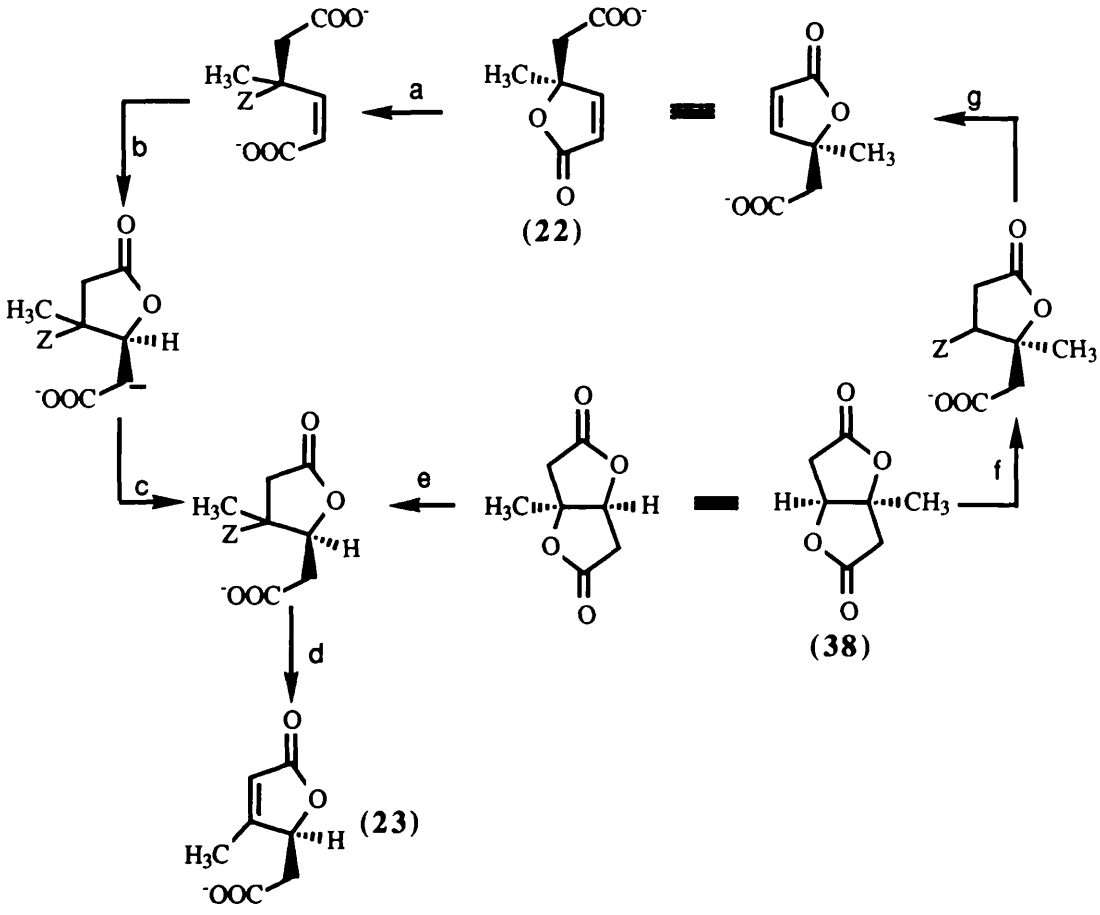
for the extreme sensitivity of the enzyme to heavy metals and thiol reagents. However the stereochemistry and mechanism for closure and opening of the dilactone (**38**) remained to be elucidated.



Scheme 24.

In contrast with the mechanism of Bruce *et al*, Pieper *et al.* (Ref. 44) postulated somewhat different mechanism for this interconversion of 4-methyl- to 3-methyl-muconolactone catalysed by the similar isomerase isolated from their organism *Pseudomonas* sp B13 (pFRC20P). They excluded the possibility of involving either methylmuconate (**21**) or the methyldilactone (**38**) as *free* intermediates, because (1) 3-methyl-*cis,cis*-muconate (**21**) was not a substrate for the enzyme, and (2) the 1-methyldilactone (**38**) was converted more slowly into 3-methylmuconolactone (**23**) than was 4-methylmuconolactone (**22**) itself. Kinetic data also showed that non-covalently

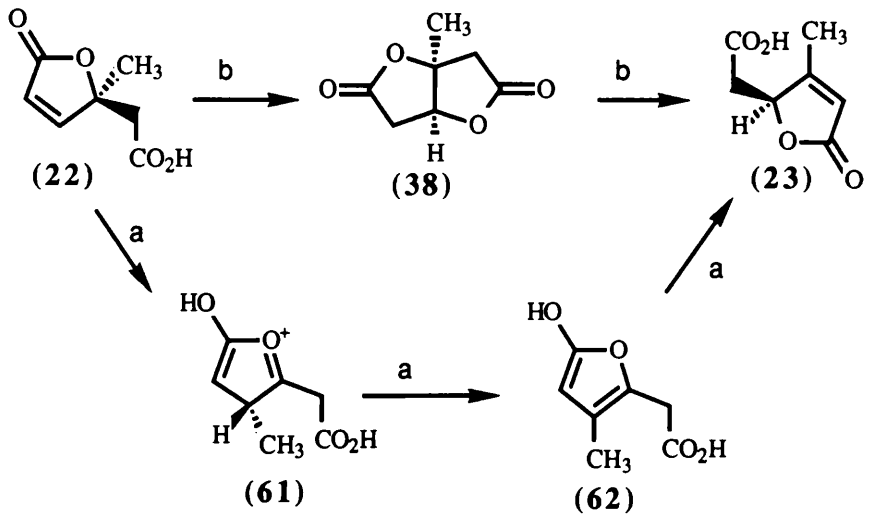
enzyme-bound 1-methyldilactone is unlikely to be an intermediate in the overall reaction. By analogy with the analysis of Ngai *et al.* (Ref. 49), they suggested that the enzymic conversion of 4-methyl into 3-methyl muconolactone, (**22**) ⇒ (**23**), takes place through the reaction steps a-d (Scheme 25), similar to those described by Ngai *et al.* for the cycloisomerization of *cis, cis*-muconate (**7**) to corresponding muconolactone (**8**). The 1-methyldilactone (**38**) could be directly isomerized to 3-methylmuconolactone (**23**) (step e-d) or transformed into 4-methylmuconolactone (**22**) (step f and g). Z represents an OH group or an enzyme nucleophile, perhaps the thiol group.



Scheme 25.

1.4 THE PROPOSED RESEARCH PROJECT

The novelty of the methylisomerase reaction (**22**) to (**23**) prompted us to investigate the stereochemistry and mechanism of this isomerization using the enzyme from *Rhodococcus rhodocrous*. Two mechanistically distinct classes of pathway merit consideration for this enzyme-catalysed isomerization of (4*S*)-4-methylmuconolactone (**22**) as shown in Scheme 26.

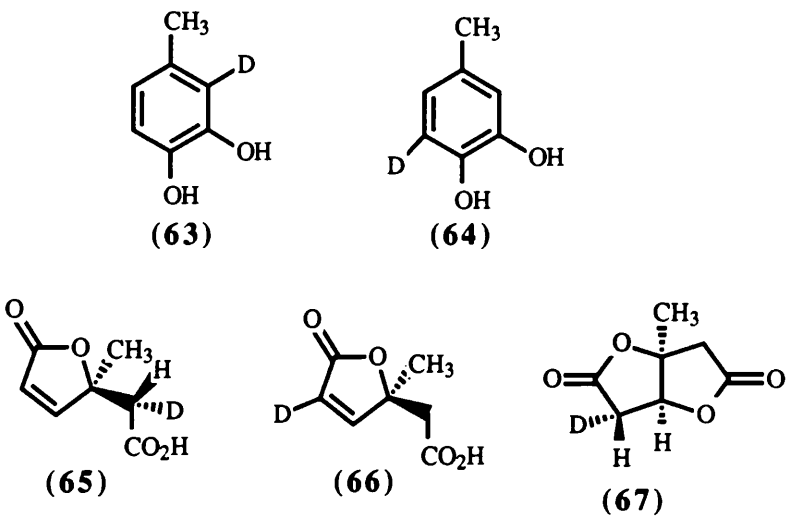


Scheme 26.

In pathway a, protonation of the lactone carbonyl group of the substrate (**22**) might be followed by a 1,2-shift of the methyl group from C-(4) to C-(3) to give the oxygen-stabilised carbocation (**61**). Loss of a proton from C-(3) would then generate the hydroxyfuran (**62**) [a reverse 1, 2-shift of the hydrogen would produce the inappropriate *R*- configuration at C-(4)]. Enzyme-catalysed reprotonation of (**61**) at C-(4) would afford the lactone tautomer (**23**). The alternative pathway b cannot involve initial ring opening to give either *cis,cis*- or 2-*cis*,4-*trans*-3-methylmuconic acid as a free intermediate, since, as explained before, neither acid is a substrate for the enzyme (Ref. 45). Consequently the simplest pathway not involving an enzyme-bound intermediate proceeds by initial ring

closure to give the dilactone (**38**). Ring-opening in the alternative sense would then give the lactone (**23**) having the (4*S*)-configuration demanded by a *cis*-ring fused intermediate. In support of pathway b, the dilactone (**38**) was shown to be an effective substrate for the enzyme, as was the parent dilactone (**59**) as described in the proceeding Sec. 1.3.3. Also, the dilactone (**38**) was transformed faster than the substrate (**22**).

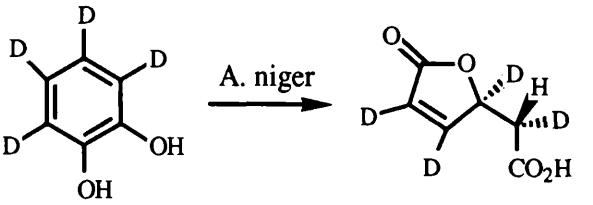
To explore the mechanism and stereochemistry involved in the muconic acid pathways, the present investigation therefore had as its first priority the design of experiments to distinguish unambiguously between the two classes of pathway illustrated in Scheme 26 and to establish the relative stereochemistry (*syn* or *anti*) of lactone ring closure and opening diagnostic of the pathway b. To this end, the deuterium labelled compounds (**63**) and (**64**) were fed to *Pseudomonas putida* to give the optically active lactones (**65**), and (**66**) (Scheme 27). The latter was chemically converted into dilactone (**67**). These three compounds (**65**), (**66**), and (**67**) were used as substrates for the 4-methylisomerase.



Scheme 27.

In a separate study, the stereochemistry of *cis,cis*-muconic acid cycloisomerase in fungi was determined for the first time, to complete the study of the set of 6

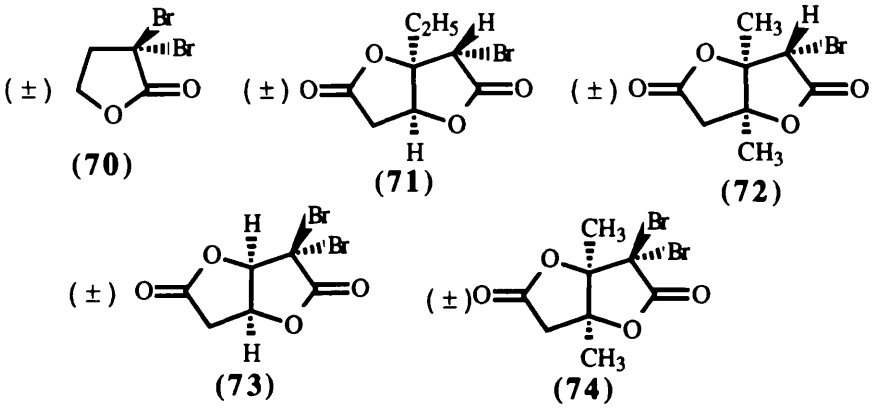
cycloisomerase enzymes (3 in bacteria and 3 in fungi). As a consequence, the fully deuteriated pyrocatechol (**68**) was fed to the mutant fungus *Aspergillus niger*, which accumulates muconolactone.



(68) **(69)**

Scheme 28.

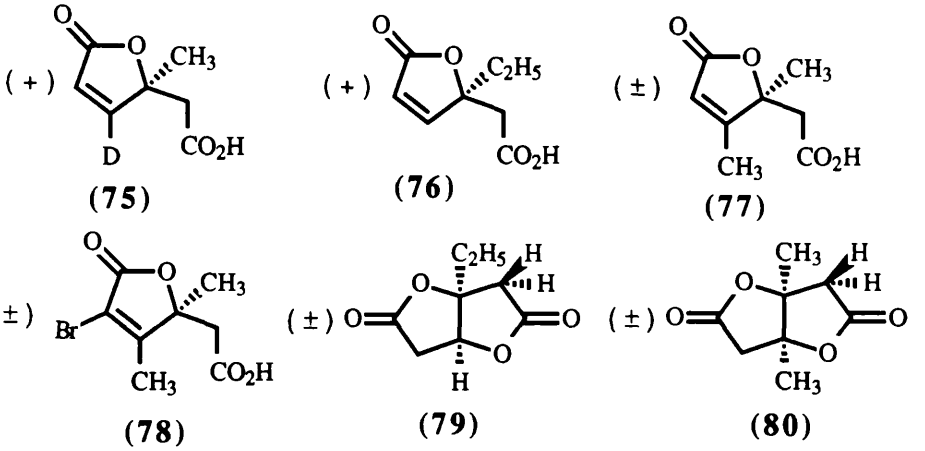
As described in Sec. 1.2.3 and Sec. 1.3.3, the dibromodilactone (**60**) is an irreversible inhibitor of the methylisomerase, and the inhibition probably involves a thiol group in the enzyme, which can be inhibited by heavy metal ions. The following compounds, shown in Scheme 29, were designed and prepared to be tested as new inhibitors. The dibromo compounds (**70**), (**73**) and known inhibitor (**60**) were reacted with thiol compounds, benzyl mercaptan and cysteine, to study the inhibition mechanism chemically.



(70) **(71)** **(72)** **(73)** **(74)**

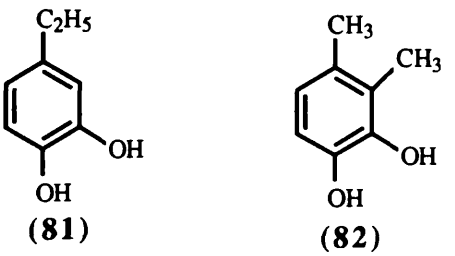
Scheme 29.

Some other structurally modified substrates (**75**) to (**80**) were also made to explore the shape and the size of the active site of the methylisomerase.



Scheme 30.

The compounds (**81**) and (**82**) (Scheme 31) were prepared to test as precursors for both the bacterium *Pseudomonas putida* and the fungus *Aspergillus niger* this time, to explore the shape and size of the active site of the dioxygenase and cycloisomerases.



Scheme 31.

Finally, the non-enzymic cyclization of the muconolactone (**8**) and 4-methyl-muconolactones (**22**) was reported (Refs. 20, 68). The same phenomenon was observed for the 4-ethylmuconolactone (**76**) by the author. The cyclization of 3,4-dimethyl-muconolactone (**77**) under various conditions was studied as well.

CHAPTER TWO

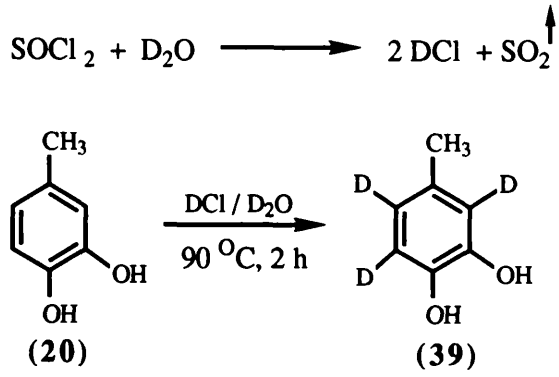
RESULTS AND DISCUSSIONS

This chapter presents the results and discussions of experiments designed to elucidate the muconic acid pathways. Chemical synthesis of the substrates and intermediates required for biological investigations is discussed in Sec. 2.1. Sec. 2.2 then describes a set of optically active muconolactones prepared biologically from the pyrocatechol derivatives using both bacteria and fungi. In Sec. 2.3, attention is focused on the elucidation of the mechanism and stereochemistry of the muconic acid pathways by using the data from the experimental results.

2.1 CHEMICAL SYNTHESIS

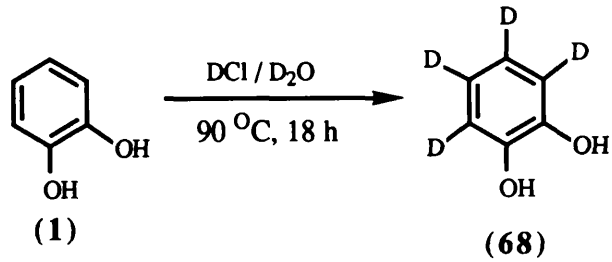
2.1.1 Full Deuteriation of Pyrocatechol

Deuterium chloride is a useful agent which can replace hydrogen with deuterium in activated aromatic rings. Cain *et al.* (Ref. 51) reported a simple method to fully deuteriate 4-methylpyrocatechol (**20**), by exchange in 4 M deuterium chloride solution at 90 °C for 2 h (Scheme 32). The deuterium chloride solution was prepared in a similar way to that described by Thomas *et al.* (Ref. 59). The required amount of thionyl chloride was added to an access of deuterium oxide at 0 °C, and the sulphur dioxide which formed was swept out of the solution by dry nitrogen.



Scheme 32.

Likewise, pyrocatechol (**1**) itself was deuteriated in 4.1 M deuterium chloride solution at 90 °C overnight (Scheme 33). The purity of the product was determined by ^{1}H NMR spectroscopy. The spectra of the starting material (**1**) and product (**68**) were obtained at the same concentration and the integration of the residual aromatic proton showed that the 3,4,5,6-tetradeuteriopyrocatechol (**68**) was almost fully deuteriated.

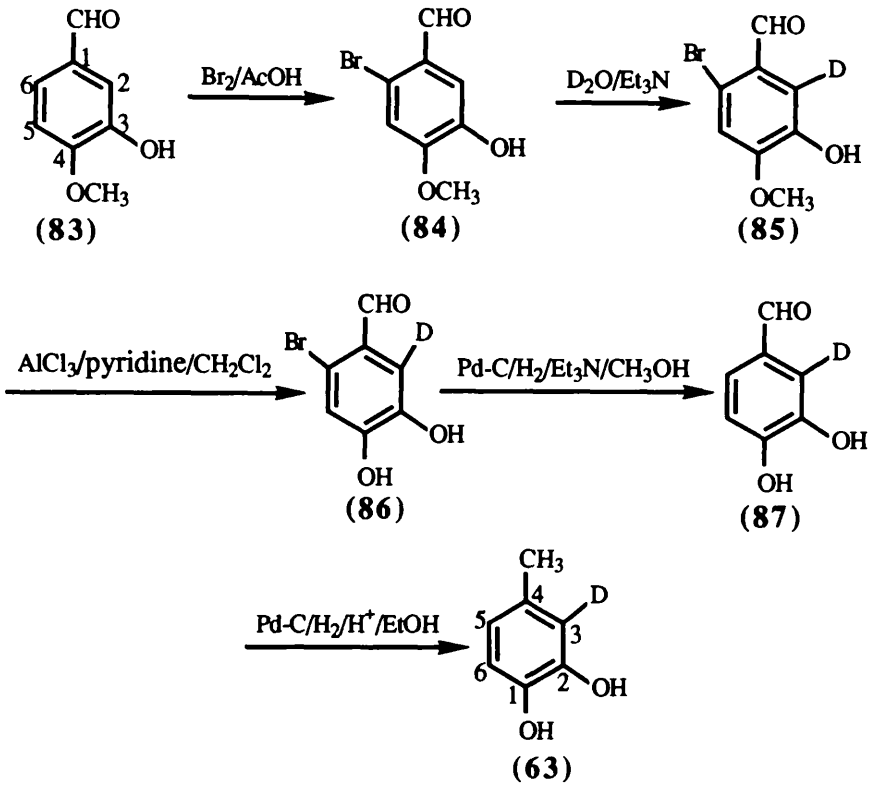


Scheme 33.

2.1.2 Three Monodeuteriated 4-Methylpyrocatechols

(1) 4-Methyl-[3-^{2}H]pyrocatechol (63)

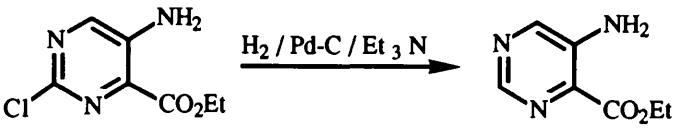
4-Methyl-[3-^{2}H]pyrocatechol (**63**) was prepared from isovanillin (**83**) as follows (Scheme 34). Although in isovanillin (**83**) the positions *ortho* and *para* to the phenolic



Scheme 34.

hydroxyl group are strongly deactivated by the the aldehyde group towards electrophilic substitution, the deuteriation under the normal conditions still occurred, but was slow. The exchange was more rapid at position 2 than at position 6 and was not observed at position 5 (Ref. 60). To obtain pure 2-deuterio-product the position 6 was protected by bromination. The bromination of isovanillin (**83**) by bromine in acetic acid gave 2- and 6-bromoisovanillin, which were separated by repeated recrystallisation (Ref. 61). Then 6-bromoisovanillin (**84**) was deuteriated with deuterium oxide containing triethylamine as catalyst under gentle reflux under nitrogen overnight. The ^1H NMR spectrum of the product showed that complete deuteriation had occurred (Ref. 60). The deuteriated compound (**85**) was demethylated with aluminium chloride in dichloromethane containing pyridine under reflux overnight to give 2-deuterio-6-bromoprotocatechuic

aldehyde (**86**) (Ref. 62). As Gallemaers *et al.* (Ref. 63) found that compound (**88**) was dechlorinated by hydrogenation over palladium-carbon and triethylamine under atmosphere pressure (Scheme 35), 2-deuterio-6-bromoprotocatechuic aldehyde (**86**) was likewise debrominated in similar conditions to yield 2-deuterio protocatechuic aldehyde (**87**) in good yield without significant loss of deuterium. Hydrogenation of compound (**87**) over palladium-carbon in ethanol containing a catalytic amount of hydrochloric acid gave the target product, 4-methyl-[3-^{2}H]pyrocatechol (**63**).

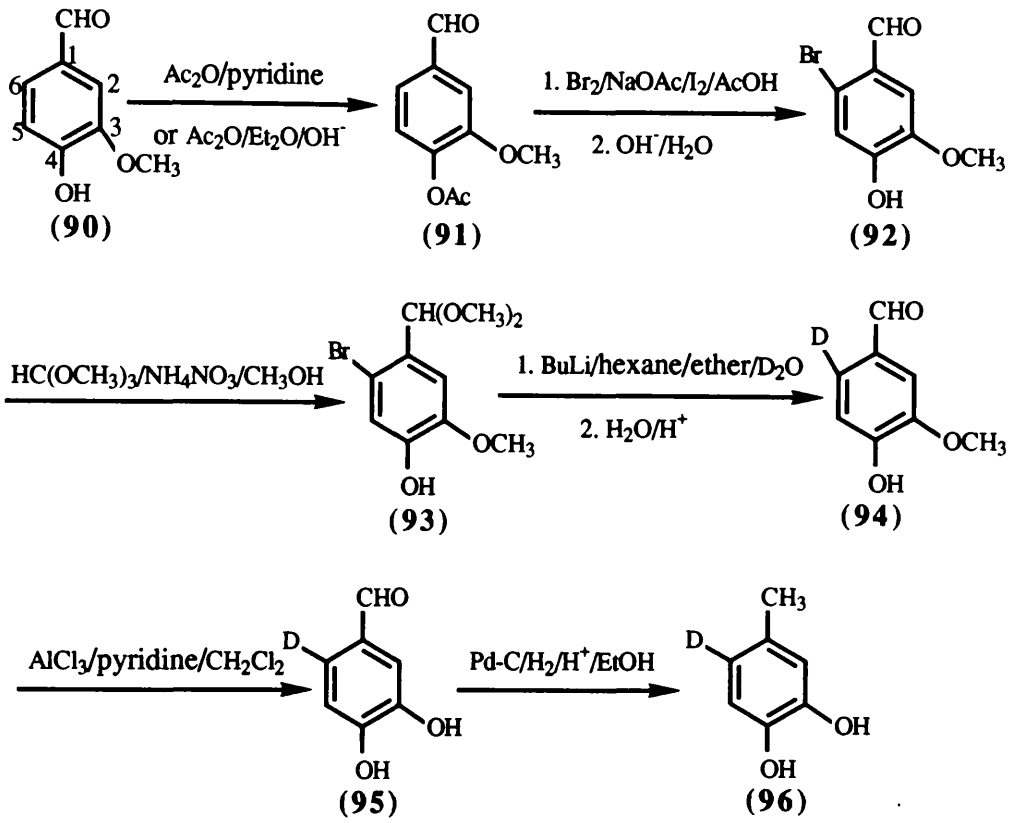


(**88**) (**89**)

Scheme 35.

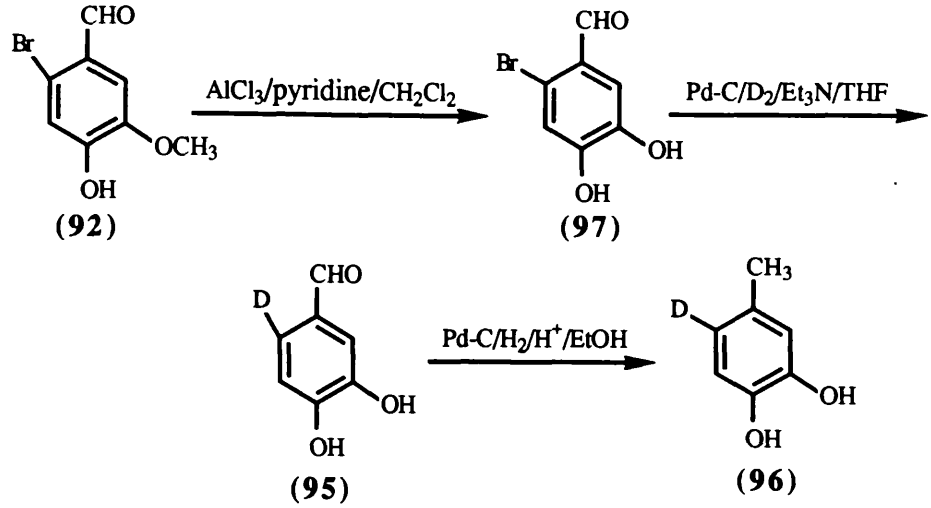
(2) 4-Methyl-[5-^{2}H]pyrocatechol (96)

The first attempt at the preparation of 4-methyl-[5-^{2}H]pyrocatechol (**96**) involved the route outlined in Scheme 36. Freudenberg *et al.* (Ref. 64) prepared [6-^{2}H]vanillin (**94**) as an intermediate in the synthesis of deuteriated coniferyl alcohol. This compound was prepared in the same way using vanillin (**90**) as starting material. Successive acetylation and bromination of vanillin (**90**) gave 6-bromovanillin (**92**) (Ref. 65), which was refluxed with methanolic trimethyl orthoformate in the presence of ammonium nitrate to yield the dimethyl acetyl (**93**). The compound (**93**) was then treated with butyllithium in ether and the lithiated intermediate was decomposed with deuterium oxide. Hydrolysis then gave [6-^{2}H]vanillin (**94**) (Ref. 66). Demethylation and hydrogenation of [6-^{2}H]vanillin (**94**) under the normal conditions yielded 4-methyl-[5-^{2}H]pyrocatechol (**96**). However, the deuteriation of compound (**93**) was difficult to control and gave a poor yield of rather impure product (**94**).



Scheme 36.

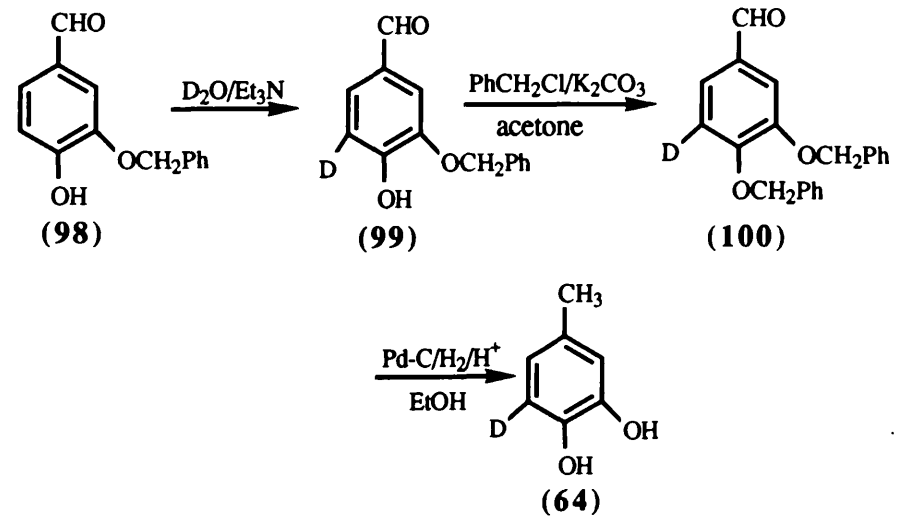
To overcome these disadvantages and to shorten the procedure, [6-^{2}H]protocatechuic aldehyde (**95**) was prepared directly from 6-bromovanillin (**92**) (Scheme 37). 6-Bromovanillin (**92**) was demethylated under the standard conditions to give 6-bromo-protocatechuic aldehyde (**97**). 6-Bromoprotocatechuic aldehyde (**97**) was dissolved in dry tetrahydrofuran and deuterium oxide containing 1 equiv. of triethylamine and debrominated by freshly prepared deuterium gas [from clean lithium metal and deuterium oxide] over 10% palladium-carbon at room temperature under atmospheric pressure. The product (**95**) was obtained in good yield and purity. This was again converted into the required product (**96**), without loss of deuterium.



Scheme 37.

(3) 4-Methyl-[6-^{2}H]pyrocatechol (64)

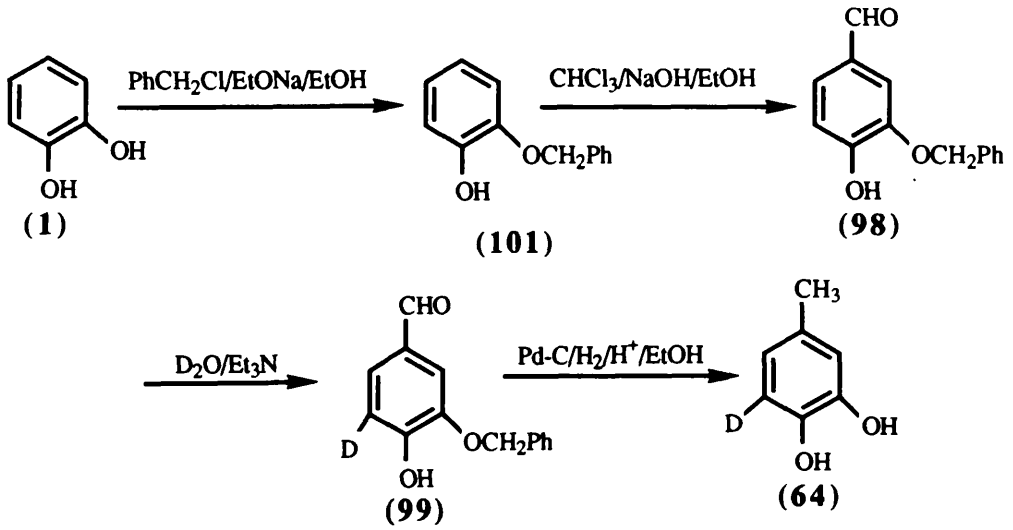
This compound (**64**) had been prepared earlier by Kirby and Ogunkoya (Ref. 60) by hydrogenation of the benzylated intermediate (**100**) (Scheme 38), which was needed for another synthetic target. To determine the stability of nuclear deuterium under the normal conditions for benzylation and debenzylation of phenolic hydroxyl groups, they converted the monobenzylprotocatechuic aldehyde (**98**) into the monodeuteriated derivative(**99**) by refluxing it with deuterium oxide containing triethylamine. The resulting com-



Scheme 38.

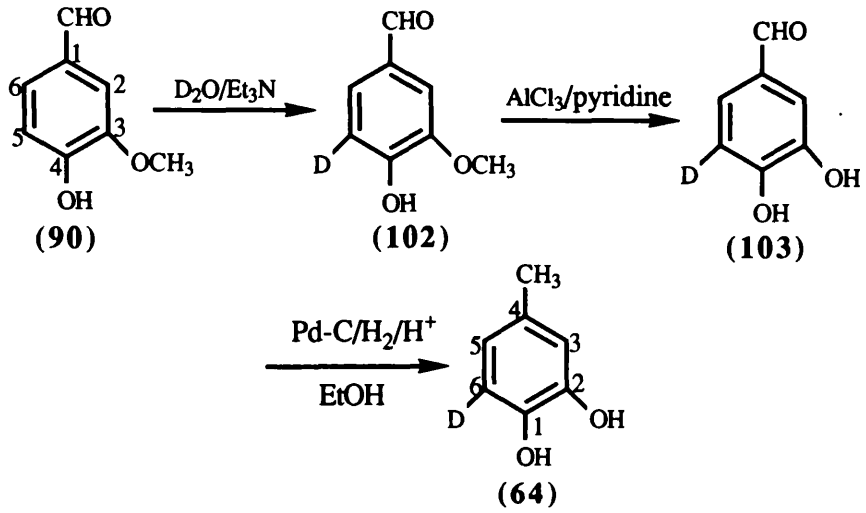
pound (**99**) was benzylated with benzyl chloride and potassium carbonate to give the corresponding benzyl ether without loss of deuterium. Hydrogenation over palladium-carbon in ethanol containing a catalytic amount of hydrochloric acid then yielded 4-methyl-[5-^{2}H]pyrocatechol (**64**).

A similar route was designed for the preparation of the pyrocatechol (**64**) using pyrocatechol (**1**) as starting material (Scheme 39). Pyrocatechol (**1**) reacted with 1 equivalent each of benzyl chloride and sodium ethoxide to give the monobenzyl ether (**101**) (Ref. 67), which was separated from its diether by careful fractional distillation. 3-*O*-Benzylprotocatechuic aldehyde (**101**) was prepared by Reimer-Tieman formylation (Refs. 68, 69, 70). Treatment of pyrocatechol *O*-monbenzyl ether (**101**) with chloroform under alkaline condition gave the aldehyde (**98**), which was separated from the *O*-monobenzyl ether (**101**) by chromatography. However the product was obtained in poor yield (19%). Deuteriation of this aldehyde (**98**) in deuterium oxide containing triethyl-amine, followed by hydrogenation over palladium-carbon in ethanol containing a catalytic amount of hydrochloric acid, under the usual conditions, gave the target product (**64**).



Scheme 39.

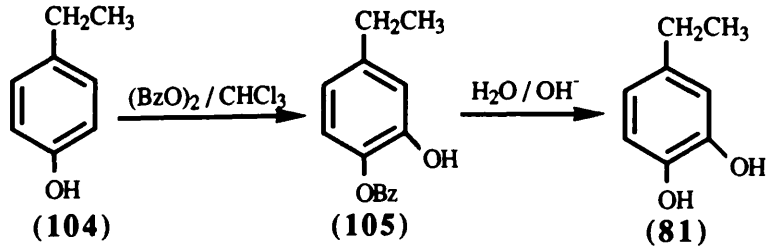
To shorten this route and get a better yield, the compound (**64**) was also synthesized from vanillin (**90**) (Scheme 40). Deuteriation of vanillin (**90**) in the usual way gave [5-^{2}H]vanillin (**102**), which was demethylated by anhydrous aluminium chloride in dichloromethane in the presence of pyridine to give [5-^{2}H]protocatechuic aldehyde (**103**). Hydrogenation of compound (**103**) yielded 4-methyl-[5-^{2}H]-pyrocatechol (**64**).



Scheme 40.

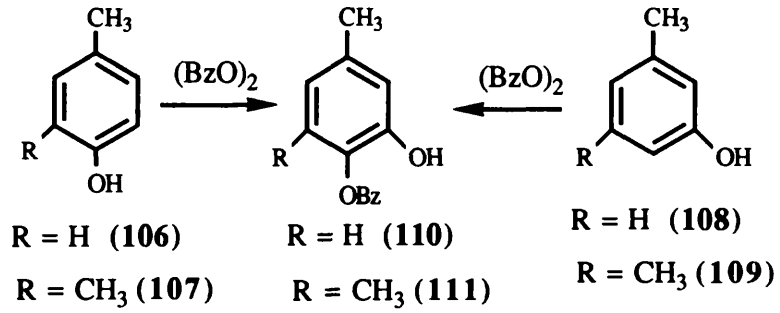
2.1.3 Ethylpyrocatechol (81)

4-Ethylpyrocatechol (**81**) had been prepared earlier by Dr. D. R. Jaap (Ref. 71). Several different routes were considered for its preparation. Among them, one seemed most promising (Scheme 41).

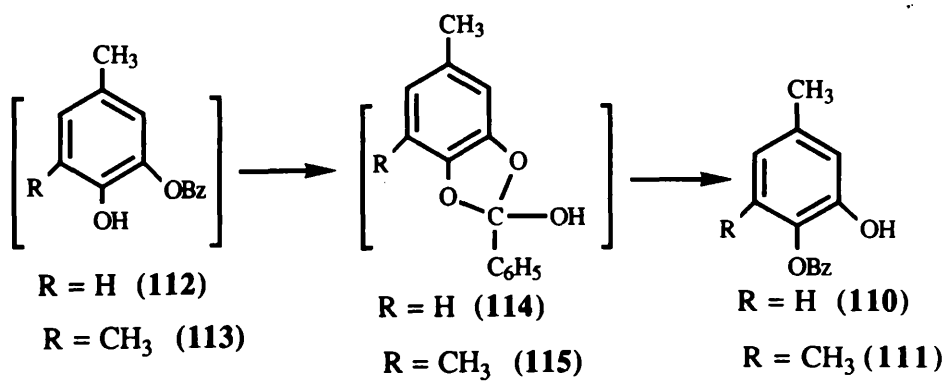


Scheme 41.

Cosgrove and Waters (Ref. 72) showed that a variety of monohydric phenols reacted with benzoyl peroxide in boiling chloroform to give monobenzoates of pyrocatechol derivatives (Scheme 42). Furthermore they showed that *para*- (**106**) and *meta*- (**108**) cresol gave the corresponding esters (**110**) and (**111**). The same products, (**110**) and (**111**), were obtained from *meta*-4- (**108**) and *meta*-5- (**109**)xylenol. They confirmed the structures of the products by independent synthesis. They suggested that with *p*-cresol (**106**) and *m*-4-xylenol (**109**) the *para*-substituted esters, (**110**) and (**111**), are formed by molecular rearrangement of the initially formed *meta*-substituted esters (**112**) and (**113**), possibly *via* the intermediate (**114**) and (**115**) (Scheme 43).



Scheme 42.



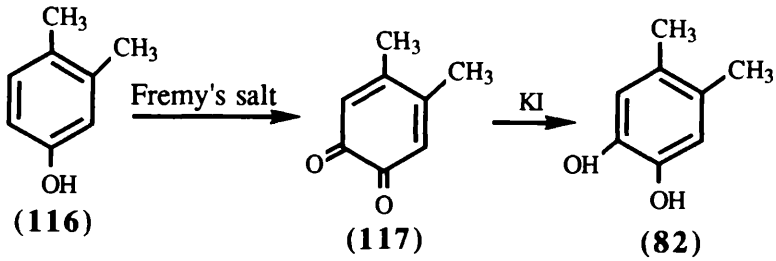
Scheme 43.

By reference to Dr. Jaap's work, 4-ethylphenol (**104**) likewise was heated under reflux in chloroform in the presence 1 equiv. of benzoyl peroxide for 6 h to give the

monoester (**105**), which was then hydrolysed using aqueous sodium hydroxide. Pure 4-ethylpyrocatechol (**81**) (Ref. 75) was obtained by Kugelrohr distillation (Scheme 43).

2.1.4 4,5-Dimethylpyrocatechol (82)

Dimethylpyrocatechol (**82**) was prepared by the oxidation of 3,4-dimethylphenol (**115**) with Fremy's salt, followed by the reduction of the quinone (**116**) with potassium iodide, as shown in Scheme 44.



(116) **(117)** **(82)**

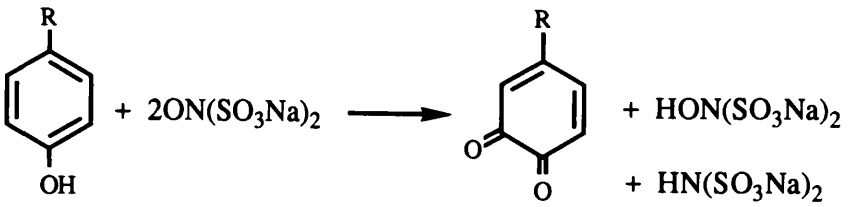
Scheme 44.

Fremy's salt (potassium nitrosodisulfonate) (**118**) was first prepared by Fremy about 150 years ago (Ref. 74). The modern methods for its preparation consist essentially of preparing the sodium or potassium salts of hydroxylamine disulfonic acid followed by oxidation with potassium permangante (Ref. 74a) (Scheme 45). Zimmer *et al.*(Ref. 75) systematically reviewed oxidations with Fremy's salt. The overall stoichiometry of the oxidation of phenols was shown to be 1 equivalent of phenol with 2 equivalents of Fremy's salt to give 1 equivalent of benzoquinone and 1 equivalent of dipotassium hydroxyimidodisulfate and 1 equivalent of dipotassium imidodisulfate (Scheme 46). The mechanism of this reaction has been studied in some detail (Refs. 76, 77).

$$NaNO_2 + NaHCO_3 + SO_2 \longrightarrow HON(SO_3Na)_2 \xrightarrow{[O]} ON(SO_3K)_2$$

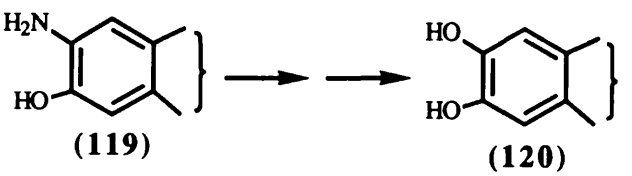
(118)

Scheme 45.



Scheme 46.

Stubenrauch and Knuppen (Ref. 78) reported that conversion of the 2-aminoestrone (**119**) to the corresponding 2-hydroxyestrone (**120**) was effected by oxidation with sodium metaperiodate in acetic acid and 0.1 N hydrochloric acid to give the corresponding *ortho*-quinone, which was immediately reduced with potassium iodide in acetic acid and chloroform without prior isolation (Scheme 47).



(119) **(120)**

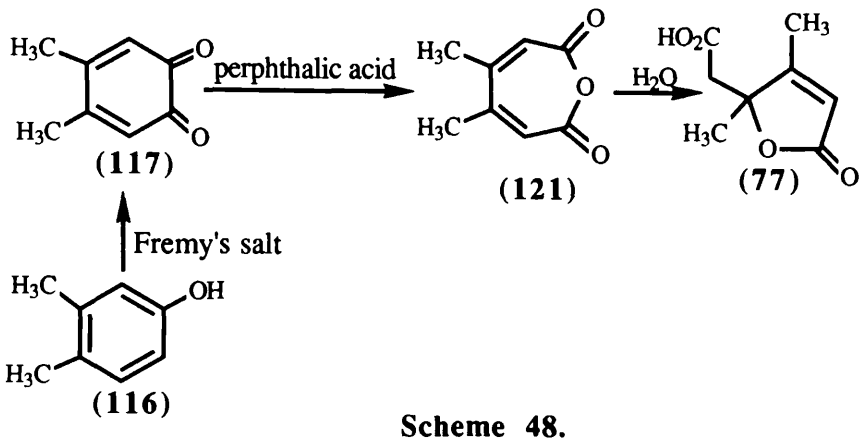
Scheme 47.

Accordingly, 3,4-dimethylphenol (**116**) was oxidised with Fremy's salt to give 4,5-dimethyl-1,2-benzoquinone (**117**) (Ref. 78), which was reduced by shaking with potassium iodide in acetic acid-chloroform for 2 min. to form 4,5-dimethylpyrocatechol (**82**) (Scheme 44) (Ref. 78a)

2.1.5 3,4-Dimethylmuconolactone (77)

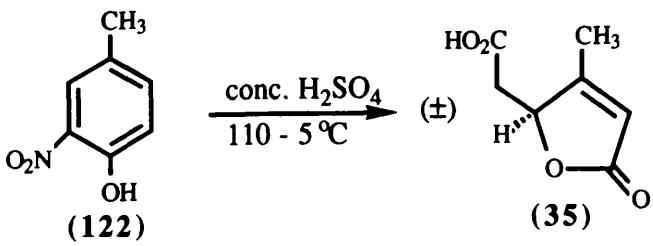
Brassard *et.al.* (Ref.79) synthesized 3,4-dimethylmuconlactone (**77**) in 1960 from 4,5-dimethyl-1,2-benzoquinone (**117**), which was prepared from 3,4-dimethyl-phenol (Scheme 48). Oxidation of the 1,2-benzoquinone (**117**) in ether with a 10% excess of perphthalic acid for 4 d at 0 °C yielded *cis, cis*-3,4-dimethylmuconic anhydride

(**121**). Heating the anhydride (**121**) with water to 50 °C formed 3,4-dimethylmuconolactone (**77**).

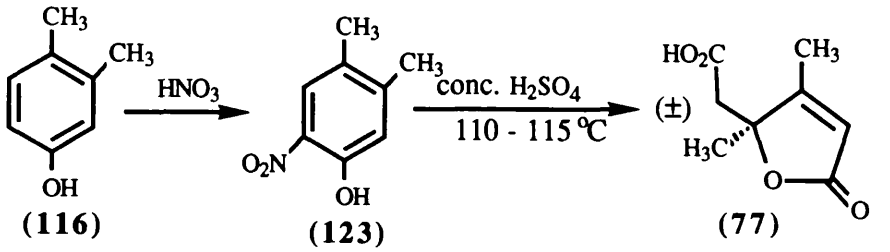


Scheme 48.

Pauly *et al.*(Ref. 80) reported the preparation of racemic 3-methylmuconolactone (**35**) from the nitro compound (**122**) (Scheme 49). Thus the benzene ring of 2-nitro-*p*-cresol (**122**) can be directly cleared by concentrated sulphuric acid to form 3-methylmuconolactone (**35**). A similar route was adopted to prepare 3,4-dimethylmuconolactone (**77**) from 3,4-dimethylphenol (**116**) (Scheme 50). 3,4-Dimethylphenol (**116**) was carefully nitrated with concentrated nitric acid in acetic acid (Ref. 81). The 3,4-dimethyl-6-nitrophenol (**123**) was separated from the reaction mixture containing a dinitro compound by column chromatography (Ref. 82). The nitro-compound (**123**) was heated with concentrated sulphuric acid at 110-115 °C for 2 h to form 3,4-dimethylmuconolactone(**77**), which was obtained by continuous extraction with

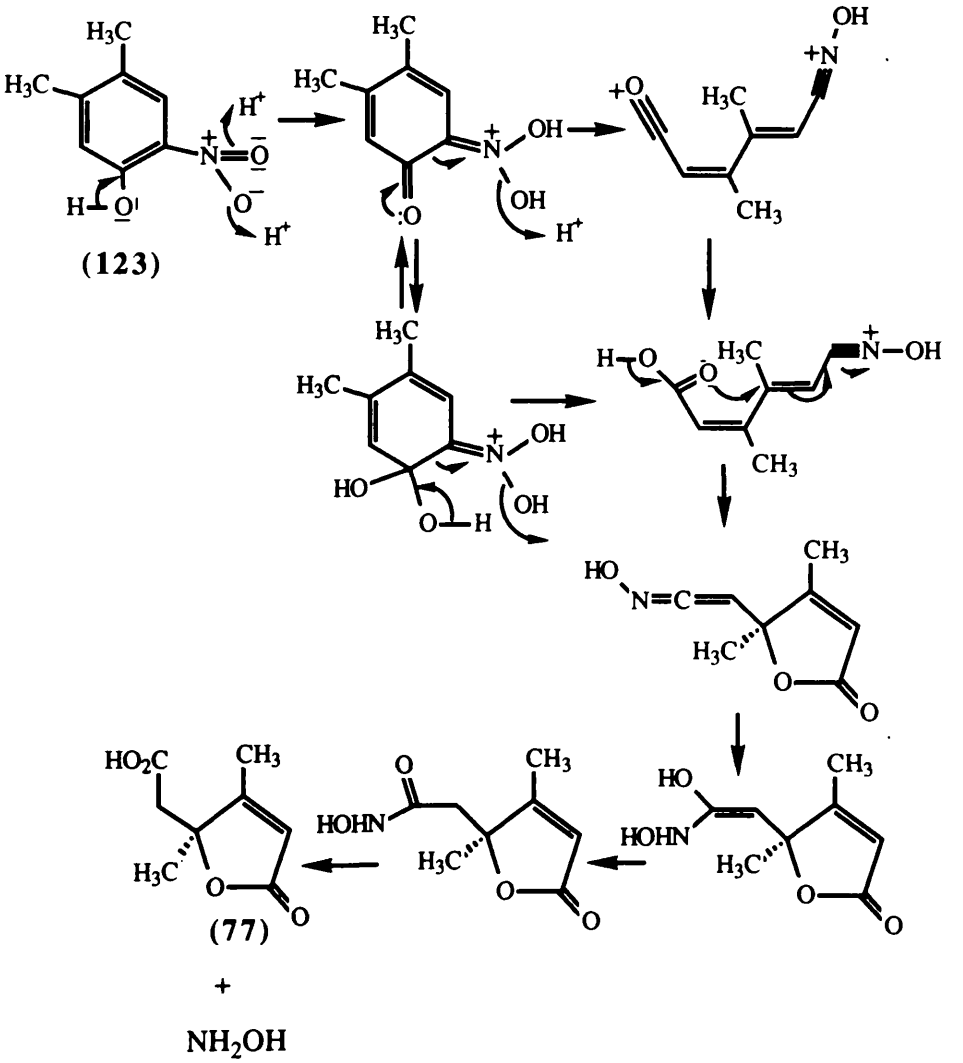


Scheme 49.



Scheme 50.

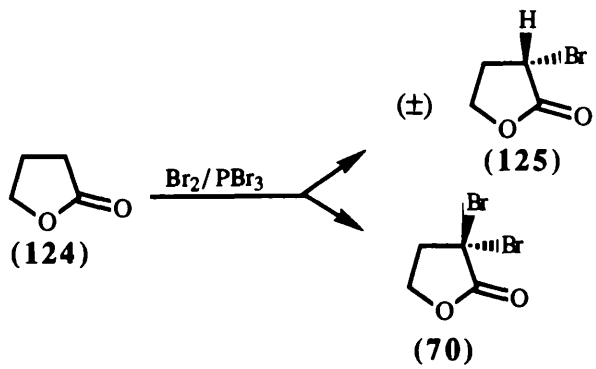
ether after the reaction mixture had been diluted with water. A possible mechanism is shown in Scheme 51.



Scheme 51.

2.1.6 2-Bromo-(125) and 2,2- Dibromo-butyrolactone (70)

These two products, (**125**) and (**70**), were prepared by a variation of Daremon's methods (Ref. 83) to get the pure compounds (Scheme 52). Butyrolactone (**124**) was heated to 100-130 °C with a catalytic amount of phosphorus tribromide. To it, 0.85 mol equivalent of bromine was added very slow during 4 h. The resulting mixture was heated between 100-130 °C for another 5 h. Repeated fractional distillation gave pure liquid 2-bromobutyrolactone (**125**). In a similar way, 3 mol equivalents of bromine was used to prepare 2,2-dibromobutyrolactone (**70**), the temperature being kept between 170-190 °C overnight. The quantities of bromine were critical, since it was possible to separate the lactone (**124**) from its monobromo derivative (**125**) by distillation, but impossible to separate the mono (**125**) and dibromo (**70**) derivatives in this way. The dibromo derivative (**70**) was crystalline.

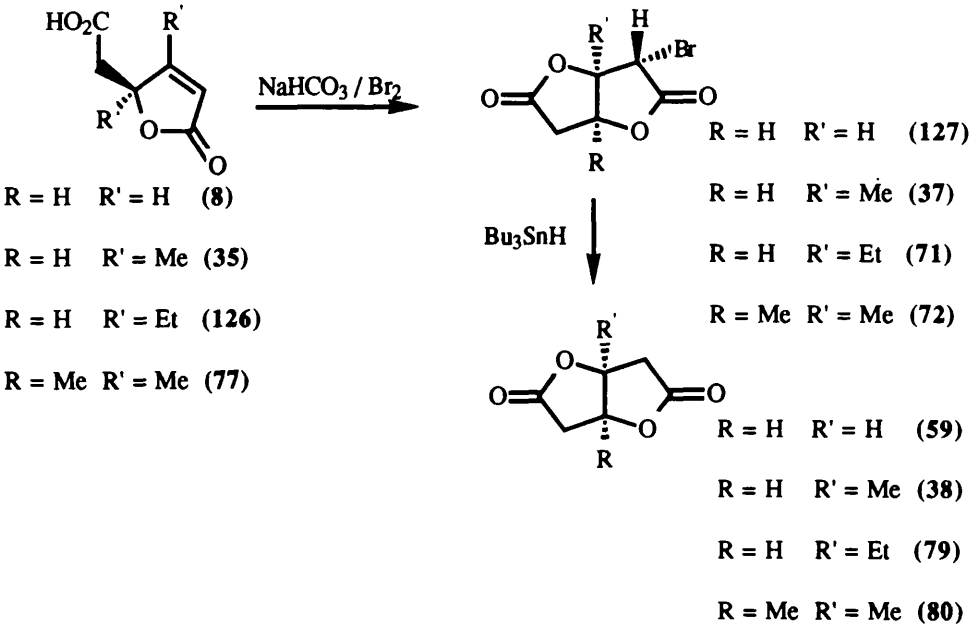


Scheme 52.

2.1.7 The synthesis of dilactones

Unlike the unsubstituted muconolactone but like 3-methylmuconolactone, 3-ethylmuconolactone and 3,4-dimethylmuconolactone also could not be cyclised by acid catalysis to give the corresponding bicyclic dilactones. Instead bromodilactones were synthesised (Scheme 53). The lactones were dissolved in ice-cold water by addition of sodium hydrogen carbonate (1mol equiv.). Bromine (1mol equiv.) in dichloromethane was added and the two-phase mixture was stirred at room temperature for 4 h. Any traces

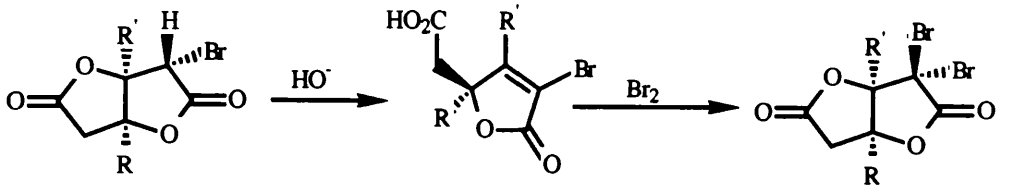
of unreacted bromine were removed by addition of aqueous sodium thiosulphate. The dichloromethane layer and dichloromethane extracts of the aqueous layer were combined and dried. Evaporation of the organic solvents gave the bromodilactones in good yield. These were then suspended in dry benzene containing azoisobutyronitrile (0.1mol equiv.) under nitrogen at room temperature. Tri-n-butyltin hydride (1.2mol equiv.) was added and the mixtures were stirred and warmed briefly to initiate reaction. After *ca.* 15 min. the bromodilactones had dissolved and the products had begun to crystallise out. After 1 h the crystals were collected and recrystallised from chloroform-hexane. The bromodilactones (**127**), (**37**), (**71**), and (**72**) and dilactones (**59**), (**38**), (**79**), and (**80**) were all prepared in this way. All were racemic.



Scheme 53.

Bromodilactones can be converted to the dibromodilactones (**73**), (**60**) and (**74**) *via* the intermediate bromolactones (**128**), (**129**), and (**78**), which need not be isolated (Scheme 54). The bromodilactones were suspended in an aqueous solution of sodium hydrogen carbonate (1 mol equiv.). The suspensions were stirred at room temperature for

ca 1.5 h to produce clear solutions of the sodium salts of the bromolactone (**128**), (**129**), (**78**). Then bromine (1mol equiv.) in dichloromethane was added in the usual way to give dibromodilactones (**73**), (**60**), (**74**).



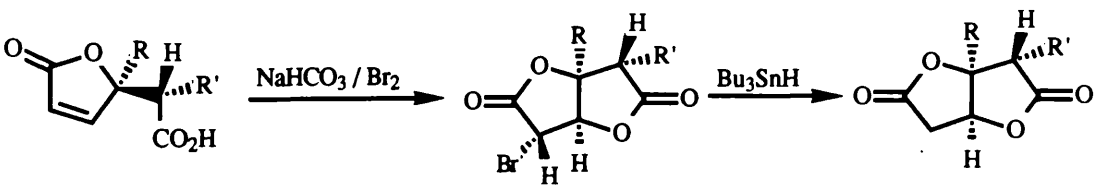
R = H R' = H (**127**) R = H R' = H (**128**) R = H R' = H (**73**)

R = H R' = Me (**37**) R = H R' = Me (**129**) R = H R' = Me (**60**)

R = Me R' = Me (**72**) R = Me R' = Me (**78**) R = Me R' = Me (**74**)

Scheme 54.

Two other dilactones (**65**) and (**76**) were also prepared, for different purposes, from optically pure 4-substituted muconolactones (Scheme 55), following the same procedures as described above for the preparation of racemic dilactones.



R = CH_3 R' = D (**65**) R = CH_3 R' = D (**130**) R = CH_3 R' = D (**67**)

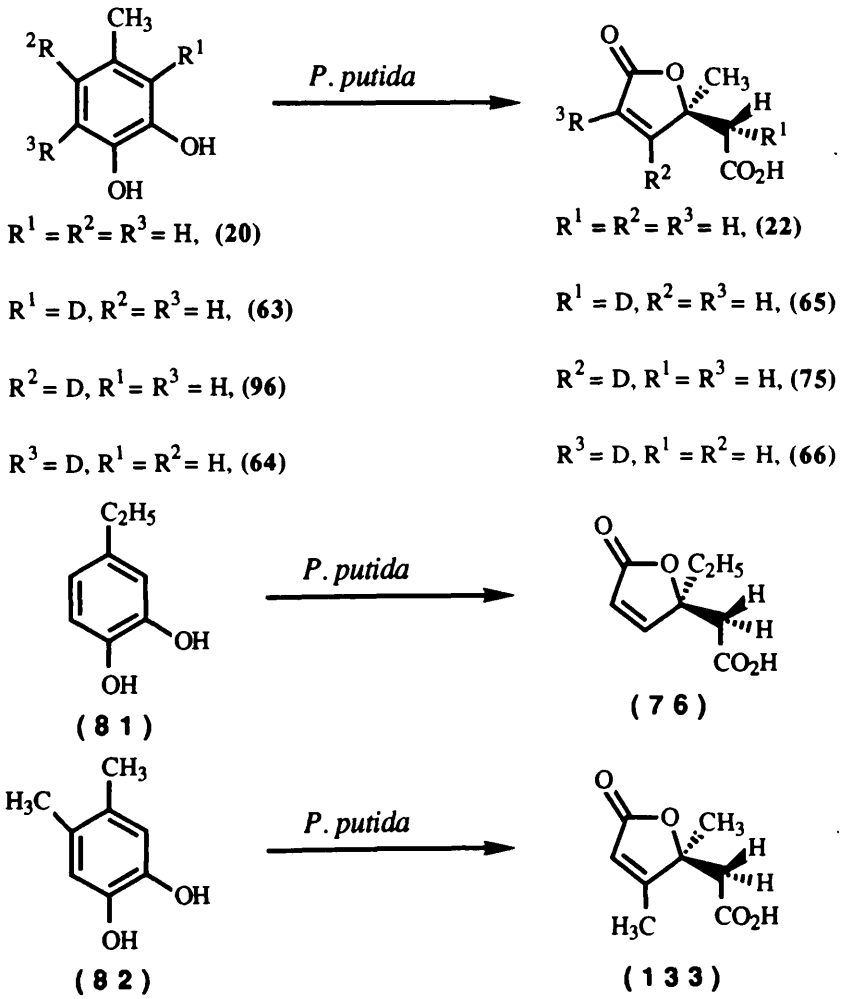
R = C_2H_5 R' = H (**76**) R = C_2H_5 R' = H (**131**) R = C_2H_5 R' = H (**132**)

Scheme 55.

2.2 BIOSYNTHESIS OF MUCONOLACTONES

Various pyrocatechol derivatives (Scheme 56) were fed to cultures of *Pseudomonas putida* for two reasons. First, the deuteriated 4-methylpyrocatechols (**20**),

(**63**), (**96**) and (**64**) gave a set of optically pure labelled 4-methylmuconolactones, (**22**), (**65**), (**75**), and (**66**), required to elucidate the stereochemistry and mechanism of the methylisomerase-catalysed reaction in *Rhodococcus rhodocrous*. Second, the ethyl (**81**) and dimethyl (**82**) derivatives were tested as substrates for the dioxygenase and cycloisomerase enzymes in *Pseudomonas putida* cultures by isolation of the corresponding derived muconolactone, (**76**), (**133**).



Scheme 56.

2.2.1 *Pseudomonas putida*

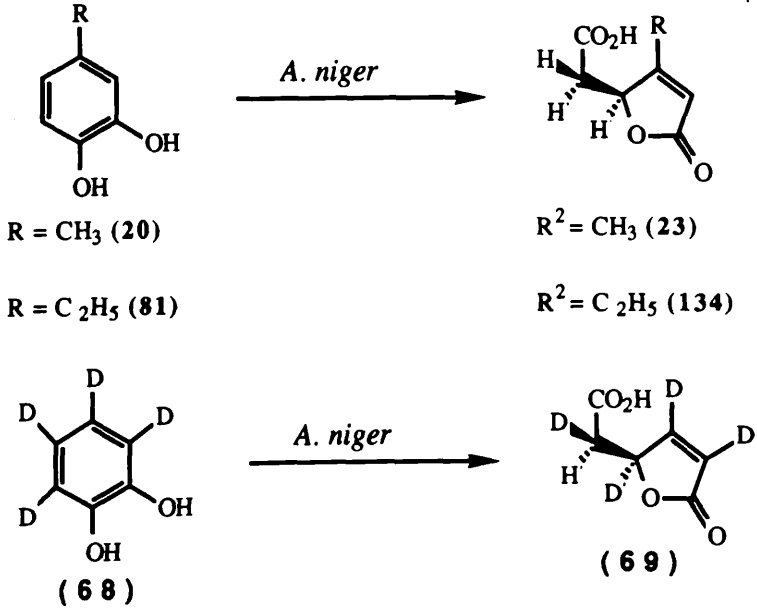
All the pyrocatechol derivatives were fed to the culture of *Pseudomonas putida* under the conditions employed successfully with the methyl pyrocatechol (Ref. 10) (Scheme 56). All substrates were fed, in three batches, to 3 day old cultures of *Pseudomonas putida*. After each batch, incubation was continued until a ferric chloride test showed that the pyrocatechol had been consumed. After 30 h, a ferric chloride test of the medium showed that there was negligible pyrocatechol left. The entire culture media were extracted at pH 7.5 with ether and the ether extracts were analysed by ^{1}H NMR spectroscopy. This showed that a small amount of non-acidic material, mainly pyrocatechols, had been removed. Then the culture media were adjusted to pH 2.5 and again extracted with ether. The ether extracts were analysed by ^{1}H NMR spectroscopy also. The substituted muconolactones were identified as major products. The results of the feeding experiments are given in Table 1.

Table 1. Incubation in cultures of *Pseudomonas putida*

	4-Methyl-pyrocatechols				Other pyrocatechols	
	(**20**)	3-D-(**63**)	5-D-(**96**)	6-D-(**64**)	4-ethyl- (**81**)	4,5-dimethyl (**82**)
Amount of catechol fed	800 mg	300 mg	373 mg	390 mg	450 mg, 560 mg	560 mg
Incubation time	30 h	30 h	30 h	30 h	30 h	30 h
Derived lactones	(**22**)	(**65**)	(**75**)	(**66**)	(**76**)	(**133**)
Yield of lactone	447mg 44%	130mg 34 %	90 mg 19%	170 mg 34%	2*290mg, 53%,42%	70 mg 20%

2.2.2 *Aspergillus Niger*

Several pyrocatechols were also fed to *Aspergillus niger* under the same conditions employed in Ref. 23b for 4-methylpyrocatechol (**20**), which can be converted into 3-methylmuconolactone (**23**) (Scheme 57). The ethyl derivative (**81**) was tested as a substrate for the dioxygenase and cycloisomerase enzyme, and the fully deuteriated pyrocatechol (**68**) was used to determine, for the first time, the stereochemistry of the cycloisomerase enzyme in a fungus. The flasks containing fungi culture were incubated. The utilization of glucose was monitored by UV spectrometry (Ref. 84). When almost all the glucose had been consumed, the substrates were fed, in two batches, to the cultures of *A. niger*. The pH was remained at 5.5. After about 44 h, the cultures were adjusted to pH 7.2 and extracted with ether to remove non-acidic materials. Then the cultures were adjusted to pH 2.0 and again extracted with ether. The ether extracts were analysed by ^1H NMR spectroscopy. 3-Substituted muconolactones or muconolactone itself were the major products (Table 2).



Scheme 57.

Table 2. Incubations in cultures of *Aspergillus niger*

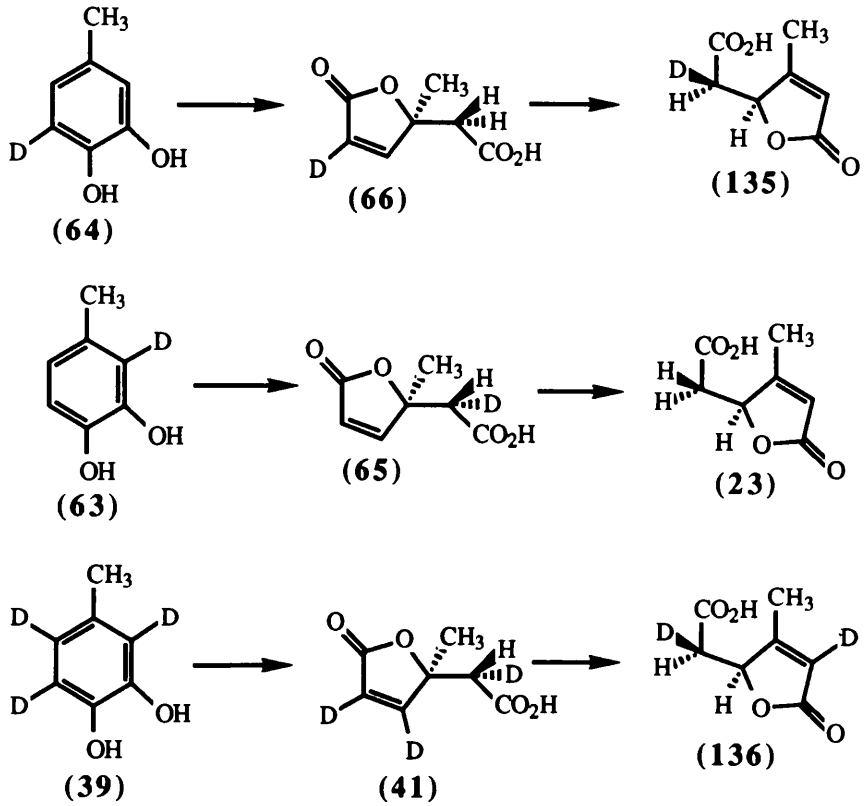
	[3,4,5,6-^{2}H]-pyrocatechol (**68**)	4-Methylpyrocatechol (**20**)	4-Ethylpyrocaterchol (**81**)
Amount of pyrocatechol fed	515 mg	481 mg	475 mg
Incubation time	46 h	44 h	44 h
Muconolactones	(**69**)	(**23**)	(**134**)
yield of lactone	290 mg 56 %	60 mg 13 %	30 mg 7 %

2.3 NEW STEREOCHEMISTRY AND MECHANISM OF MUCONATE PATHWAYS

2.3.1 4-Methylmuconolactone (22) to 3-Methylmuconolactone (23)

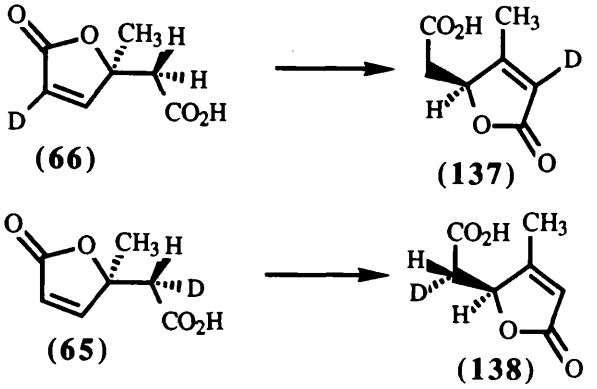
To clarify the two, mechanistically distinct mechanisms proposed in Sec. 1.4., a set of deuteriated 4-methylpyrocatechols was synthesised to serve as precursors for the correspondingly labelled 4-methylmuconolactones (Scheme 58). The preparation of (**64**) and (**63**) was discussed in Sec. 2.1.1. These two precursors, (**64**) and (**63**), were fed separately to cultures of *Pseudomonas putida* to afford the lactones (**66**) and (**65**) respectively. The monodeuterio 4-methylmuconolactone (**66**) and (**65**) were designed to test the possibility of methyl migration (Scheme 27, pathway a) during the isomerisation (**22**) ⇒ (**23**). The lactones (**66**) and (**65**) were taken to Professor Cain's laboratory in

Newcastle by Dr. G. V. Rao, who carried out incubation studies with the isomerase enzyme from *Rhodococcus rhodocrous*, with the following results.



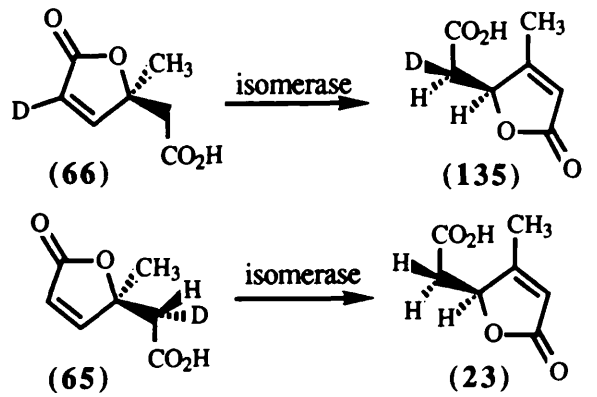
Scheme 58.

If methyl migration were to occur, as indicated in Scheme 27, pathway a, compound (**66**) would give the 2-deuterio derivative (**137**) [although, conceivably, deuterium loss might occur by exchange from the medium], and the compound (**65**) would give the (5*S*)-5-deuterio derivative (**138**) (Scheme 59). However, these products (**137**) and (**138**) were not identified in the actual methylisomerase tests. When the 2-deuteriomuconolactone (**66**) was incubated with the isomerase enzyme, isomerisation proceeded, essentially without loss of deuterium, to afford the (5*R*)-5-deuteriolactone (**135**) (Scheme 60). The ^{1}H NMR spectrum of (**135**) showed a strong signal, δ 2.50 (dt, $J_{4,5}$ 8.5 Hz $J_{H,D}$ 2.4 Hz), arising from the 5-*pro*-*S*-proton in (**66**), and only a



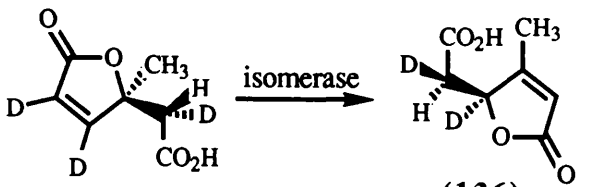
Scheme 59.

weak signal, δ 2.98 (dd, *J* 16.3 and 2.4 Hz), arising from the 5-*pro*-*R*-proton in the unlabelled lactone due to the incomplete deuteriation of the pyrocatechol. Because the stereochemical assignment of methylene signals in the ^{1}H NMR spectra of the lactone (**66**) and (**135**) had previously been established by unambiguous methods, it followed that the newly formed lactone ring in (**135**) must have arisen by *anti* addition of the carboxyl group to the 2,3-double bond of the substrate (**66**). Again, incubation of the (5*S*)-5-deuteriolactone (**65**) with the isomerase gave the unlabelled lactone (**23**), consistent with *anti* elimination to form the 2,3-double bond of the product. However, there remain the possibility that enzyme-catalysed exchange of deuterium in the lactone (**65**) had proceeded by ring opening.



Scheme 60.

Finally the trideuteriolactone (**41**), prepared by Dr. G.V. Rao, gave the dideuterio product (**136**) (Scheme 61). The ^1H NMR spectrum showed signals at δ 2.49 (br s, 5-*pro-S*-H), and 5.87 (br s, 2-H). Thus, the deuterium labelling pattern confirmed the conclusions drawn from experiments with the monodeuteriuolactones (**66**) and (**65**).



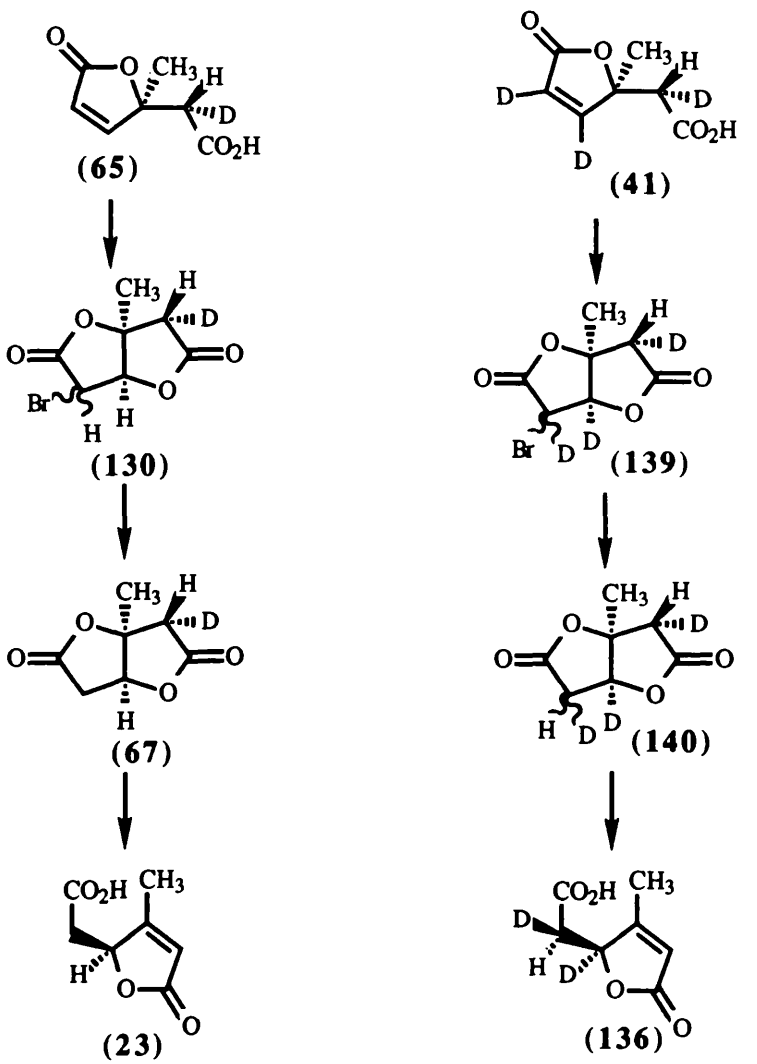
(**41**) (**136**)

Scheme 61.

Clearly, these results exclude any possibility of isomerisation by a 1,2-methyl shift (*e.g.* pathway a, Scheme 27). On the other hand, if the dilactone (**38**) is an intermediate on the alternative pathway b, then the stereochemistry of hydrogen loss from the 8-methylene group should match that observed for the lactone (**65**) (Scheme 62). To test this, the monolactone (**65**) was converted chemically into the (8*S*)-8-deuteriodilactone (**67**)*via* the corresponding deuterio derivative of the bromodilactone (**130**). Incubation of the dilactone (**67**) with the isomerase gave the unlabelled lactone (**23**). Similarly the trideuteriomonolactone (**41**), prepared by Dr. G.V. Rao gave the corresponding bromodilactone (**139**), which was debrominated to afford the trideuteriodilactone (**140**). This was a mixture of C-4 epimers since, as expected, radical debromination was not stereospecific. Consequently, incubation of (**140**) gave the (5*R*)-4,5-dideuteriolactone (**136**) mixed with smaller amount of its (5*S*) epimer. Deuterium was again lost specifically from the 8-position of the dilactone (**140**) (Scheme 62).

To summarise, experiments with the deuteriated 4-methylmuconolactones showed that the enzymic isomerisation (**22**) to (**23**) cannot proceed *via* 1,2-migration of the methyl group (Scheme 27, pathway a). An alternative pathway (Scheme 27, pathway b) involving the dilactone or some equivalent, enzyme-bound intermediate, is indicated.

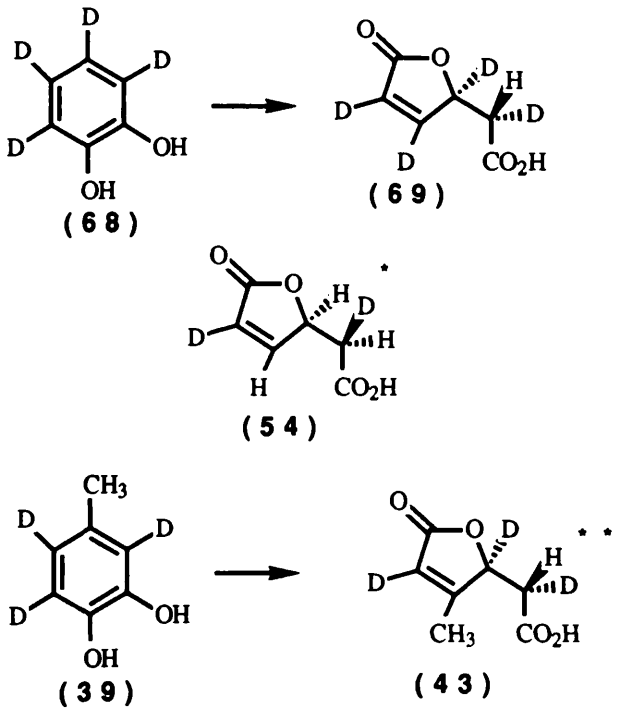
Irrespective of the timing of steps, closure and opening of the lactone rings proceeds stereospecifically (> 95%) by *anti* addition and elimination reactions. Interestingly, this conclusion contrasts with the *syn* addition observed for the cyclisation of 3-methyl-*cis,cis*-muconic acid, in *Pseudomonas putida* to give 4-methylmuconolactone (**22**) and in *Aspergillus niger* to give 3-methylmuconolactone (**23**). The observed loss of deuterium from the dilactones (**67**) and (**140**) is consistent with their putative role as intermediates. However, there is at present no direct proof that the dilactone is a free intermediate in the isomerisation (**22**) to (**23**).



Scheme 62.

2.3.2 Stereochemistry of the Muconic Acid Cycloisomerase Reaction in the Fungus *Aspergillus niger*

The first stereochemical study of the muconic acid pathway was reported in 1969. Using the cycloisomerase enzyme from the bacterium *Pseudomonas putida*, Avigad and Englard (Ref.46) showed that the cyclisation of *cis,cis*-muconic acid (**7**) in tritiated water proceeded by *syn* addition of the carboxyl group to the double bond to form tritiated (4*S*)-muconolactone (Scheme 11). For the sake of completeness, it was decided to determine whether the same stereochemistry applied in a typical fungus (Scheme 63). The *Aspergillus niger* that accumulates muconolactone (**23**) was selected. The tetradeuteriopyrocatechol (**68**), prepared from pyrocatechol (**1**) in Sec. 2.1.1., was fed to



*. ^{1}H NMR spectrum: δ(200 MHz, D_2O, tert-butanol at 1.22) 7.54 (1H, s, 3-H), 5.32 (1H, d, *J* 8.6Hz, 4-H), 2.30 (1H, d, *J* 8.6 Hz, 5-H) cited from Ref. 56.

**. 5-H at δ2.76 (t, J 2.3 Hz) (D_6-Acetone) reported by Dr.G.V.Rao

Scheme 63.

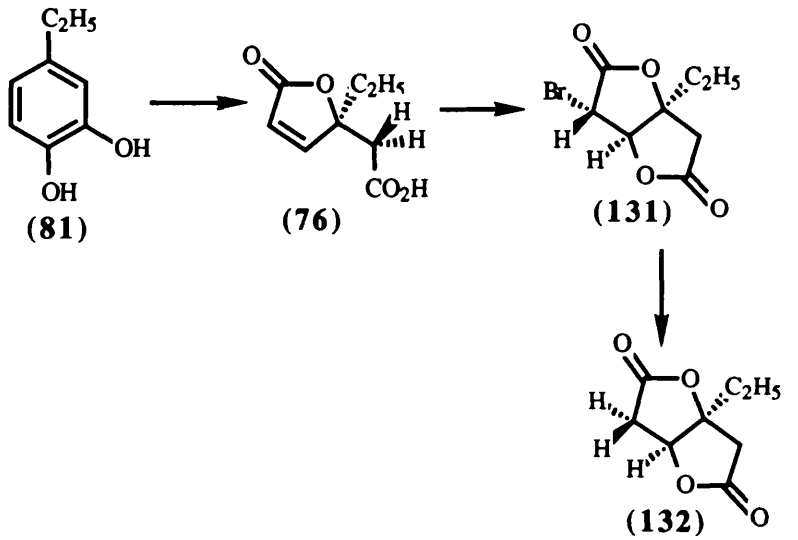
the culture of *Aspergillus niger* as described in Sec. 2.2.2. The ^1H NMR spectrum of the product (**69**) showed only a singlet at δ2.69 (pH7-8) with fine splitting. This was compared with that of (4*S*, 5*R*)-2,5-dideuteriomuconolactone (**54**), which was described by Chari *et al.* (Ref. 58). This showed that the lactone (**69**) obtained was (4*S*,5*S*)-[2,3,4,5-$^2H_4$]muconolactone, *i.e.* the cyclisation of *cis,cis*-muconic acid (**7**) proceeds by *syn* addition in the fungus. The product (**43**), obtained by Dr. G.V.Rao by feeding trideuterio 4-methylpyrocatechol (**39**) to the same organism, provided good evidence of the above observation (Scheme 63) (Ref. 53). The establishment of the absolute stereochemistry of compound (**43**) was discussed in Sec. 1.2.3.

2.3.3 The Influence of Substituents on the Enzymic Reaction in the bacterium *Pseudomonas putida*

In preliminary experiments, Dr. D.R.Jaap (Ref. 73) fed 4-ethylpyrocatechol (**81**) to *Pseudomonas putida* cultures and isolated 4-ethylmuconolactone (**76**) in low yield. The lactone was not crystalline, and the optical rotation, $[\alpha]_D$ 7.9 and 19.7 ° in methanol, of specimens from two separate experiments differed. Both were lower than that, $[\alpha]_D$ 32±2 ° (Ref. 22) in water, of (*S*)-4-methylmuconolactone (**22**). Consequently, this feeding experiment was repeated (see Sec. 2.2.1). The lactone (**76**) again failed to crystallise, and the optical rotation was still low, $[\alpha]_D$ 14.5 °. The ^1H NMR spectrum agreed with that reported by Dr. Jaap. To effect complete characterisation and to provide a new potential substrate for the methylisomerase enzyme, it was decided to convert this lactone (**76**) into the corresponding ethyldilactone (**132**) (Scheme 64). It is not certain that the amorphous ethyllactone (**76**) is optical pure, however the sign of rotation and that of the crystalline ethyldilactone indicate it is, as expected, predominantly the (*S*)-isomer.

4-Ethylpyrocatechol (**81**) and 4, 5-dimethylpyrocatechol (**82**) were tested in parallel with 4-methylpyrocatechol (**20**) as precursors for *Pseudomonas putida,* described in Sec.2.2.1., to explore the shape and size of the active site of the dioxygenase and

cycloisomerase. Interestingly, 4-ethylpyrocatechol (**81**) gave lactone yields of 42%, which is similar to the yield of 44% for 4-methylpyrocatechol (**20**), while 4,5-dimethylpyrocatechol (**82**) had a lower yield of 20%. These results suggest that the bulkier ethyl group does not affect the enzymic reaction, but the two methyl groups sterically hinder the cyclase enzyme. The dioxygenase appears to accept the dimethylpyrocatechol as a substrate since there was very little unchanged substrate (about 9%) recovered at the end of the incubation. The product 3,4-dimethylmuconolactone (**77**) failed to crystallise. The structure was established by comparing the ^1H NMR spectrum of the bacterial product with that of the racemic synthetic muconolactone, and more supply is required for the further analysis.



Scheme 64.

2.3.4 The Influence of Substituents on the Enzymic Reaction in the Fungus *Aspergillus niger*

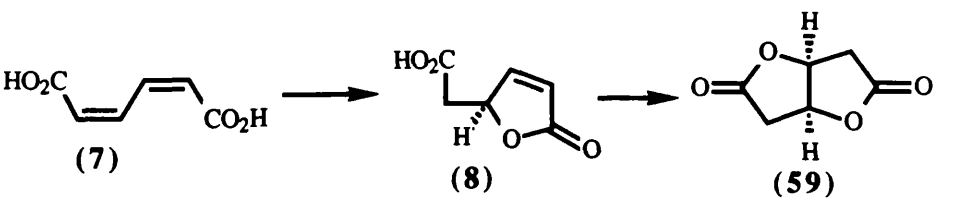
To explore the shape of the active site of the dioxygenase and cycloisomerase, 4-ethylpyrocatechol (**81**) was fed to the fungus *Aspergillus niger*. The 4-methyl-pyrocatechol (**20**) was fed in parallel to provide a comparison of the yields of the lactones. The corresponding yield for the parent muconolactone (**69**) was also a good

guide to the relative efficiencies of conversion. The results from feeding experiments showed that the yields of lactone from tetradeuteriopyrocatachol (**68**), 4-methyl-pyrocatechol (**20**), and 4-ethylpyrocatechol (**81**) in this fungus were quite different (56%, 13%, and 7% respectively) indicating that bulkier alkyl groups make the pyrocatechols poorer substrates for the cycloisomerase enzyme systems because there was not much pyrocatechols (1%, 8%, and 11% respectively) recovered at the end of the incubation.

The structure of the product 3-ethylmuconolactone (**134**) was established by comparing the ^1H NMR spectrum of fungal product with that of the racemic muconolactone (**126**). Due to the dark colour there is not enough light going through the solution, and the optical rotation could not be obtained. More analysis could not be done with such a low yield of the product.

2.3.5 The Non-enzymic Cyclisation of Muconolactones

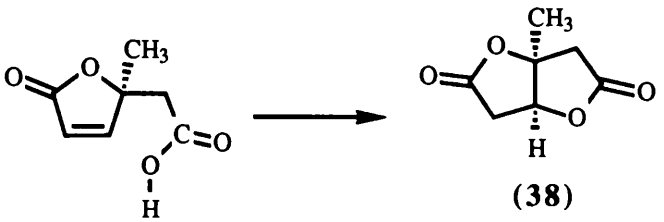
In 1950, Elvidge (Ref. 13) found that an isomeric, none-acidic compound was formed as a by-product in the study of lactonisation of *cis,cis*-muconic acid. This compound (**59**) was reproducibly prepared by either prolonged treatment of *cis,cis*-muconic acid (**7**) with sulphuric acid at room temperature or refluxing *cis,cis*-muconic acid (**7**) with 50% sulphuric acid (Scheme 65).



Scheme 65.

Catelani *et al.* (Ref. 20) investigated the dilactonisation of both 4-methyl- and 3-methylmuconolactone and found that 4-methylmuconolactone (**22**) can easily cyclise in

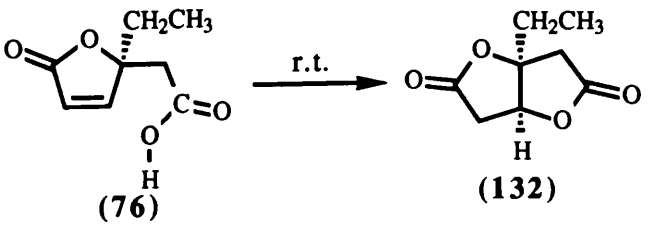
several ways (Scheme 66). Thus, distillation of the crude compound, boiling with concentrated hydrochloric acid followed by ether extraction, leaving the oily product for several weeks, or chromatography on silica, all caused cyclisation to produce the methyldilactone (**44**). But 3-methylmuconolatone (**23**) was unaffected by each of these treatments.



(22) **(38)**

Scheme 66.

During the course of preparation of optically active 4-ethylmuconolactone (**76**), the author found that this lactone (**76**) cyclised spontaneously during storage at room temperature for several weeks (Scheme 67). Proof of the dilactone structure came from the following physiochemical features. The crystalline product had $[\alpha]_D$ -105 ^{o} (*c*, 0.6, in methanol) and m.p. 138-139 ^oC. The IR spectrum showed neither bands of acidic hydroxyl groups nor those of a double bond. There was a very intense band at 1790 cm^{-1}, due to the two saturated γ-lactone groups. The ^1H NMR spectrum of compound showed the following signals; δ 1.0 (3 H, t, *J* 7Hz, CH_2-CH_3), 2.85 (2H, q, with fine splitting, *J* 7Hz, CH_2-CH_3), 2.68 and 2.98 (4 H, m, 8- and 4-H_2) and 4.85 (1 H, m, 5-H). The mass spectrum showed the molecular ion at *m/e* 170. These data were consistent with that obtained from the ethyldilactone (**132**), therefore they had the same structure.



(76) **(132)**

Scheme 67.

It was interesting to study the non-enzymic cyclisation of 3,4-dimethyl-muconolactone (**77**) (Scheme 68), which has the structural features both of 4-methyl-muconolactone (**22**), which cyclises easily, and of 3-methylmuconolactone (**23**), which does not. Also cyclisation would provide a short route to the dilactone (**80**), which otherwise must be prepared (see Sec. 2.1.7) *via* the bromodilactone. Unfortunately, It did not cyclise under the following conditions:

(a) in $CDCl_3$ containing 50 % CF_3COOH at room temperature.

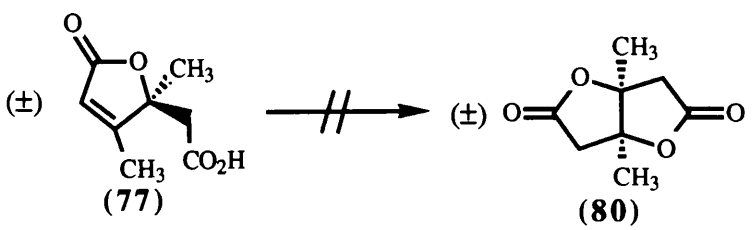
(b) in CF_3COOH with heating under reflux.

(c) in 70% H_2SO_4 with heating under reflux.

(d) in 50% aqueous ethanol containing Amberlyst-15 as catalyst, at room temperature.

(e) adsorbed on silica gel HF_{254} in EtOAc at room temperature.

Conditions (b) and (c) led to loss of material. In all other cases the lactone (**77**) was unchanged.

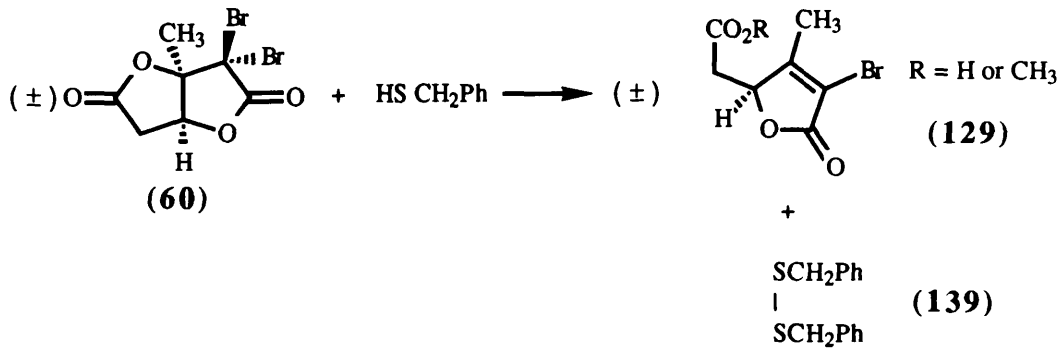


Scheme 68.

2.3.6 Chemical Model Studies for an Inhibitor of the Methylisomerase

In co-operation with Professor Cain we found that the dibromomethyldilactone (**60**) is a strong irreversible inhibitor of 4-methylmuconolactone methylisomerase. It was already known that this enzyme was also inhibited by Hg^{2+} salts, so it was deduced that the inhibition might be related to the reaction with a thiol group on the protein. Several dibromo compounds were studied to test this idea chemically.

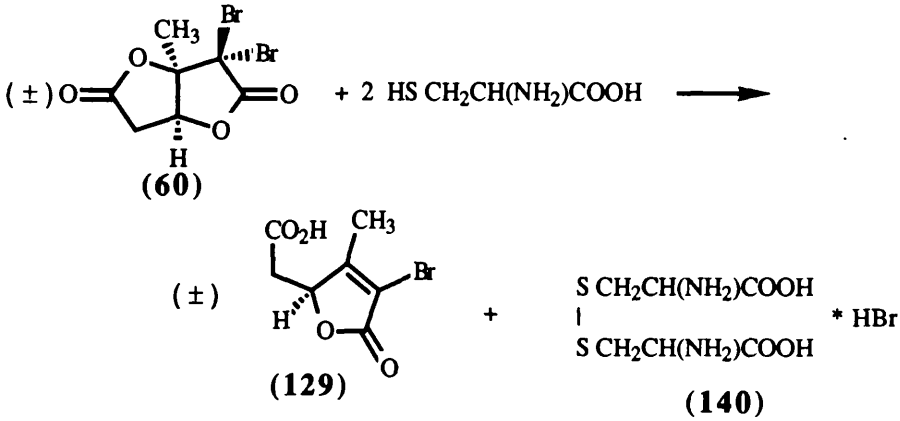
The known irreversible inhibitor, dibromomethyldilactone (**60**) was firstly studied. It was treated with 2 equivalents of benzyl mercaptan in aqueous ethanol under reflux overnight (Scheme 69). The 200 MHz ^1H NMR spectrum of the crude reaction mixture showed that it was a mixture of debrominated product, bromomuconolactone (**129**, R=H), the ethyl ester (**129**, R=C_2H_5) (the ratio is about 3:7), and disulphide (**139**). When the reaction was carried out with 0.9 equivalent of benzyl mercaptan at room temperature overnight, the spectrum showed that the reaction mixture apparently contains debrominated product (**129**, R =H), its ethyl ester (**129**, R=C_2H_5), unreacted dibromodilactone (**60**) and disulphide (**139**). Further characterisation of the reaction product was not achieved. More interesting work was carried our with the thiol amino acid, cysteine, which is described as follows.



Scheme 69.

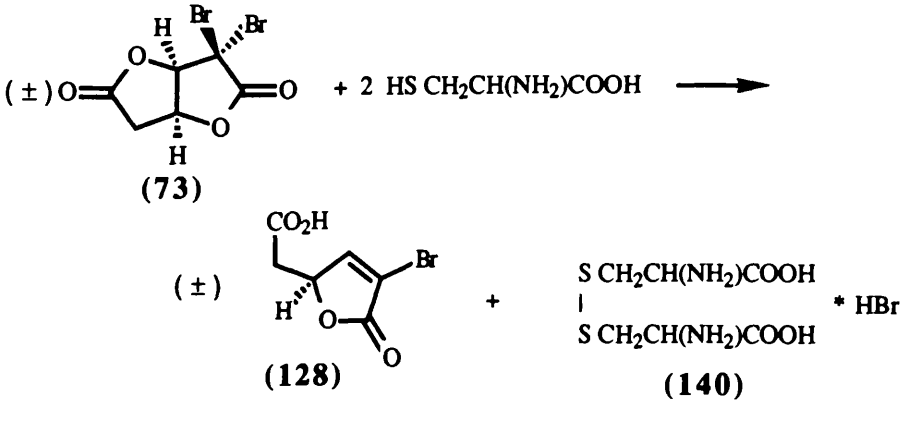
The dibromomethyldilactone (**60**) was first treated with 2 equivalents of the amino acid, cysteine (Scheme 70), in ethanol-water (1:1, v/v) at room temperature. The white precipitate that formed immediately was collected after 2 h and identified as cystine monohydrobromide (**140**) by ^1H NMR spectroscopy and mixed melting point determination. A reference specimen was prepared from cystine and hydrobromic acid. The filtrate was evaporated to dryness and the residual solid was recrystallised from chloroform. This solid was characterised as 2-*bromo*-3-*methyl*-4-*carboxymethyl*-Δ^{α}-

butenolide (**129**, R=H) from the following physiochemical features. It had the melting point 77-80 °C (from chloroform); *m/e* 235.9500 (19.5%), 233.9522. $C_7H_7BrO_4$ requires 235.9509, 233.9586. The IR spectrum has the corresponding absorbance for double bond and carbonyl group. The ^1H NMR spectrum, δ 1.62 (2H, s, 3-Me), 2.51-3.18 (2H, AB part of ABX, *J* 18, 7 and 1 Hz, 5-H_2), and 5.25 (1 H, dd, *J* 7 and 1 Hz, 4-H).



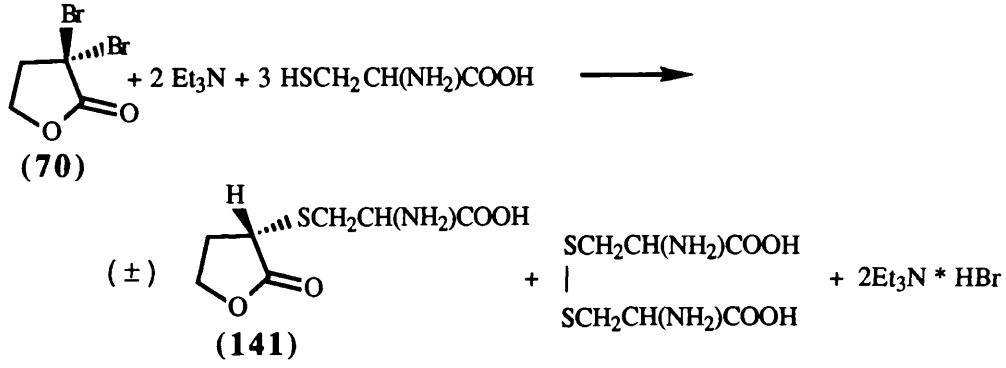
Scheme 70.

Likewise the parent dibromodilactone (**73**) was also reacted with cysteine under the same conditions (Scheme 71). Again, the reaction occurred rapidly at room temperature. The water soluble product was characterized as 2-*bromo*-4-*carboxymethyl*-Δ^{α}-*butenolide* (**128**) by comparison with an anthentic sample.



Scheme 71.

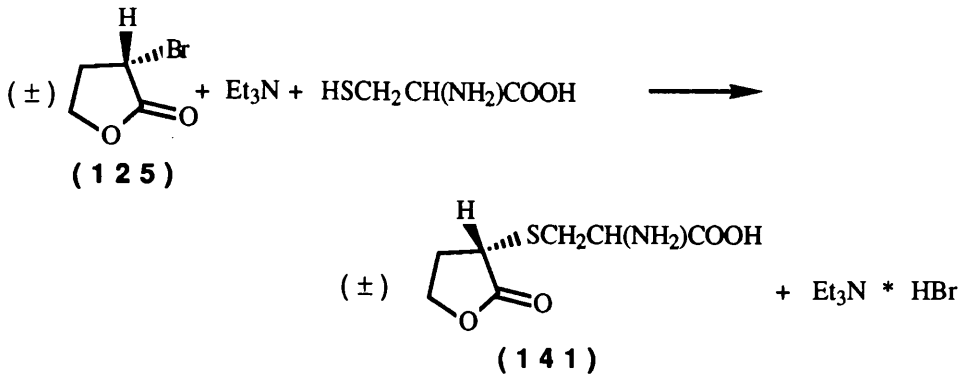
The simplest dibromo lactone (**70**) was also treated with cysteine under the same conditions. No significant reaction occurred at room temperature even overnight. Therefore triethylamine (1 equiv.) was added to generate the more reactive thiolate anion. This time a complex mixture of products was produced slowly. Finally, the dibromolactone (**70**) was treated with 3 equivalents of cysteine and 2 equivalents of triethylamine (Scheme 72). After 4 h, the precipitate was filtered off and dried. It was identified as cystine by ^{1}H NMR spectroscopy and mixed melting point determination. The fitrate was evaporated to dryness and the water soluble residue was extracted with chloroform and methanol respectively. Evaporation of the chloroform gave triethylamine hydrobromide. Evaporation of methanol gave a light coloured solid. Although it could not be fully purified, it appeared to be the substituted lactone (**141**), presumably existing as a mixture of diastereoisomers.



Scheme 72.

This was confirmed by treating 2-bromobutyrolactone (**125**) with 1 equivalent of cysteine and 1 equivalent of triethylamine (Scheme 73). The ^{1}H NMR spectrum and mass spectrum of the product closely resembled those of the foregoing product.

Presumably, the simple dibromodilactone (**70**) is also first reductively debrominated by the thiol, but the debromination is slower than the subsequent reaction of the bromolactone (**125**) with more cysteine. It is interesting that debromination with lactone ring opening, of the dibromodilactone, is much faster than the reaction of the



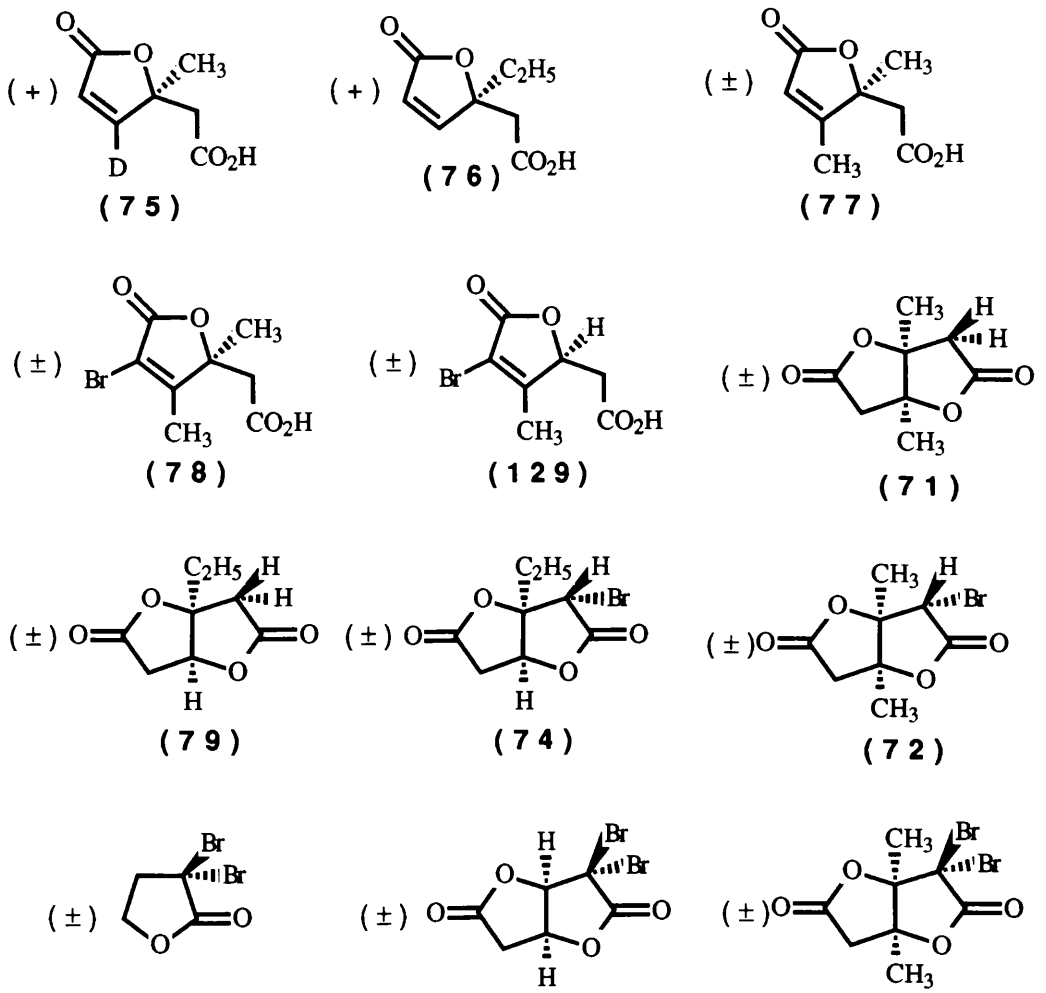
Scheme 73.

model dibromolactone (**70**). Indeed, the former reactions do not require any added base. It remains to be determined whether inhibition of the enzyme with the dibromomethyldilactone (**60**) also results in disulphide formation with liberation of the bromolactone (**129**).

2.4 CONCLUSIONS

Investigations of muconate pathways were centered on the elucidation of the mechanism of and establishment of the stereochemistry of the muconolactone 4-methylisomerisation reaction. Experiments with labelled substrates showed that the enzymic isomerisation can not proceed *via* 1,2-migration of the methyl group. An alternative pathway involving the dilactone or some equivalent enzyme-bound intermediates is indicated. The analysis of the products obtained by incubating the corresponding labelled muconolactone to the methylisomerase from *Rhodoccus rhodocrous* established the location and stereochemistry of the deuterium, thus showing that isomerisation had occurred with the formation of a new lactone ring by *anti* addition and the transformation was completed with the ring opening by *anti* elimination. The loss of deuterium from the labelled dilactone reinforces the status of the methyldilactone (**44**)

as an intermediate in the methylisomerisation reaction. The same conclusiona were reached from Dr. G.V.Rao's supplementary experiments. These conclusions contrast with the *syn* addition observed for the cyclisation of 3-methyl-*cis,cis*-muconic acid (**21**), in *Pseudomonas putida* to give 4-methylmuconolactone (**22**) and in *Aspergillus niger* to give 3-methylmuconolactone (**23**). However, direct experimental evidence is still needed to decide whether the methyldilactone (**44**) is a free intermediate in the enzymic reaction. So far only 3 substrates have been tested for the methylisomerase of *Rhodococcus rhodocrous*.



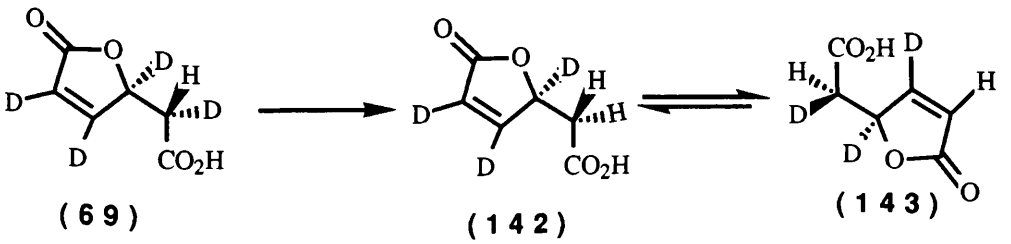
Scheme 74.

A large set of mono- and di-lactones (Scheme 74) has already been sent to Newcastle to be tested as substrates or inhibitors for the methylisomerase. Moreover, the

3-substituted monolactones, and the 3,4-dimethyllactone (**77**) will be tested with cell extracts able to degrade 3-methylmuconolactone. The dimethyllactone (**77**) is unlikely to be degraded, but it might act as a competitive inhibitor.

Attempts were made to correlate the thiol group with the enzyme inhibition chemically. The known inhibitor, dibromodilactone (**60**) can be debrominated by benzyl mercaptan. The debrominated product, bromomuconolactone (**129**) was obtained. When this dibromodilactone (**60**) was treated with cysteine under neutral conditions the debrominated product, bromomuconolactones (**129**), and the disulphide compound (**139**), cystine, were formed. The same outcome was obtained with the parent muconolactone (**73**). Whereas the simple dibromobutyrolactone did not react with cysteine under the same condition. Triethylamine was used as catalyst, the dibromobutyrolactone yielded the substituted lactone (**141**). These findings need to be confirmed by biochemical experiments.

A great deal still needs to be done to clarify the mechanism of this novel methylisomerase enzyme. For example, experiments with tritiated 4-methyl-muconolactone are planned to see if the methyldilactone can be detected as a free intermediate. Also, it will be interesting to use the tetradeuteriolactone (**69**), already prepared enzymically, to see if the enzyme will catalyse a ' virtual reaction', that is conversion of the parent muconolactone into itself (Scheme 75). Since the enzyme, fortuitously, operates in the *anti* mode, then this lactone would be converted into a 1:1 mixture of the trideuteriated products (**142**) and (**143**). The composition of this mixture could be determined unambiguously by ^1H NMR spectroscopy and mass spectrometry.



Scheme 75.

Little is known about the pathway leading from 3-methylmuconolactone (**23**) to metabolically useful products in fungi or the specialised bacteria. This will be a major area of investigation in the future.

Fully deuteriated pyrocatechol was fed, for the first time, to the fungus *Aspergillus niger* to give (4*S*,5*S*)-2,3,4,5-tetradeuterio muconolactone (**69**), which completed the study of the set of enzymes (3 in bacteria and 3 in fungi). The result demonstrated that in the fungus *Aspergillus niger*, the cyclisation of *cis,cis*-muconic acid proceeds *via syn* addition of the carboxyl group the the double bond, The same stereochemical outcome has been achieved in previous published papers summarized in Sec.1.1.3.

The biosynthetic experiments with *Pseudomonas putida* showed that pyrocatechol with the larger alkyl group like ethyl does not effect its conversion into the corresponding muconolactone. But 3,4-dimethylpyrocatechol (**82**) gives a poorer yield of the corresponding muconolactone (**133**), which may be that either pyrocatechol with more alkyl-substituents is not readily cleaved by the dioxygenase enzyme which effects their conversion into the corresponding muconic acid or subsequent cyclisation to give 4-alkyl muconolactone is also retarded by more alkyl groups. The latter point could be tested by experiments with *cis,cis*-3,4-dimethylmuconolactone.

The feeding experiments with *Aspergillus niger* showed that 4-substituted pyrocatechols with larger alkyl group are not good substrates for the enzyme system. The larger the substituent the pyrocatechol has, the poorer yield of muconolactone it produces.

Tests for the cyclisation of muconolactones into their dilactones demonstrated that like optically active 4-methylmuconolactone (**22**), optically active 4-ethylmuconolactone (**76**) can also cyclise non-enzymically under mild condition such as storage at room temperature to give the dilactone with opposite optical rotation. Whereas racemic 3,4-

dimethylmuconolactone (**80**), just like 3-methylmuconolactone (**23**), does not cyclise even under very vigorous conditions such as strong acid and heating.

CHAPTER THREE

EXPERIMENTAL

M.p.s were recorded on a Kofler hot-stage apparatus and are uncorrected. I.R. spectra were recorded on a Perkin-Elmer 580 or 257 spectrometer by Mr. G. McCulloch. ^1H NMR spectra were recorded on a Perkin-Elmer R34 (90 MHz) spectrometer, unless otherwise stated. 200 MHz spectra were recorded by Mr. J. Gall on a Bruker W.P. 200 SY instrument in the pulsed Fourier Transform (F.T.) mode. Deuteriochloroform was used as the solvent with tetramethylsilane as internal standard unless otherwise stated. All proton chemical shifts are quoted to the nearest 0.01 ppm. Low resolution mass spectra were recorded by Mr. A. Ritchie and his staff in the E.I. mode at 70 eV on an A.E.I.M.S. 12 instrument, and high resolution spectra on an A.E.I.M.S. 9 instrument coupled to a GEC-905 computer for data collection and processing. Microanalysis was performed by Mrs. K. Wilson. Microbiological tests were done by Mrs P.Tait and her colleagues. Analytical TLC was carried out on precoated Merck Kieselged GF_{254} plates of thickness 0.25 mm. Spots were viewed under a UV lamp (254 mm) and developed by iodine vapour. Column chromatography was carried out on Merck silica HF_{254} according to the method of Harwood(Ref. 82). Preparative TLC was carried out on 20 cm x 20 cm glassplates coated with a 0.75 mm layer of Merck GF_{254} silica with detection of compounds by UV light.

"Ether" refers to diethyl ether throughout. Solutions in organic solvents were dried, unless otherwise stated, over anhydrous sodium sulphate and evaporated in a Buchi Rotavapor.

3.1 CHEMICAL SYNTHESIS

3.1.1 Fully Deuteriated Pyrocatechol

Fresh deuterium chloride solution was prepared as reported by Thomas *et al.*(Ref.59). Into a 25 ml two-neck, round-bottomed flask was put deuterium oxide (10 ml). The system was cooled with ice, and freshly distilled thionyl chloride (2.62 g , 41 mmol) was added dropwise with stirring. The sulphur dioxide that formed was bubbled away by dry nitrogen. The pyrocatechol (1.10 g, 10 mmol) was then added and the mixture was kept heating at 90 °C overnight. The solvent was evaporated, and a white solid (1.0 g , 91%) was left, m.p. 103-103 °C. There was no signal on the ^{1}H NMR spectrum by comparision of it with the spectrum obtained from an equal amount of pyrocatechol, indicating the completion of the deuteriation reaction.

3.1.2 3-, 5-, 6-Deuteriated Pyrocatechols

(1) 4-Methyl-[3-^{2}H] pyrocatechol (63)

6-Bromoisovanillin (84). -To a solution of isovanillin (**83**) (10 g, 65 mmol) in acetic acid (60 ml) was added bromine (10.7 g, 67 mmol) in acetic acid (15 ml) gradually with stirring at room temperature during *ca.* 1.5 h. After another 2 h, the precipitated white crystals were filtered off. The filtrate was evaporated to half the volume to produce a further crop of crystals and finally to dryness to give a dark brown oil, which then solidified. Recrystallisation of the combined first and second crops gave 2-bromo-isovanillin as white crystals (5.0 g, 33%), m.p. 210-212 °C (from ethanol) (lit., 211-212°C) (Ref. 61). The mother liquors were evaporated to dryness and combined with the brown solid. Recrystallisation of this brown mass from ethanol-water three times, to

constant melting point, gave 6-bromoisovanillin (**84**) as its monohydrate (6.9 g, 46%), m.p. 109-110 °C (from ethanol-water) (lit., 111-112 °C) (Ref. 61).

6-Bromo-[2-^{2}H]isovanillin (85).- 6-Bromoisovanillin (**84**) monohydrate (1.14 g, 4.6 mmol), deuterium oxide (2.2 ml), and triethylamine (473 mg,4.6 mmol) were mixed and heated under reflux under nitrogen for 36 h. The reaction mixture was allowed to cool and was acidified with 6 N hydrochloric acid using Congo Red indicator (blue colour). The mixture was extracted with ether (3 x 50 ml). The ether extract was washed with water and dried. Evaporation of the ether gave 6-bromo-[2-^{2}H]-isovanillin (**85**) (0.79 g, 75%), m.p. 109-111 °C (lit., 109-112 °C). The ^{1}H NMR spectrum [$(CD_3)_2SO$] showed a singlet at δ 7.30 (1 H, 5-H) and no significant signal at δ 7.35 for 2-H.

6-Bromo-[2-^{2}H]protocatechuic aldehyde (86).- Anhydrous aluminium chloride (1.91 g, 15 mmol) was suspended in a solution of 6-bromo-[2-^{2}H] isovanillin (**85**) (3.0g, 13 mmol) in dichloromethane (20 ml). Pyridine (4.53 g, 57 mmol) was added slowly to this ice-cooled, stirred mixture under a nitrogen atmosphere. The resulting clear, light-orange solution was heated under reflux for 24 h under nitrogen. The darkened solution was cooled and acidified with 6 N hydrochloric acid until a Congo Red indicator became blue. The lower dichloromethane layer was discarded (it mainly contained unreacted starting material) and the aqueous layer was extracted with ether. The extracts were washed with water and then dried and evaporated to give the product (**86**) as a yellowish solid (2.3 g, 89%), m.p. 220-224 °C (from ethanol-water) (lit., 220 °C) (Ref. 62). The ^{1} H NMR spectrum [$(CD_3)_2SO$] showed the disappearance of the singlet at δ 2.92 (OMe) present in the starting material. There was no indication of loss of deuterium, δ 7.25 (1 H, s, 2-H).

[2-^{2}H]Protocatechuic aldehyde (87). - Triethylamine (101 mg, 1 mmol) and 10% palladium-carbon (38 mg) were added to a solution of 6-Bromo-[2-^{2}H]protocatechuic aldehyde (**86**) (216 mg, 1mmol) in methanol (350 ml). The mixture was hydrogenated with stirring at room until *ca.* 1 equivalent of hydrogen (25 ml) was absorbed. The mixture was filtered and the filtrate was evaporated . The residual brown solid was extracted with ether. Evaporation of the ether gave the product (**87**) as a yellowish solid (120 mg, 87%). The ^{1}H NMR spectrum [$(CD_3)_2SO$] showed signals at δ 6.90 and δ 7.30 (2 H, ABq, *J* 9Hz, 5-and 6-H). There was no significant signal at δ 7.25 for 2-H.

4-Methyl-[3-^{2}H] pyrocatechol (63). - [2-^{2}H]Protocatechuic aldehyde (**87**) (290 mg, 2 mmol) in ethanol containing a catalytic amount of 1 M hydrochloric acid in ethanol (0.04 ml,*ca.* 0.04 mmol) was hydrogenated over 10% palladium carbon (74 mg) with stirring at room temperature for 1 h until sufficient hydrogen (100 ml, 4.2 mmol) was absorbed. The mixture was filtered and the filtrate was neutralised with solid sodium bicarbonate. Ethanol was evaporated under reduced pressure and the residue was extracted with ether, leaving a mass of inorganic salts. Evaporation of the ether gave the product (**63**) as a brown oil (0.196 g, 77%) ; δ [D_2O (Bu^tOH as standard, δ 1.22)] 2.18 (3 H, s, Me) and 6.60 and 6.85 (2 H, ABq, *J* 9Hz, 5- and 6- H). There was no significant signal at δ 6.75 arising from a proton at C-(3).

(2) 4-Methyl-[5-^{2}H]pyrocatechol (96)

Acetylvanillin (91). - (a) Acetic anhydride (10.1 ml,*ca.* 11.1 g, 0.1 mol) in ether (100 ml) was added slowly with stirring at room temperature to a solution of vanillin (**90**) (15.4 g, 0.1 mol) and sodium hydroxide (4.0 g, 0.1 mol) in water (80 ml). The mixture was stirred for 30 min and the organic phase was separated, washed with water and dried. Evaporation of the ether gave white crystals of acetylvanillin (**91**) (17 g, 82%)

(lit., 81%) (Ref. 64). Recrystallisation gave white needles, m.p. 76-77 °C (from ethanol) (lit., 76-77 °C) (Ref.64). (b) A mixture of vanillin (**90**) (25 g, 0.18 mol), acetic acid (25 ml), and analytical grade pyridine (5 ml) was heated under reflux for 3 h. The reaction mixture was cooled and concentrated under reduced pressure. The residual oil solidified on cooling. Recrystallisation gave white crystals (28 g, 89%) (lit., 81.7%), m.p. 76-77 °C (from ether) (lit., 76-77 °C) (Ref. 64).

6-Bromovanillin (92). - A mixture of acetylvanillin (**91**) (38.8 g, 0.2 mol), anhydrous sodium acetate (38.8 g, 0.47 mol), iodine (0.25 g, 1 mmol), acetic acid (150 ml), and bromine (11 ml, *ca.* 44 g , 0.27 mol) was heated with stirring at 45 °C for 3 h. The warm reaction mixture was poured slowly into cold water (2 L) with vigorous stirring. A light orange solid precipitated after 2 h. The precipitate was collected by filtration and washed thoroughly with water and dried under vacuum. 6-Bromo-*O*-acetylvanillin, so obtained (45 g, 83%) was used without purification. The bromoacetyl compound (45 g, 0.165 mol) was dissolved in water (150 ml) containing potassium hydroxide (45 g, 0.8 mol) and heated at 80 °C for 1 h with stirring. The clear brown liquid was cooled in ice and acidified with concentrated hydrochloric acid to pH 3-4. The resulting pale-brown precipitate was collected by filtration, washed thoroughly with cold water, and dried under vacuum. Treatment of this solid with charcoal in ethanol gave 6-bromovanillin (**92**) as white crystals (28 g, 73%) , m.p. 176-177 °C (from ethanol) (lit., 176-177 °C) (Ref. 64).

6-Bromovanillin dimethyl acetal (93). - 6-Bromovanillin (**92**) (2.2 g, 96 mmol), dried and distilled methanol (50 ml), trimethyl orthoformate (2.2 g, 21 mmol), and ammonium nitrate (750 mg, 9.4 mmol) were heated under reflux under a dried nitrogen atmosphere overnight (20 h). The reaction mixture was slightly cooled and evaporated under reduced pressure. The residual oil was dissolved in anhydrous ether and passed

through Celite which was then washed thoroughly with ether. Evaporation of the ether solution gave a light coloured oil, which was allowed to stand under high vacuum to yield a white solid (2.3 g , 88%). Recrystallisation from light petroleum (b.p. 60-80 °C) gave the acetal (**93**), m.p. 42-43 °C.

[6-^{2}H]Vanillin (94). - 6-Bromovanillin dimethyl acetal (**93**) (625 mg, 2.3 mmol), in anhydrous ether (10 ml) (dried over sodium hydride) was cooled with solid carbon dioxide-acetone to -50 °C and stirred under dry nitrogen. Butyl-lithium in hexane (1.6 M, 8 ml, 12.8 mmol) was added over a period of 10 min. The solution was allowed to warm to room temperature. Deuterium oxide (98%, 0.3 ml, 15 mmol) was added slowly over 2 h. The mixture was refluxed gently for 1 h, water (5 ml) was added, and the aqueous layer was adjusted to pH 3-4 with concentrated hydrochloric acid. The mixture was refluxed for another 10 min. The ether-hexane layer was separated and the aqueous layer was saturated with sodium chloride and then extracted with ether. The combined ether solutions were treated with charcoal and dried. Evaporation of the ether gave a dark oil, which was separated by chromatography on a short GF_{254} silica column. Elution with chloroform gave [6-^{2}H]vanillin (**94**) as a white solid (56 mg, 17%); δ 3.92 (3 H, s, 4-OMe), 6.78 (1 H, br s, 5-H) , 7.26 (1 H, s, 2-H) and 9.18 (1 H, s, CHO). There is also a weak peak at δ 7.38 for 6-H, which indicated incomplete deuteriation.

[6-^{2}H]Protocatechuic aldehyde (95). - [6-^{2}H]Protocatechuic aldehyde (**95**), m.p. 154-156 °C (lit., 153-154 °C) (Ref. 60), was obtained (83%) by demethylation of [6-^{2}H]vanillin (**94**) under the conditions described for demethylation of (**85**). The ^{1}H NMR spectrum confirmed that demethylation had occurred without loss of deuterium.

The compound (**95**) was also prepared from 6-bromoprotocatechuic aldehyde (**97**) in good yield (99%) and purity by reduction in tetrahydrofuran by deuterium gas (see Fig.1). Deuterium gas was prepared by adding deuterium oxide slowly to sliced

clean lithium with vigorous stirring, and then was passed through a trap cooled with solid carbon dioxide-acetone to get rid of moisture. A mixture of 6-bromoprotocatechuic aldehyde (**97**) (1.10 g, 5 mmol), 10% palladium-carbon (190 mg), triethylamine (505 mg, 5 mmol), dry tetrahydrofuran (69 ml) and deuterium oxide (0.69 ml, 1% v/v of THF) was debrominated with stirring at room temperature until 1 equivalent of newly-prepared deuterium gas was absorbed. The reaction mixture was worked up as usual. A yellowish solid was obtained (0.7 g, 99.7%); δ [$(CD_3)_2SO$] 6.95 (1 H, s, 2-H), 7.25 (1 H, s, 5-H), and 9.65 (1 H, s, CHO). there was no signal at about δ 7.35 arising from a proton at C-(6).

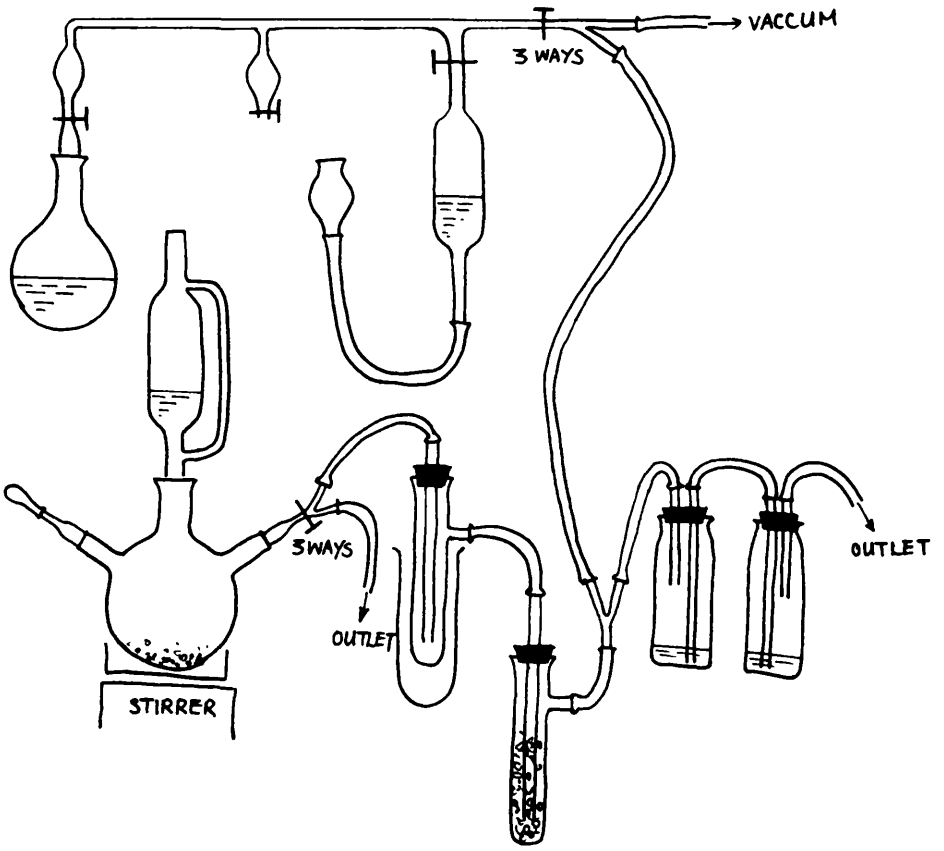


Fig.1. Apparatus Skeleton for Deuterium Debromination

6-Bromoprotocatechuic aldehyde (97). - 6-Bromoprotocatechuic aldehyde (**97**), m.p. 222-224 °C (lit., 220 °C) (Ref. 62) was obtained *via* demethylation of 6-bromovanillin (**92**) by anhydrous aluminium chloride and pyridine according to the procedure for the preparation of compound (**86**).

4-Methyl-[5-^{2}H]pyrocatechol (96). - This compound (**96**) was prepared from [6-^{2}H]protocatechuic aldehyde (**95**) (86%) in a similar way to the preparation of compound (**63**); δ [D_2O (Bu^tOH as standard, δ 1.22)] 2.25 (3 H, s, 4-CH_3), 6.70 (1 H, s, 3-H), and 6.75 (1 H, s, 6-H).

(3) 4-Methyl-[6-^{2}H]pyrocatechol (64)

Pyrocatechol monobenzyl ether (101). - Sodium ethoxide, prepared from sodium (18.4 g, 0.8 mol), in dry ethanol (280 ml) was added dropwise with stirring to pyrocatechol (**1**) (8 g, 0.8 mol) in ethanol (280 ml) under nitrogen at room temperature. Benzyl chloride (102 g, 0.806 mol) was added slowly to the dark orange solution, which was then stirred for a further period of 30 min. The mixture was heated under reflux for 2 h to complete the reaction. The solution was allowed to cool and was acidified with 5 N hydrochloric acid. The precipitated sodium chloride was filtered off and the filtrate was kept in the refrigerator overnight to allow the dibenzyl ether to precipitate. This was then filtered off. The filtrate was evaporated and the residue was extracted with ether. The ether was evaporated under reduced pressure and fractional distillation of the residue gave the monobenzyl ether (**101**) as a pale yellow liquid (87 g, 54%), b.p. 158 °C (2-3 mmHg) [lit., 173 °C (13 mmHg)] (Ref.69).

3-*O*-Benzylprotocatechuic aldehyde (98). - Sodium hydroxide (39.9 g, 0.99 mol) in water (42 ml) was added to the pyrocatechol monobenzyl ether (**101**) (10.5 g,

0.053 mol) in ethanol (88 ml) with stirring at room temperature. Then chloroform (31.5 g, 0.26 mol) was added slowly with stirring to the mixture. The mixture was stirred for 1.5 h and then heated under reflux for 30 min. Solvent were evaporated and the residue was acidified with 5 N hydrochloric acid. The dark brown oil which formed was extracted with ether (3 x 250 ml). The combined ether extracts were washed with water, dried and evaporated. The residual dark oil was chromatographed on a silica gel, GF 254 column. Elution with benzene gave the *O*-benzylpyrocatechol (**101**) and elution with benzene-chloroform (1:1) gave the 3-*O*-benzylprotocatechuic aldehyde (**98**) (2.3 g, 19%), m.p. 112-113 °C (from benzene) (lit., 113-114 °C) (Ref. 70).

3-*O*-Benzyl-[5-^{2}H]protocatechuic aldehyde (99). - The compound (**99**), m.p. 110-113 °C (lit., 112-114 °C) (Ref. 60), was obtained (87%) from (**98**) by exchange in deuterium oxide and triethylamine as described before (Ref. 62).

[5-^{2}H]Vanillin (102). - [5-^{2}H]Vanillin (**102**), m.p. 77-79 °C (from water) (lit., 79-81 °C) (Ref.), was obtained from vanillin (**90**) by deuterium oxide/triethylamine as described before (Ref. 62).

[5-^{2}H]Protocatechuic aldehyde (103). - This compound (**103**), m.p. 153-156 °C (lit., 153-154 °C) (Ref. 60), was prepared from its *O*-methyl ether, [5-^{2}H]vanillin (**102**) (82%) by following the procedure described before (Ref. 62).

4-Methyl-[6-^{2}H] pyrocatechol (64). - The compound (**64**) was prepared from 3-*O*-benzyl-[5-^{2}H]protocatechuic aldehyde (**99**) (81%) and from [5-^{2}H]protocatechuic aldehyde (**103**) (88%) by hydrogenolysis as described in the preparation of compound (**63**), δ [D_2O (Bu^tOH as standard, δ 1.22)] 2.15 (3 H, s, 4-Me), 6.65 (1 H, s, 6-H), and 6.75 (1 H, s, 2-H).

3.1.3 4-Ethylpyrocatechol (81)

4-Benzoyloxy-3-hydroxyethylbenzene (105). - A mixture of 4-ethylphenol (**104**) (6.1 g, 0.05 mol), benzoyl peroxide (12.11 g, 0.05 mol) and dried chloroform (50 ml) was heated to reflux for 6 h. After being cooled, the resultant brown solution was washed with saturated aqueous sodium bicarbonate until alkaline pH indicated that all the benzoic acid that had formed had been removed. The chloroform layer was dried and evaporated to dryness under reduced pressure. The residual oil was scratched under hexane. The resulting orange coloured solid was collected and recrystallised from chloroform-hexane to give the cream coloured benzoyl ester (4.5 g, 37%), 4-*benzoxyloxy*-3-*hydroxy-ethylnenzene*, m.p. 126-128 °C (Found: C, 74.21; H, 5.76%. $C_{15}H_{14}O_3$ requires C, 74.36; H, 5.82%); δ 1.10 (3 H, t, *J* 7Hz, 4-CH_2-CH_3), 2.56 (2 H, q, *J* 7Hz, CH_2-CH_3), 6.68-8.30 (9 H, m, Ar-H and -OH, 1 H exch. with D_2O).

4-Ethylpyrocatechol (81). - The ester (2.2 g, 10 mmol) was added slowly with stirring to dilute aqueous sodium hydroxide solution (100 ml) containing sodium hydroxide (0.8 g, 20 mmol) under nitrogen. The solution, which became dark in colour, was stirred overnight and then acidified with concentrated hydrochloric acid, and saturated with sodium chloride. The ethylpyrocatechol (**81**) that had formed was extracted with ether and the combined ether extracts were washed with saturated sodium bicarbonate to remove benzoic acid and then water. The dark ether solution was dried and evaporated. The residual dark mass was distilled (Kugelrohr distillation) and a clear pale yellow liquid was obtained which then solidified (0.95 g, 76%), b.p. 125 °C (13 mmHg) [lit., 172 -175 °C (35 mmHg)]. m.p. 36-38 °C (lit., 39 °C) (Ref. 75). δ 1.12 (3 H, t, *J* 7Hz, 4-CH_2-CH_3), 2.52 (2 H, q, *J* 7Hz, 4-CH_2-CH_3), 6.54-6.84 (3 H, m, Ar-H).

3.1.4 4,5-Dimethylpyrocatechol (82)

Potassium nitrosodisulfonate (Fremy's salt) (118). - The procedure given by Singh (Ref. 74) was followed with minor changes. In a 5 L three neck-round-bottomed flask were placed distilled water (1 litre) and crushed ice (500 g), sodium bicarbonate (79.2 g, 0.94 mol), sodium nitrite (66 g, 0.84 mol). The mixture was thoroughly mixed with a mechanical stirrer and was externally cooled to - 5 °C by a mixture of salt and ice. Sulphur dioxide was then passed into the mixture rapidly, and its temperature was controlled at - 5 °C to - 2 °C. When the colour changed to orange-brown (approx. 50 min), the passage of sulphur dioxide was immediately stopped. The mixture was stirred for an additional 10 min., during which time it became colourless. Clean nitrogen was then bubbled through the solution for 5 min. and the pH of the solution was adjusted to 10.1 with 18% ammonium hydroxide (50 ml). The solution was stirred at - 2 °C for 30 min, and 1N potassium permanganate (120 g in 760 ml water), cooled to - 3 °C, was then added portion-wise at a rate which kept the temperature below 5 °C. The mixture was stirred for 1 h and then allowed to stand at the same temperature for 1 h. The dark-brown slurry was filtered through a Celite bed into a saturated solution of potassium chloride (500 g in 140 ml water at 30 °C). On cooling, the solution yielded an orange crystalline precipitate which was collected by Buchner funnel, washed with saturated potassium chloride and ammonial methanol (containing about 10% concentrated ammonia), dried under vacuum over calcium oxide, in the presence of ammonium carbonate in a separate dish to provide an ammonia atmosphere, and the product (**118**) (170 g, 72%) stored in a refrigerator.

4,5-Dimethyl-1,2-benzoquinone (117).- Sodium dihydrogen phosphate (1.88 g) was dissolved in water (525 ml) in a 1 L conical flask, with stirring. To this solution was added potassium nitrosodisulfonate (Fremy's salt) (12 g, 41 mmol). The mixture was

stirred to give a clear, purple solution. A solution of 3,4-dimethylphenol (**116**) (2.0 g, 16.3 mmol) in ether (44 ml) was added quickly to this purple solution. The mixture was stirred for another 20 min. The colour of the solution changed to red-brown. The *o*-quinone thus formed was subsequently extracted three times with chloroform (total 130 ml). The combined extracts were dried and evaporated under reduced pressure at room temperature. The residual, oily, red-brown crystals were slurried with ice-cold ether (2 ml). The resulting dark-red crystals (1.2 g, 51%) had m.p. 99-101 °C (lit., 102 °C) (Ref.78); δ 2.15 (6 H, s, 4- and 5- Me) and 6.21 (2 H, br s, 3- and 6-H).

4,5-Dimethylpyrocatechol (82). - This compound was prepared by reference of Stubeurauch and Knuppen's work. 4,5-Dimethyl-1,2-benzoquinone (**117**) (1.2 g, 8.8 mmol) was dissolved in chloroform (120 ml) in a separating funnel. Potassium iodide (4.0 g, 24 mmol) in acetic acid (250 ml) was added to this dark red solution, and the mixture was shaken for 2 min. Iodine was removed by washing with 5% sodium thiosulphate solution and then water. The organic layer was dried and evaporated. The residual oil was distilled (Kugelrohr distillation) to give white solid (750 mg, 63 %), m.p. 84-86 °C; δ 2.10 (6 H, s, 4- and 5-Me) , 6.65 (2 H, s, 3- and 6-H) and 5.70 (2 H, br s, 1- and 2-OH, exch. with D_2O).

3.1.5 Monolactones

2-Bromobutan-4-olide (125). - A mixture of freshly distilled butan-4-olide (**124**) (17.2 g, 0.2 mol) and phosphorus tribromide (0.2 ml) was heated to 100 °C under nitrogen. Bromine (28 g, 0.18 mol) was added to it slowly over a peroid of 5 h at a rate which kept the temperature between 100 °C-130 °C. The reaction mixture was kept at 130 °C overnight. The mixture was allowed to cool and nitrogen was bubbled through it for 1 h. After three-round fractional distillations, pure 2-bromobutan-4-olide (**125**) was

obtained, b.p. 76 °C (0.5 mmHg) [lit., 125 °C (12 mmHg)] (Ref. 85); δ 2.34-3.16 (2 H, m, 3-H), 4.36-4.78 (3 H, m, 2-H and 4-CH_2).

2, 2-Dibromobutan-4-olide (70). - A mixture of freshly distilled butan-4-olide (**124**) (17.2 g, 0.2 mol) and phosphorus tribromide (0.2 ml) was heated to 170 °C under nitrogen. Bromine (96 g, 0.6 mol) was added slowly to it at a rate which kept at a temperature ≤ 190 °C. The resultant mixture was kept at about 190 °C overnight. The work-up of the product was the same as that described in the preparation of 2-bromobutan-4-olide (**125**). The colourless liquid, 2, 2-dibromobutan-4-olide (**70**), b.p. 65 °C (0.1mmHg) [lit., b.p. 115 °C (8mmHg)] (Ref. 85) was collected and the liquid crytallized on standing. m.p. 47-48 °C; δ 3.25 (2 H, t, *J* 7 Hz, 3-CH_2), 4.44 (2 H, t, *J* 7 Hz, 4-CH_2).

3.1.6 Dimethylmuconolactones

3,4-Dimethyl-6-nitrophenol (123). - 3,4-Dimethylphenol (**116**) (6.0 g, 0.049 mol) in acetic acid (60 ml) was added rapidly to concentrated nitric acid (9 ml, d 1.42, 0.202 mol) in acetic acid (30 ml), which was cooled using a mixture of ice and salt. The resultant mixture was diluted at once with water and the oily precipitate was scratched to induce crystallisation. The dark orange solid was collected and washed thoroughly with water. 3,4-Dimethyl-6-nitrophenol (**123**) (2.4 g, 29%) was separated from the solid mixture, which contained approx. 40 % 2, 6-dinitro compound by chromatography on a column of silica gel HF_{254}, which was eluted with chloroform-hexane (3:7, v/v). The product had m.p. 84-86 °C (lit., 87 °C) (Ref. 83); δ 2.20 (3 H, s, Me), 2.25 (3 H, s, Me), 6.70 (1 H, s, 2-H) and 7.70 (1 H, s, 5-H).

3,4-Dimethylmuconolactone (77). - 3,4-Dimethyl-6-nitrophenol (**123**) (4.0 g, 0.024 mol) was added in portions to concentrated sulphuric acid (15 ml) at 110-115 °C during the course of 2 h with stirring. After another 30 min stirring, the black mixture was allowed to cool to room temperature and was carefully poured onto crushed ice. When the ice had melted, the aqueous solution was saturated with sodium chloride and extracted continuously with ether for 48 h. The ethereal extract was dried and evaporated to give a yellowish solid, which was recrystallised from benzene giving white crystals of 3,4-dimethylmuconolactone (**77**) (1.6 g, 48.5%), m.p.103-104 °C (lit., 103-104 °C) (Ref. 81); δ [200MHz] 1.54 (3 H, s, 4-Me), 2.08 (3 H, s, 3-Me), 2.71 and 2.92 (2 H, ABq, *J* 8Hz, 5-CH_2), 5.78 (1 H, s, 2-H) and 8.70 (1 H, br s, COOH, exch. with D_2O).

2-Bromo-3,4-dimethylmuconolactone (78). - The title compound (**78**) (79%) was prepared from (±)-1,5-dimethyl-8-bromodilactone (**72**). The bromodilactone (**72**) (125 mg, 0.5 mmol) was dissolved in dichloromethane (2 ml) containing triethylamine (50.5 mg, 0.5 mmol) and the solution was stirred at room temperature for 1 h. The disappearance of bromodilactone (**72**) was followed by TLC. The evaporation of dichloromethane gave the 2-bromo-3,4-dimethylmuconolactone triethylamine salt. The salt was dissolved in methanol and stirred with acid resin, Ambulite 200, for 30 min. and the resin was filtered off and washed thoroughly with methanol. The combined methanol solution was evaporated and the free acid of 2-bromo-3,4-dimethylmuconolactone (**78**), *2-bromo-3,4-dimethyl-4-carboxylmethyl-*Δ^{α}*-butenolide*, was given. The product had m.p. 103-105 °C (chloroform-hexane); (Found: C, 38.40; H, 3.64%. $C_8H_9BrO_4$ requires C, 38.58; H, 3.64%); ϑ_{max} 1790 and 1650 cm^{-1}(-CO-); δ (CD_3OD) 1.58 (3 H, s, 4-Me), 2.15 (3 H, s, 3-Me), and 2.94 (2 H, s, 5-CH_2); m/e, 250 and 248 (1:1)

3.1.7 Dilactones

(1) The muconodilactones

(±)-4-Bromomuconodilactone (127).- (±)-Muconolactone (**8**) (142 mg, 1 mmol), prepared by Dr. G.V. Rao was dissolved in aqueous sodium hydrogen carbonate (84 mg in 3 ml water). Bromine (160 mg, 1 mmol) in dichloromethane (5 ml) was added at 0 °C with stirring. The mixture was allowed to warm to room temperature and was stirred for 7 h to complete the reaction. Aqueous sodium thiosulphate was added to remove any traces of bromine. The mixture was extracted with dichloromethane (3 x 10 ml) and the extracts were dried ($MgSO_4$). Evaporation of the solvents gave the bromodilactone (**127**) as a white solid, which recrystallised from chloroform-hexane as white crystals (200 mg, 90.5%). (±)-4-Bromo-dilactone (**127**) had m.p. 137-139 °C; δ 2.98 (2 H, dd, *J* 4 and 2 Hz, 8-H_2), 4.46 (1 H, d, *J* 1 Hz, 4-H), 5.12 (1 H, dd, *J* 4 and 1 Hz, 5-H), 5.42 (1 H, br s, 1-H).

(±)-4,4-Dibromomuconodilactone (73). - The 4-bromomuconodilactone (**127**) (110 mg, 0.5 mmol) was dissolved in water containing sodium bicarbonate (42 mg, 0.5 mmol) and the mixture was stirred for 1.5 h to get a clear solution. To this, bromine (80 mg, 0.5 mmol) in dichloromethane (3 ml) was added and left stirring for another 3 h. The dichloromethane layer was separated and the aqueous solution was extracted with dichloromethane. The combined organic layers were washed with saturated sodium bicarbonate and water, and then dried. Evaporation of the solvent gave 4,4-*dibromo*-3,7-*dioxo*-2,6-*dioxabicyclo*-(3, 3, 0)-*octane* (**73**) as a white solid. Recrystallisation from ethyl acetate-hexane gave rods (135 mg, 90%), m.p. 128.5-130 °C (Found: C, 24.21; H, 1.26%. $C_6H_4Br_2O_4$ requires C, 24.03; H, 1.34%); $\vartheta_{max}(CHCl_3)$ 1807cm^{-1} (-CO-); δ 3.10 (2 H, d with fine splitting, 8-CH_2), 5.28 (1 H, d, *J* 4Hz, 5-H), 5.46 (1 H, br m, 1-

H); *m /e* 301.8429 (0.2%), 299.8482 (0.3%), and 297.8507 (0.2%). $C_6H_4{}^{81}Br_2O_4$, $C_6H_4{}^{81}Br^{79}BrO_4$, and $C_6H_4{}^{79}Br_2O_4$ require 301.8420, 299.8507, and 297.8537.

(±)-Muconodilactone (59). - To a suspension of the bromodilactone (**127**) (110 mg, 0.5 mmol) in dry benzene (2 ml) azoisobutyronitrile (7 mg, 10% mmol of dilactone) was added under nitrogen and the flask was sealed with a subaseal. Tributyltin hydride (0.27 ml, *ca*, 0.6 mmol) was injected into this mixture, which was then warmed to 30-40 °C to get clear solution. The white solid was precipitated 5 min. later and the mixture was heated for another 15 min. and then allowed to cool to room temperature with a further 1 h stirring. The solvent was evaporated and The residue was triturated with hexane. The white solid was collected and recrystallised from chloroform-hexane as a white solid (65 mg, 92%), m.p. 131-133 °C (lit., 129-130 °C) (Ref. 12); δ 2.98 (4 H, d, 4- and 8-H), 5.20 (2 H, br s, 1- and 5-H). This compound had been fully characterised by Dr. G. V. Rao.

(2) Methyl-substituted muconodilactones

(±)-1-Methyl-8-bromomuconodilactone (37). - This compound (**37**) (89%) was prepared from (±)-3-methylmuconolactone (**35**) supplied by Dr. G.V. Rao following the same methods used for the parent bromomuconodilactone (**127**). It had m.p. 88-89 °C, δ 1.76 (3 H, s, 1-Me), 2.94 (2 H, m, 4-H), 4.45 (1 H, s, 8-H), and 4.96 (1 H, m, 5-H). The m.p. and spectrum agreed with those obtained by Dr. G. V. Rao.

(±)-1-Methyl-8,8-dibromodilactone (60). - (±)-1-Methyl-8,8-dibromodilactone (**60**) (93%) was prepared from (±)-1-methyl-8-bromomuconodilactone (**37**) following the procedure described for the parent dibromomuconodilactone (**73**). The dilactone (**60**) had m.p. 153-155 °C (from ethyl acetate-hexane); δ (200MHz) 1.87 (3 H, s, 1-Me),

2.75-3.20 (2 H, AB part of ABX, *J* 18.6 Hz, 3.7Hz and 1.1Hz, 4-H_2), and 5.10 (1 H, X part of ABX, *J* 3.7 Hz and 1.1Hz, 5-H). This compound had been fully characterised by Dr. G. V. Rao.

(±)-1-Methylmuconodilactone (38). - The (±)-1-methyldilactone (**38**) (91%) was obtained *via* debromination of (±)-1-methyl-8-bromomuconodilactone (**37**) by tributyltin hydride and AIBN in benzene following the procedure used in the preparation of parent dilactone (**59**). The dilactone had m.p. 90-91 °C (from chloroform-hexane); δ (D_6-acetone) 1.65 (3 H, s, 1-Me), 2.55-3.45 (4 H, m, 4- and 8-C$\underline{H}_2$), and 5.15 (1 H, m, 5-H). The m.p. and spectrum agreed with those obtained by Dr. G. V. Rao.

1-methyl-4-bromo-[8-^{2}H]-muconodilactone (130). - The crude (4*S*,5*S*)-4-methyl-[5-^{2}H]muconolactone (**65**) (20mg, 0.13 mmol), obtained by feeding [3-^{2}H]-4-methylpyrocatechol (**63**) to *P. putida*, was dissolved in a solution of sodium hydrogen carbonate (11.0 mg, 0.13 mmol) in water (0.25 ml). Bromine (22.0 mg, 0.13 mmol) in dichloromethane (0.4 ml) was added at 0 °C with stirring. The mixture was allowed to warm to room temperature and was stirred for 7 h to complete the reaction. Aqueous sodium thiosulphate was added to remove any traces of bromine. The mixture was extracted with dichloromethane (3x10ml) and the extracts were dried ($MgSO_4$). Evaporation of the solution gave the bromodilactone (**130**), 4-*bromo*-1-*methyl*-3,7-*dioxo*-2,6-*dioxabicyclo*-(3, 3, 0)-*octane* (**130**), as an orange coloured solid (20 mg); δ [$(CD_3)_2CO$] 1. 89 (3 H, s, 1-C$\underline{H}_3$), 3.05 (1 H, br s, 8-C$\underline{H}$), 4.82 (1 H, s, 4-H) and 5.22 (1 H, s, 5-H). The procedure was that carried out by Dr. G. V. Rao with the unlabelled material.

1-Methyl-[8-^{2}H]-muconodilactone (67). - Azoisobutyronitrile (AIBN) (1.2 mg, 0.0086 mmol) was added under dry nitrogen to a suspension of the bromo-

muconodilactone (**130**) (20 mg, 0.086 mmol) in dry benzene (0.18 ml) and the flask was sealed with a subaseal. Tributyltin hydride (0.03 ml, *ca.* 0.011 mmol) was injected into this mixture, which was then warmed to 30-40 °C. The solution was stirred for 1 h and evaporated. The residue was triturated with hexane and the residue solid was recrystallised from chloroform-hexane to yield the dilactone (**67**), 8-*deuterio*-1-*methyl*-3,7-*dioxo*-2,6-*dioxabicyclo*-(3, 3, 0)-*octane* (**67**), as white solid (2 mg), m.p. 107-108 °C; δ 1.70 (3 H, s, Me), 2.65 and 3.45 (3 H, m, 4-H_2 and 8-H), and 5.20 (1 H, br s, 5-H).

(3) Ethyl-substituted Muconodilactones

(±)-1-Ethyl-8-bromomuconodilactone (71). - The title compound (**71**) (88%) was prepared from (±)-1-ethylmuconolactone (**126**) supplied by Dr. D.R.Jaap following the procedure described in the preparation of the parent bromodilactone (**127**), 4-*bromo*-1-*ethyl*-3,7-*dioxo*-2,6-*dioxabicyclo*-(3, 3, 0)-*octane* (**71**) had m.p. 101-102 °C (chloroform-hexane) (Found: C, 38.79; H, 3.64%. $C_8H_9BrO_4$ requires C, 38.58; H, 3.64%); δ 1.10 (3 H, t, 1-CH_2-CH_3), 1.53-2.40 (2 H, q with fine splitting, 1-CH_2-CH_3), 2.98 (2 H, m, 4-H_2), 4.45 (1 H, s, 8-H), and 4.96 (1 H, m, 5-H).

(±)-1-Ethyldilactone (79).- The (±)-1-ethyldilactone (**79**) (89%) was prepared from the bromodilactone (**71**) by debromination following the same procedure as the parent dilactone. 1-*Ethyl*-3,7-*dioxo*-2,6-*dioxabicyclo*-(3, 3, 0)-*octane* (**79**) had m.p. 121-123 °C (chloroform-hexane) (Found (%), C, 56.19; H, 6.08; $C_8H_{10}O_4$ requires (%), C, 56.46; H, 5.92); δ 1.10 (3 H, t, *J* 7 Hz, 1-CH_2-$C\underline{H}_3$), 1.86 (2 H, q ,*J* 7 Hz, 1-$C\underline{H}_2$-CH_3), 2.65-3.05 (4 H, m, 4- and 8-$C\underline{H}_2$), and 4.85 (1 H, t, 5-H).

(-)-1-Ethyl-4-bromomuconodilactone (131). - (-)-1-Ethyl-4-bromodilactone (**131**) was prepared from optically active (+)-4-ethylmuconolactone (**76**) obtained by feeding 4-ethylpyrocatechol (**81**) to the bacteria *Pseudomonas putida* described in 3.2.1. The product bromodilactone, 1-*ethyl*-4-*bromo*-3,7-*dioxo*-2,6-*dioxabicyclo*-(3, 3, 0)-*octane* (**131**) was semi-solid. $[\alpha]_D$ - 54 ° (*c* 1 in methanol); δ 1.12 (3 H, t, *J* 7 Hz, 1-CH_2-C$\underline{H}_3$), 1.89-2.45 (2 H, q with fine splitting, 1-C$\underline{H}_2$-CH_3), 2.94 and 3.18 (2 H, ABq, *J* 19Hz, 4-C$\underline{H}_2$), 4.85 (1 H, br s, 5-H) and 5.25 (1 H, s, 8-H).

(-)-1-Ethylmuconodilactone (132). - (-)-1-Ethyldilactone (**132**) was prepared from (-)-1-ethyl-4-bromodilactone (**131**) by the debromination in benzene with tributyltin hydride and AIBN. 1-*Ethyl*-3,7-*dioxo*-2,6-*dioxabicyclo*-(3, 3, 0)-*octane* (**132**) had m.p. 137-138.5 °C (chloroform-hexane); $[\alpha]_D$ - 104 ° (*c* 0.8 in methanol); δ 1.09 (3 H, t, *J* 7Hz, 1-CH_2-C$\underline{H}_3$), 2.85 (2 H, q, *J* 7Hz, 1-C$\underline{H}_2$-CH_3), 2.58-3.08 (4 H, m, 4- and 8-C$\underline{H}_2$), and 4.85 (1 H, m, 5-H). The m.p., optical rotation and ^{1}H NMR spectrum agreed with the corresponding dilactone obtained during storage.

(4) Dimethyl-substituted Muconodilactones

(±)-1,5-Dimethyl-4-bromomuconodilactone (72). - (±)-1,5-Dimethyl-4-bromomuconodilactone (**72**) (87%) was prepared from (±)-3,4-dimethylmuconolactone (**77**) following the procedure described in the preparation of the parent bromomuconodilactone (**127**). 4-*bromo*-1,5-*dimethyl*-3,7-*dioxo*-2,6-*dioxabicyclo*-(3,3,0)-*octane* (**72**) had m.p.103-5°C (chloroform-hexane); ν_{max} 1786 and 1678 cm^{-1} (-CO-); δ [200MHz] 1.73 (3 H, s, 1-*Me*), 1.75 (3 H, s, 5-*Me*) , 2.20-3.05 (2 H, m, *J* 18.6 and 1.1 Hz, 8-H2), and 4.49 (1 H, d, *J* 1.1Hz, 4-H); *m /e* 248 (0.6%) and 250 (0.6%).

(±)-1,5-Dimethyl-4,4-dibromodilactone (74). - (±)-1,5-Dimethyl-4,4-dibromomuconodilactone (**74**) (93%) was prepared from (±)-1,5-Dimethyl-4-bromomuconodilactone (**72**) following the procedures described for the parent dibromomuconodilactone (**73**). Recrystallisation from chloroform-hexane gave 4,4-*dibromo*-1,5-*dimethyl*-3,7-*dioxo*-2,6-*dioxabicyclo*-(3, 3, 0)-*octane* (**74**) as neddles, m.p. 174-175 °C (chloroform-hexane) (Found: C, 29.31; H, 2.41%. $C_8H_8Br_2O_4$ requires C, 29.30; H, 2.46%); $\vartheta_{max.}$1795 cm^{-1}(-CO-); δ 1.89 (6 H, s, 1-Me and 5-Me), 2.78-3.08 (2 H, ABq,*J* 18Hz, 8-H); *m /e* 327.8784 (1.1%), and 325.8772 (0.5%). $C_8H_8{}^{79}Br^{81}BrO_4$, $C_6H_4{}^{79}Br_2O_4$ requires 327.8797, and 325.8754.

(±)-1,5-Dimethylmuconodilactone (80). - The title compound (**80**) (89%) was prepared from (±)-1,5-dimethyl-4-bromomuconodilactone (**72**) *via* debromination by AIBN and tributyltin hydride in benzene. 1,5-*Dimethyl*-3,7-*dioxo*-2,6-*dioxabicyclo*-(3, 3, 0)-*octane* (**80**) had m.p. 143-145 °C (chloroform-hexane) (Found: C, 56.34; H, 5.79%. $C_8H_{10}O_4$ requires C, 56.46; H, 5.92%); ϑ_{max}1786 cm^{-1}, (-CO-); δ 2.05 (6 H, s, 1-Me and 5-Me), 2.78-3.08 (4 H, ABq, *J* 18 Hz, 4- and 8-H_2); *m/e* 170.

3.1.8 Cyclization Test of 3, 4-Dimethylmuconolactone

The cyclisation of 3,4-dimethylmuconolactone (**77**) was tested under various conditions and monitored by ^1H NMR spectrometer. The ^1H NMR spectrum showed that 3, 4-dimethylmuconolactone did not cyclise under these different conditions.

Table 3. Cyclisation of dimethylmuconolactone (77)

Conditions	Time (h)	^{1}H NMR
a. dissolved in $CDCl_3$, add 50% v/v CF_3COOH	0.5	no change
	1	no change
	24	no change
b. refluxed in CF_3COOH	24	no change
c. refluxed in 70% H_2SO_4	24	no change
d. stirred in 50% aqueous ethanol, Amberlyst-15 as catalyst	24	no change
e. dissolved in EtOAc, then absorbed on silica gel HF_{254}	24	no change
	72	no change

3.1.9 The Reaction of Dibromolactones with Thiol Compounds

(1) 1-Methyl-8,8-dibromomuconolactone with Benzyl Mercaptan

(a). Into a round-bottomed flask were put benzyl mercaptan (12.4 mg, 0.1 mmol) and ethanol (0.6 ml). The dibromomuconolactone (**60**) (15.6 mg, 0.05 mmol) was added to this solution and the reaction mixture was refluxed overnight. The solvents were evaporated and the residue was dried under high vaccum. A white solid was obtained. This was assumed to be a mixture of 2-bromomuconolactone (**129**), by comparision of its ^{1}H NMR spectrum with authentic sample, its ethyl ester, and disulphate (**139**). (b). The dibromodilactone (**60**) (25.2 mg, 0.0806 mmol) was added to a solution of benzyl mercaptan (9.0 mg, 0.0726 mmol) in ethanol (1 ml), the mixture was stirred overnight at room temperature. The solvents were evaporated to dryness and dried. A white solid was obtained. The same results were obtained and unreacted dibromodilactone was observed in the spectrum.

(2) 1-Methyl-8,8-dibromodilactone with cysteine

The dibromodilactone (33mg, 1mmol) was dissolved in 50% aqueous ethanol (7ml) to get a clear solution. To this a clear solution of cysteine (27mg, 2mmol), in 5 ml of 50% aqueous ethanol) was added, at room temperature, with stirring. A white solid precipitated immediately after the addition. The reaction mixture was left stirring for another 2h. The TLC showed that the reaction was complete. The white solid was collected.The ^1H NMR and mixed melting point with cystine monohydrobromide prepared showed that it is cystine hydrobromide. The filtrate was evaporated giving white solid. Recrystallisation of this from chloroform gave white crystals, m.p. 78-79 ^oC, ϑ_{max} 1785 cm^{-1}, 1725 cm^{-1}, 1722 cm^{-1}, δ (CD_3OD, TMS) 1.62 (3 H, s, 3-Me), 2.51-3.18 (2 H, AB part of ABX, *J* 18Hz 7Hz 1Hz, 5-H), 5.25 (1H, dd,*J* 7Hz 1Hz, 4-H); *m/e*, 235.07 ($C_7H_7BrO_4$ requires 235.01). So this compound was characterized as *2-bromo-3-methylmuconolactone* (**129**).

(3) 4, 4-Dibromomuconodilactone (73) with Cysteine

The reaction of 4,4-dibromomuconodilactone (**73**) with cysteine followed the same procedure as described in (2). The chloroform soluble white solid was chacterized as *2-bromomuconolactone* (**128**) which has been fully characterised by Dr. G.V.Rao. δ 2.80 (2 H, d, *J* 7Hz, 5-$C\underline{H}_2$), 5.42 (1H, dt, *J* 7Hz 2Hz, 4-H), 7.83 (1 H, d, *J* 2Hz, 3-H).

(4) Simple α,α-dibromo-γ-butyrolactone (70) with cysteine

(a). The dibromolactone (**70**) (242 mg, 1 mmol) was dissolved in 50 % ethanol (15 ml) and cysteine (242 mg, 2 mmol) was also dissolved in 50 % ethanol (25 ml). The two clear solutions were mixed together and stirred overnight at room temperature. The solvent was evaporated to dryness and the residue was extracted with chloroform. The ^1H NMR spectrum showed that most of the lactone had been recovered. (b) The cysteine

(242 mg, 2 mmol) was dissolved in 50 % ethanol (25 ml) and triethylamine (101 mg, 1 mmol) was added (this helps cysteine to dissolve) and this clear solution was added slowly to the solution of dibromo compound (**70**) (242 mg, 1 mmol) in 50% ethanol (15 ml). A precipitate formed slowly and the mixture was stirred overnight. The work up followed the same procedure as before. The ^{1}H NMR spectrum showed that some unreacted lactone was still present. (c). Conditions were the same as in (b) but three equivalents of cysteine (363 mg, 3 mmol) and two equivalents of triethylamine (202 mg, 2 mmol) were used. The precipitate formed within 2 min and the mixture was stirred for a further 3 h. The precipitate was filtered and dried (154 mg). It was characterized as cystine by an ^{1}H NMR spectrum. Evaporation of the aqueous solution gave some white solid which was extracted with chloroform. The chloroform soluble portion (375 mg) was identified as triethylamine hydrobromide by ^{1}H NMR. The residue left (170 mg) was water soluble. m.p. 185-213 °C. TLC showed that it was a mixture and the mass spectrum showed that it contained the product (**141**), *m/e* 205.0413 ($C_7H_{11}NO_4S$ requires 205.0409), and cysteine *m/e* 121.0200 ($C_3H_7NO_2S$). The same product (**141**) was observed by reacting 1 equivalent of 2-bromobutyrolactone (**125**), cysteine and triethylamine. Attempts to isolate them failed in both cases.

3.2 BIOSYNTHESIS

3.2.1 *Pseudomonas Putida*

Maintenance: *Pseudomonas putida* (ATCC 12633) (Ornston & Stanier, 1966) was a stock laboratory culture originally obtained from Torry Reseach Station. It was maintained on 1% yeast extract agar slope test-tube at 4 ^oC .

Chemicals: 4-Methylpyrocatechol was purchased from BDH company and all the other substrates used were prepared by the author.

General Procedure:

Culture Conditions. - The nutrient medium was prepared as Stanier and Ornston with some modifications.

Nitriloacetic acid	220mg
Magnesium sulphate hetahydrate	580 mg
Calcium chloride dihydrate	88.5 mg
Ferrous sulphate	7 mg
Ammonium sulphate	1.0 g
Potassium dihydrogen orthophosphate	6.8 g
Disodium hydrogen orthophosphate	7.1 g
Sodium benzoate	1.44 g
Hunter' metal solution '44 ' *	1 ml
Deionised water	1 litre

* Hunter's Metals "44" Solution for O &S Medium.

Ethylene Diamine Tetra-Acetate Disodium Salt (E.D.T.A. $Na_2 \cdot 2H_2O$)	318 mg
Zinc Sulphate Hydrate	1095 mg
Manganese Sulphate Hydrate	154 mg
Copper Sulphate Hydrate	39.2 mg
Cobalt Nitrate Hydrate	24.8 mg
Sodium Tetraborate	17.7 mg
Ammonium Molybdate Hydrate	20 mg
Deionised Water	100 ml

Procedures. - The *P. putida* (ATCC 12633) slants were maintained on 1% yeast extract agar for 3 d at 25 °C. A water suspension of the bacteria was prepared by adding 2ml of sterile water to the slope and scratching the surface gently to form a suspension. The suspension was used to inoculate 3 conical flasks each containing 100 ml of the O/S culture medium. The flasks were incubated at 30 °C for 3d with a shaking speed of 160 r.p.m.. This three day-old culture was then used as inoculum for a different number flasks depending on different substrates. Each flask contains 100 ml O/S culture medium. These flasks were then incubated at 30 °C with the same shaking speed for 18 h . The first batch of the pyrocatechol in sterile deionised water was fed to those flasks. The flasks were shaken for 4 h at 30 °C. A ferric chloride test showed that all the pyrocatechol had been metabolised. A second batch of the pyrocatechol was then fed. The flasks were left shaking overnight (18 h) . The ferric chloride test was again negative after this second addition, and a third batch of the pyrocatechol in water was fed. After another 4h, the cultures were adjusted to pH 7.5 with aq. ammonia and extracted with ether to remove non-acidic material. Then the cultures were adjusted to pH 2.5 with orthophosphoric acid, saturated with sodium chloride, and extracted with ether to obtain the lactone. The ether extracts were filtered through Celite, dried, and evaporated to give the target lactone.

(1) Biosynthesis of 4-methylmuconolactones

(+)-(4*S*)-4-Methylmuconolactone (22). - 4-Methylpyrocatechol (**20**) (800mg) in sterile deionised water (6ml) was fed to 18h-grown cultures of *Pseudomonas putida* (20 flasks) in three batches following the general procedures. The evaporation of ether extracts at pH 2.5 gave the target lactone, (+)-(4*S*)-4-methylmuconolactone (**22**) (447mg, 44%) as oil which failed to crystallise; $[\alpha]_D$ = 31.5 °(c, 0.9 in methanol); δ 1.57 (3 H,

s, 4-C$\underline{H}_3$), 2.68 (1 H, d, *J* 15.9 Hz, 5-H_R), 2.92 (1 H, d, *J* 15.9 Hz, 5-H_S), 6.06 (1 H, d, *J* 5.7Hz, 2-H), 7.66 (1 H, d, *J* 5.7Hz, 3-H), and 11.0 (1 H, s, COOH).

(4*S*, 5*S*)-4-Methyl-[5-^{2}H]muconolactone (65). - 4-Methyl -[3-^{2}H]pyrocatechol (**63**) (100mg) in sterile deionised water (2ml) was fed to 18h grown cultures of *Pseudomonas putida* (10 flasks) in three batches and the flasks were shaken for 4h. A ferric chloride test showed that all pyrocatechol had been metabolised. The colour of the solution had changed to violet-red. One of the flasks was adjusted first to pH 7.5 and then to 2.5 with orthophosphoric acid and extracted with ether successively at each pH value. The ^{1}H NMR spectrum of the extracts at pH 2.5 showed that the product was the required monodeuteriated lactone (**65**). The rest of pyrocatechol (200mg) in water (4ml) was fed to the remaining 9 flasks, and the ether extract at pH 2.5 was evaporated to dryness giving (4*S*, 5*S*)-4-methylmuconolactone (**65**) (130 mg, 34%). δ 1.56 (3 H, s, 4-C$\underline{H}_3$), 2.75 (1 H, br s, 5- H $_S$), and 6.10 and 7.65 (2 H, AB_q, *J* 6Hz, 2-H and 3-H).

(4*S*)-4-Methyl-[2-^{2}H]muconolactone (75). - 4-Methyl-[6-^{2}H]pyrocatechol (**96**) (373 mg) was fed in the same way described in general procedure to give the crude (**75**) (90 mg, 19%). Recrystallisation from ethyl acetate-hexane gave the product as a white solid; δ 1.57 (3 H, s, 4-C$\underline{H}_3$), 2.65 and 3.00 (2 H, AB_q, *J* 16Hz, 5-C$\underline{H}_2$), 6.08 (1 H, s, 3-H).

(4*S*)-4-Methyl-[3-^{2}H]muconolactone (66). - 4-Methyl-[6-^{2}H]pyrocatechol (**64**) was fed to 18h grown cultures of *Pseudomonas putida* (10 flasks) following the general procedure to give the crude lactone, (4*S*)-4-methyl-[3-^{2}H] muconolactone (**64**) (170 mg, 34 %) as an oil which failed to crystallise; δ 1.58 (3 H, s, 4-C$\underline{H}_3$), 2.55 and 2.95 (2 H, AB_q, *J* 16Hz, 5-C$\underline{H}_2$), 7.65 (1 H, s, 3-H).

(2) Biosynthesis of 4-ethylmuconolactone

(+)-(4*S*)-4-Ethylmuconolactone (76). - (+)-(4*S*)-4-Ethylmuconolactone (**76**) (290 mg in both cases) was obtained in the yield of (53%) and (42%) respectively by feeding 4-ethylpyrocatechol (**76**) (450 mg, 560 mg respectively) in sterile deionized water (10 ml) to 18h grown *Pseudomonas putida* (10 and 15 flasks respectively) following the general proecdure. The product was an oil which failed to crystallise; $[\alpha]_D$ = 14.5° (c, 0.94 in methanol), δ 0.86 (3 H, t, *J* 7Hz, CH_2-$C\underline{H}_3$), 1.95 (2 H, q, with fine splitting,*J* 7Hz, $C\underline{H}_2$-CH_3), 2.71 and 2.99 (2 H, AB_q, *J* 16Hz, 5-$C\underline{H}_2$), 6.15 and 7.58 (2 H, AB_q, *J* 6Hz, 2- and 3-H). The first batch was stored in the fridge while the second batch was stored at room temperature. The latter batch cyclised spontaneously to give good crystals of the dilactone (**132**). The crystals were collected (40mg from 260 mg oil) and recrystallised from ethyl acetate-hexane to give colourless crystals (10 mg); m.p. 138-139 °C, $[\alpha]_D$ = 105.2 ° (c, 0.6 in methanol), ν_{max} (KBr) 1790cm^{-1}, (-CO-); Found C 56.39, H 6.11 ($C_8H_{10}O_4$ requires C, 56.46; H, 5.92), *m/e* at 170 (11.6%), δ 1.0 (3 H, t, *J* 7Hz, CH_2-$C\underline{H}_3$), 2.85 (2 H, q, with fine splitting, *J* 7Hz, $C\underline{H}_2$-CH_3), 2.68 and 2.98 (2 H, AB_q, *J* 20 Hz, 8-$C\underline{H}_2$), 2.90 (1 H, d, *J* 3 Hz, 4-H), 4.85 (1 H, t, *J* 3Hz, 5-H). All the data were consistent with the optically pure dilactone (**132**) prepared chemically from (4*S*)-4-ethylmuconolactone (**76**).

(3) Biosynthesis of 3,4-dimethylmuconolactone

3,4-Dimethylmuconolactone (77). - 4,5-Dimethyl-pyrocatechol (**82**) (560 mg) in water (12 ml) was fed to 18h grown cultures of *Pseudomonas putida* (10 flasks containing 100ml medium each) following the general procedure to give 3,4-dimethylmuconolactone (**77**) (70 mg, 20%) as an oil which failed to cryatallise, δ1.57 (3 H, s, 4-Me), 2.12 (3 H, s, 3-Me), 2.71, 2.96 (2 H, dd,*J* 8Hz, 5-H) and 5.78 (1 H,

s, 2-H). The structure was established by comparing the spectrum with that of the racemic muconolactone prepared from 3,4-dimethyl-6-nitrophenol (**123**).

3.2.2 *Aspergillus Niger*

Maintenance: *Aspergillus niger* EM 32 mutant was originally obtained from University of Newcastle-upon-tyne. It was maintained at 4 ^{o}C on *Difco bact* potato dextrose agar slopes in 10ml rubber-sealed bottles. Strains of *Aspergillus niger* were subcultured every six months after which time conidial viability was observed to decrease significantly.

Chemicals: 4-methylpyrocatechol was purchased from BDH company and all the other chemicals were prepared by the auther.

General Procedure:

Culture Condition. - Fungi were grown in liquid culture in a basal salts medium containing:

Ammonium sulphate	0.5g
Potassium dihydrogen orthophosphate	1g
Magnesium sulphate heptahydrate	50mg
Trace elements solution *	1ml
Deionised water	1L
Glucose	1.8g

* Trace elements solution:

Ferrous sulphate heptahydrate	0.054g
Manganous sulphate tetrahydrate	0.04g
Zinc sulphate heptahydrate	0.02g
Copper sulphate pentahydrate	.004g
Cobaltous chloride hexahydrate	0.004g

Potassium iodide	0.03g
Sodium molybdate dihydrate	0.005g
Calcium chloride dihydrate	0.662g
Sodium chloride	1g
Deionised water	100ml

The basal salts medium, made up to volume with deionised water, was adjusted to pH 5.5 with 1M sodium hydroxide solution and autoclaved at 15ib/in^2, 120^{o}C.

Procedure. - Fresh conidia were obtained by inoculating slope cultures stored onto the surface of the fresh potato dextrose agar at 21^{o}C for 3 d. The conidia were washed off with Tween 80 solution and suspended in sterile deionised water. The water suspension was used to inoculate. A 1 ml inoculum was put into each flask which contained 100 ml of the medium. The flasks were incubated at 30 ^{o}C for 46 h with a shaking speed of 160 r.p.m. The utilization of glucose was monitored by U.V. spectrometer.** When the glucose almost disappeared, the first batch of catachol was fed and the PH was kept at 5.5 by the addition of sterile 0.5 M dipotassium hydrogen orthophosphate. The flasks were kept at 30 ^{o}C overnight with the same shaking speed. A ferric chloride test showed that all the catechol had been metabolised. A second batch of the catechol was fed and pH was adjusted to 5.5 by adding sterile 0.5 M dipotassium hydrogen orthophosphate and left shaking for another 26 h. The cultures were adjusted to pH 7.2 with saturated sodium bicarbonate and extracted with ether to remove non-acidic material. The cultures were adjusted to pH 2.0 with orthphosphoric acid saturated with sodium chloride and extracted with ether. Evaporation of ether gave some lactone and the aqueous solution was then extracted continously for 48 h to yield more lactone.

**** Colormetric method for determination of concentration of glucose**

Glucose is determined by colorimetric method in phenol and concentrated sulphuric acid reported by Landa (Ref. 84). The amount of glucose in culture medium was determined by reference to the standard curve described as follows.

Standard curve: -The initial concentration of 180 mg/100 ml was prepared. It was diluted to get a different concentration and then 1 ml of aqueous solution was put into 10 ml volumetric flask. 1 ml of 5% phenol solution was added and mixed. Blanks are prepared with 1 ml of water instead of glucose solution. From a fast-flowing pipet (a portion of the tip was removed), 5 ml of 95% sulfuric acid was added to each flask so that the steam hits the liquid surface directly to produce good mixing and even heat distribution. Each flask was shaken during the acid addition, and exactly the same mixing procedure was practised throughout. The yellow-orange colour was stable for several hours. Absorbances were scanned from 300-700 nm using 0.2 cm x 1.5 cm cell. The average absorbance of the blanks is subtracted. The maximum absorbance was located at 487 nm.

Table 4. UV absorbance of glucose at 487 nm

Concentration (mg/100 ml)	Absorbance at 487 nm
90	3.040
72	2.562
54	1.729
36	1.069
18	0.783

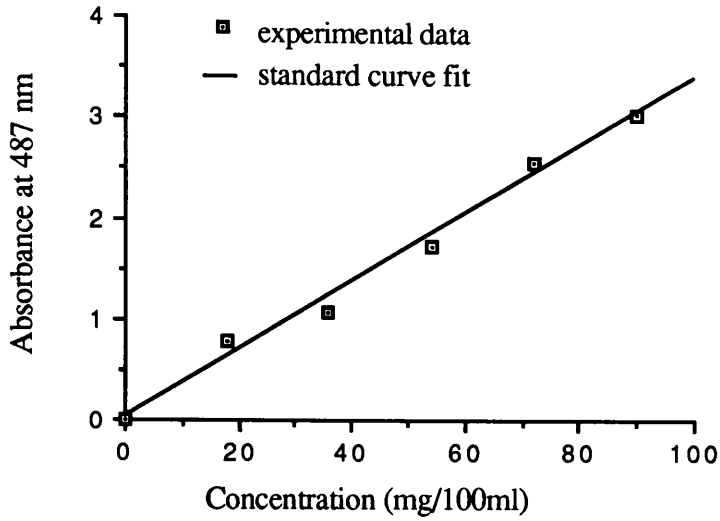


Fig. 2 UV Standard Curve for Glucose

(1) 2,3,4,5-Tetradeuteriomuconolactone (69). - This compound (**69**) (290 mg, 56 %) by feeding 2,3,4,5-tetradeuteriopyrocatechol (**68**) (515 mg) in water (4 ml) in two batches to the cultures of *A. niger*.(15 flasks) δ (200 MHz) 2.69 (s, 5-H) at pH 7-8.

(2) 3-Methylmuconolactone (23). - 4-Methylpyrocatechol (**20**) (481 mg) in water (4 ml) was fed to 3 d old *A. niger* (15 flasks) in two batches to give 3-methylmuconolactone (**23**) (60 mg, 13 %) as dark grey semisolid (13 %). δ (D_2O, t-butanol at 1.22) 2.12 (3 H, s, $C\underline{H}_3$), 2.40-3.10 (2 H, m, 5-$C\underline{H}_2$), 5.25 (1 H, m, 4-H), 5.89 (1 H, br s, 2-H). TLC (isopropyl ether/ formic acid/ water at 200:7:3 by volume) showed the same R_f (0.25) as that was reported (Ref. 22).

(3) 3-Ethylmuconolactone (134). - 3-Ethylmuconolactone (**134**) (30 mg, 7%) was obtained, by feeding 4-ethylpyrocatechol (**81**) (475 mg) in water (10 ml) to 3 d old *A. niger* (15 flasks) in two batches, as a dark oil, which failed to crystallise. δ (D_2O, t-butanol at 1.22) 1.18 (3 H, t, $CH_2C\underline{H}_3$), 2.25-2.34 (2 H, m, $C\underline{H}_2CH_3$), 2.52 (2 H, m, 5-$C\underline{H}_2$), 5.22 (1 H, m, 4-H), and 5.80 (1H, br s, 2-H).

3.2.3 EXPERIMENTS WITH 4-METHYLMUCONOLACTONE METHYL-ISOMERASE FROM *RHODOCOCCUS RHODOCROUS* (carried out by Dr. G.V.Rao at University of Newcastle upon Tyne, June, 1989)

General Procedure. - Frozen cells of *Rhodococcus rhodocrous* N75 (LA 1069) (10 g), grown on *p*-toluate, were thawed at 4 °C, suspended in tri buffer (50 mM Tris hydrochloride, pH 7.0, 10 ml) containing dithiothreitol (0.5 mM), then sonicated at 0 °C for 3 min with occasional interruptions to prevent overheating. The resulting suspension was centrifuged at -4 °C for 20 min at 16000*γ* to remove cell debris. The supernatant was frozen for storage. Portions of this cell extract were heat treated, immediately prior to use, at 65 ±0.1 °C for 15 min. Precipitated protein was then removed by centrifugation as before (Ref. 44).

(1) (4*S*)-2-Deuterio-4-methylmuconolactone (66). - (4*S*)-2-Deuterio-4-methylmuconolactone (**66**) (11 mg) in sodium phosphate buffer (pH 7.0) (10 ml) was incubated with the foregoing, heat treated extract (0.5 ml) at 30 °C. Aliquots (100 µl) of the mixture were taken after 0, 5, 10, 20, and 30 min and injected into orthophosphoric acid (20 µl). The mixtures were diluted with water to a final volume of 1 ml then were centrifuged, as before, to remove precipitated protein. The supernatant solutions were analysed by HPLC on a Merck RP-18 LiChrocart column eluted with acetonitrile-water (1:9) containing orthophosphoric acid (1 g/l) with a flow rate of 1 ml/min. The muconolactones were detected by their UV absorption at λ215 nm, the 4-methylmuconolactone (**66**) being eluted a little after the product, (4*S*,5*R*)-5-deuterio-3-methylmuconolactone (**135**). The isomerisation was essentially complete in 10 min. The mixture was adjusted to pH 1-2 with orthophosphoric acid, saturated with sodium chloride, then extracted continuously with ether for 48 h. The ^{1}H NMR spectrum (see the

Table) of the total extract (10 mg) showed it to be largely (4*S*,5*R*)-5-*deuterio*-3-*methylmuconolactone* (**138**) (*anti* addition). The mass spectrum confirmed the presence of one deuterium atom; *m/z* 157 (5.7%) ($C_7H_7DO_4$ requires *M*, 157), 111 (64.5), 97 (47.8), 69 (95.0), and 41 (100). The other incubations were carried out similarly, as follows.

(2) **(4*S*, 5*S*)-5-Deuterio-4-methylmuconolactone (65).** - The substrate (**65**) (5 mg) gave (4*S*)-3-methylmuconolactone (**23**), which contained no significant amount of deuterium (*anti* elimination).

(3) **(1*S*,5*S*,8*S*)-8-Deuterio-1-methylmuconolactone (67).**- (1*S*,5*S*,8*S*)-8-deuterio-1-methylmuconolactone (**67**) (1 mg), prepared from the foregoing 5-deuterio-muconolactone (**65**), likewise gave 3-methylmuconolactone (**23**) with complete loss of deuterium.

(4) **(4*S*,5*S*)-2,3,5-Trideuterio-4-methylmuconolactone (41).** - (4*S*,5*S*)-2,3,5-trideuterio-4-methylmuconolactone (**41**) (52mg) gave (4*S*,5*R*)-4,5-dideuterio-3-methylmuconolactone (**136**) (57mg) (*anti* addition and elimination); *m/z* 158 (1.8%), ($C_7H_6D_2O_4$ requires *M*, 158), 111 (28.5), 98 (22.1), 70 (27.6), 42 (34.8), 18 (100).

(5) **(1*S*,4*R*,5*S*,8*S*)-4,5,8-Trideuterio-1-methylmuconolactone (140).** - (1*S*,4*R*,5*S*,8*S*)-4,5,8-Trideuterio-1-methylmuconolactone (140) (16 mg) (containing some of the 4*S* epimer), prepared from the foregonig trideuteriomuconolactone, gave (4*S*,5*R*)-4,5-dideuterio-3-methylmuconolactone (**136**) (15 mg) (containing *ca.* 20% of the 5S epimer); m/z 158 (0.4%), 93 (34.8) and 39 (100). In this experiment the cell extract was not heat treated before use.

Table 5. (-)-β-Methylmuconolactones from *R. rhodocrous* Isomerase Incubation

No. of Product	Me	5-H_S	5-H_R	4-H	2-H	Note
(135) from **(66)**	2.13	2.50(dt, *J* 8.5 & 2.4Hz)	2.98(dd, *J*16.3&3.9Hz)	5.26 (d, *J* 9.2Hz)	5.85 (m)	* weak
(23) from **(65)**	2.13 (q, *J* 0.7 Hz)	2.50(dd, *J* 16.2&8.5Hz)	2.99(dd, *J*16.3&3.9Hz)	5.26 (m)	5.84 (q, *J* 1.5Hz)	
(136) from **(41)**	2.13 (d, *J* 1.5 Hz)	2.49 (br s)	2.89* (d,*J* 16.4Hz)	5.27* (d, *J* 8.5Hz)	5.87 (m)	* weak
(136) from **(41)** ^2H in Me_2CO; proton decoupled		2.47 (r.i.,4.3)	2.93 (s) (r.i. 111)	5.22 (s) (r.i. 100)	5.84* (r.i. 4.9)	* weak, r.i.=relative intensity, also 6.03 r.i. 4.8
(136) from **(140)**	2.13 (d, *J* 1.5 Hz)	2.49 (br s)	2.98* (d,*J* 16.4Hz)	5.28** (br d, *J* 7.9Hz)	5.87 (q, *J* 1.5Hz)	* weak, ** very weak also ** at 2.97
(136) from **(140)** ^2H in Me_2CO; proton decoupled		2.47 (r.i. 16.2)	2.94 (s) (r.i. 79.6)	5.22 (s) (r.i. 100)	5.85 (r.i. 4.1)	major C(5) epimer, as shown, ratio of epimer 4.9:1
(23) from **(67)**	2.13 (q, *J* 0.7 Hz)	2.50(dd, *J* 16.2&8.5Hz)	*	5.29 (m)	5.83 (m)	* signal obscured by HO; 2-H is about 90% of 4-H.

REFERENCES

1. N.N. Sen Gupta, *J. Agric. Sci.*, 1921, **2**, 136.
2. F.C. Happold, *Biochem. J.*, 1930, **24**, 1737.
3. W.C. Evans, *Biochem. J.*, 1947, **41**, 373.
4. F.M. Collins and C.M. Sims, *Nature, Lond.*, 1956, **178**, 1073.
5. L.N. Ornston and R.Y. Stanier, *Nature, Lond.*, 1964, **204**, 1279.
6. G.D. Hegemann and S.L. Rosenberg, *Ann. Rev. Microbiol.*, 1970, **24**, 429.
7. W. Reineke and H.J. Knackmuss, *Ann. Rev. Microbiol.*, 1988, **42**, 263.
8. R.Y. Stanier and C.N. Ornston, *Advances in Microbial Physiology,* eds. A.H. Rose and D.W. Tempest, Academic Press, London, 1973, **9**, 89.
9. J.M. Sala-Trepat, K. Murray, and P.A. Williams, *Eur. J. Biochem.*, 1972, **28**, 347.
10. L.N. Ornston, R.Y. Stanier. *J. Biol. Chem.* 1966, **241**, 3766.
11. O. Hayaishi, K. Hashimoto, *J. Biochem.* (Japan) 1950, **37**, 371
12. J.A. Elvidge, R. P Linstead, P Sims, H. Baer and P. B. Pattison, *J. Chem. Soc.*, 1950, 2228.
13. J. A. Elvidge, R. P Linstead, P Sims and B.A. Orkin, *J. Chem. Soc.*1950, 2235.
14. W.C. Evans and B. S. W. Smith, *Biochem. J.*, 1951, **49**, x.
15. W.C. Evans, B.S. Smith, R.B. Linstead, and J.A. Elvidge, *Nature,* 1951, **168**, 772.
16. W.R. Sistrom, R.Y. Stanier, *J. Biol. Chem.*, 1954, **210**, 82
17. D.L. MacDonald, R.Y. Stanier, J.L. Ingraham, *J. Biol. Chem.*, 1954, **210**, 809.
18. S.R. Gross, R.D.Gafford, E.l.Tatum. *J. Biol. Chem.*, 1956, **219,** 781.
19. J.B. Powlowsk, J. Ingebrand, and S. Dagley, *J. Bacteriol.*, 1985, **163**, 1136.

20. D. Catelani, A. Fiecchi, and E. Galli, *Biochem. J.*, 1971, **121**, 89.

21. H.J. Knackmuss, M. Hellwizg, H. Lackner, and W. Otting, *Eur. J. Appl. Microbiol.*, 1976, **2**, 267.

22. D.J. Miller, *Actinomycetes, Zbl. Bakt. Suppl. II*, 1981, 355.

23. a. D.H. Pieper, K.H. Engesser, R.H. Don, K.N. Timmis, and H.J. Knackmuss, *FEMS Microbiology Letters*, 1985, **29**, 63.

b. E. F. Ahlquist and R. B. Cain, unpublished observations; E. F. Ahlqiust, Ph.D. Thesis, Unversity of Kent in Canterbury, 1977.

24. N.C. Bruce and R. B. Cain, *FEMS Microbiology Letters*, 1988, **50**, 233.

25. O.Hayaishi, M. Katagiri and S Rothberg, *J. Biol. Chem.*, 1957, **229**, 905.

26. J.M. Varga, H.Y. Neujahr, *Eur. J. Biochem.*, 1970, **12**, 427.

27. A. Zakazawa, Y. Kojima,T. Taminchi, *Biochim. Biophys. Acta.*, 1967, **147**, 89.

28. Y.Kojima, H. Fujisawa, A. Nakazawa, F. Kanetsuna, H. Taninchi, M. Nozaki,and O. Hayaishi. *J. Biol. Chem.*, 1967, **242**, 3270.

29. H.Fujisawa and O. Hayaishi. *J. Biol. Chem.*, 1968, **243**, 3673.

30. M. C. Ludwig, L. Weber and D. P. Ballon, *J. Biol. Chem.*, 1984, **259**, 14840.

31. L.N. Ornston, *J. Biol. Chem.*, 1966, **241**, 3800

32. J. L. Canovas and R.Y. Stanier, *Eur. J. Biochem.*, 1967, **1**, 289.

33. M.B. Kemp and G.D. Hegeman, *J. Bacteriol.*, 1968, **96**, 1488.

34. R.B. Meagher and L.N. Ornston, *Biochemistry*, 1973, **12**, 3523.

35. A. Goldman, D.L. Ollis, T.A. Steitz, *J. Mol. Biol.*, 1987, **194,** 143.

36. D. J. Neidhart, G. L. Kenyon, J. A. Gerlt, G. A. Petsko, *Nature*, 1990, **347,** 692.

37. B. A. Katz, D. L. Ollis and H. W. Wyckoff, *J. Mol. Biol.*, 1985, **184**, 311.

38. L.N. Ornston. *J. Mol. Biol.*, 1966, **241,** 3887.

39. R.N. Patel, R.B. Meagher, and L.N. Ornston, *Biochemistry*, 1973, **12**, 3531.

40. D. Parke, R.B. Megher, and L.N. Ornston, *Biochemistry*, 1973, **12**, 3537.

41. B. F . Johnson, R. Y. Stanier.*J. Bacteriol*, 1971, **167**, 476.

42. D. L. Ollis, K. L. Ngai, *J. Biol. Chem.*, 1985, **260**, 9818.

43. D. R. Thatch and R. B. Cain, *Eur. J. Biochem.*, 1974, **48**, 549.

44. D. H. Pieper, K. S. Fritzsche, H-J. Knackmuss, K-H., Engesser, N. C. Bruce and R. B. Cain, *Biochem. J.*, 1990, **271**, 529.

45. N.C. Bruce, R.B. Cain, D.H. Pieper, and K.H. Engesser, *Biochem. J.*, 1989, **262**, 303.

46. G. Avigad, S. Englard, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 1969, **28**, 345.

47. K.L. Ngai, L.N. Ornston and R.G. Kallen, *Biochemistry*, 1983, **22**, 5223.

48. G.W. Kirby, G.J. O'Loughlin and D.J. Robins, *J. Chem. Soc. Chem. Commun.* 1975, 402.

49. R.V.J. Chari, G.P. Whitman, J.W. Kozarich, K.L. Ngai, and L.N. Ornston, *J. Am. Chem. Soc.*, 1987, **109**, 5514.

50. R.B. Cain, A.A. Freer, G.W. Kirby, and G.V. Rao, *J. Chem. Soc. Perkin Trans. I*, 1989, 202.

51. R.B. Cain, G.W. Kirby, and G.V. Rao, *J. Chem. Soc. Chem. Commun.* 1989,1629.

52. H. Pauly, R. Gilmour and G. Will, *Annalen,* 1914, **403,** 119.

53. P.A. von der Muhll, G. Settimj, H. Weber, and D. Arigoni, *Chimia*, 1965, **19**, 595.

54. R.A. Hill, G.W. Kirby, and D.J. Robins, *J. Chem. Soc. Chem. Commun.* 1977, 459.

55. D. R. Thatcher and R. B. Cain, *Biochem. J.,* 1970, **120**, 28P.

56. R.V.J. Chari, G.P. Whitman, J. W. Kozarich, Analysis, *J. Am. Chem. Soc.*, 1987, **109**, 5520.

57. J. P. Klinman and I. A. Rose, *Biochemistry,* 1971, **10**, 2259.

58. S. R. Elseden and H. J. Peel, *Ann. Rev. Microbiol.*, 1958, **12**, 145.

59. P.N. Thomas, S. Eduardo and L.W. Christopher, *J. Chem. Soc.,* 1939,1194.

60. G. W. Kirby, and L. Ogunkoya, *J. Chem. Soc.,* 1965, 6914.

61. T. A. Henry and T. M. Sharp, *J. Chem. Soc.,* 1930, 2279.

62. R. .G. Lange, *J. Org. Chem.,* 1962, **27**, 2037.

63. J. P. Gallemaers, D. Christophe, and R. Promel, *Tetrahedron lett.,* 1976, 693.

64. K. Freudenberg and V. Jovanonic and F. T. Topfmeier, *Chem. Ber.,* 1963, **96**, 2178.

65. J. H. Boyer L. R. Morgen, *J. Org. Chem.,* 1961, **26**, 1654.

66. L. C. Raiford and W. C. Stoesser, *J. Am. Chem. Soc.,* 1927, **49**, 1077.

67. J. Druey, *Bull. Soc. Chim.*, 1935, **3**, 1737.

68. D. H. R. Barton, G. W. Kirby, J. B. Taylor and G. M. Thomas, *J. Chem. Soc.,* 1963, 4545.

69. Schering, in "Fortschritte der Theerfarbenfabrikation" (ed. Friendlaender), Julius Springer, Berlin, Vol. IV, p1282.

70. H. Wynberg, *Chem. Rev.,* 1960, **60**, 169.

71. D. Jaap, Ph. D. Thesis, Glasgow University, 1989.

72. S. L. Cosgrose and W. A. Waters, *J. Chem. Soc.,* 1949, 3189.

73. *Beilstein,* 6, I, 442.

74. E. Fremy, *Ann. Chim. Phys.*, 1845, **15**, 1408.

74a. R. P. Singh, *Can. J.. Chem.,* 1966, **44**, 1994.

75. H. Zimmer, D. C. Lankin, S. W. Horgan, *Chem. Rev.,* 1971, **71**, 229.

76. H. J. Teuber and W. Ran, *Chem. Ber.,* 1953, **86**, 1036.

77. H. J. Teuber, *Angew. Int . Ed. Engl.,* 1965, **4**, 871.

78. *Org. Syn.* 52, 88.

78a. G. Stubeurauch and R. Knuppen, *Steroid,* 1976, 739.

79. P. Brassard and P. Karrer, *Helv. Chim. Acta.,* 1960, **XLIII**, 263.

80. H. Pauly, R. Gilmour, and G. Will, *Annalen,* 1914, **403**, 119.

81. E. Diepolder, *Chem. Ber.*, 1909, **42**, 2916.

82. L. M. Harnood, *Aldrichimica Acta*, 1985, **1**, 18.

83. C. Daremon and R. Rambaud, *Bull. Soc. Chim. de France*, 1971, **1**, 294.

84. S. Landa and J. Eliasek, *Chemicke Listy*, 1956, **50**, 1834.

PART TWO

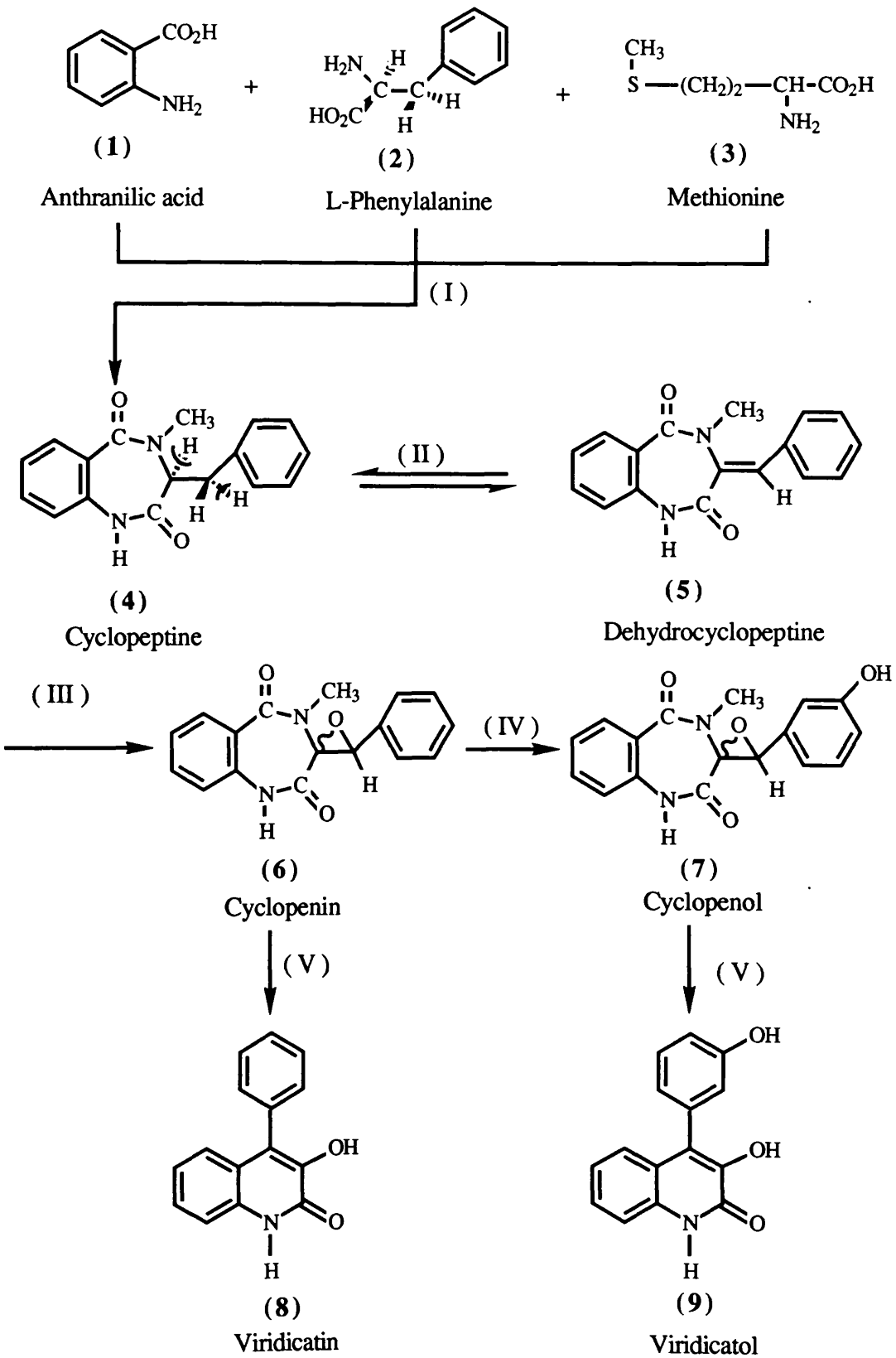
BIOTRANSFORMATION OF PHENYLALANINES INTO BENZODIAZEPINE ALKALOIDS: CYCLOPENIN AND CYCLOPENOL

CHAPTER ONE
INTRODUCTION

Part II of the thesis attempts to explore some mechanisms of the biosynthesis of the cyclopenin group of benzodiazepine alkaloids in *Penicillium cyclopium*. This chapter will present a general introduction, which has been divided into three sections. Sec. 1.1 gives a brief review of biosynthetic pathways of benzodiazepine alkaloids. Sec. 1.2 then discusses properties of organofluoro compounds with a few examples to highlight the specific utility of fluorine in chemistry. The last section, Sec. 1.3, outlines the aims of the present project.

1.1 A REVIEW OF THE BIOSYNTHESIS OF THE CYCLOPENIN GROUP OF BENZODIAZEPINE ALKALOIDS IN *PENICILLIUM CYCLOPIUM*

Molds of the genus *Penicillium cyclopium* synthesize the cyclopenin group benzodiazepine alkaloids, which are formed in only a few species of section Asymetrica, subsection Fasciculate. The first correct structure of the benzodiazepine alkaloids, namely, those of cyclopenin (**6**) and cyclopenol (**7**) (Scheme 1), were published in 1963


(1)
Anthranilic acid
(2)
L-Phenylalanine
(3)
Methionine
(I)
(II)
(4)
Cyclopeptine
(5)
Dehydrocyclopeptine
(III)
(IV)
(6)
Cyclopenin
(7)
Cyclopenol
(V)
(V)
(8)
Viridicatin
(9)
Viridicatol

Scheme 1.

by Mohammed and Luckner (Ref. 1). The biosynthetic pathway was investigated in surface cultures of *Penicillium cyclopium* by the radio tracer technique. From feeding experiments with ^{14}C labelled phenylalanine, anthranilic acid, or [methyl-^{14}C]-methionine, and subsequent degradation of the formed cyclopenin and cyclopenol, it was found that all the carbon atoms come from anthranilic acid (**1**) and L-phenylalanine (**2**) and the methyl group of methionine (**3**) (Refs. 2, 3, 4). The incorporation of ^{15}N-labelled precursors revealed that N-1 and N-4 of the diazepine ring are derived from the nitrogen atoms of anthranilic acid (**1**) and L-phenylalanine (**2**), respectively (Ref. 5). L-Phenylalanine (**2**), but neither *m*-tyrosine, dopa nor tyrosine, proved to be a precursor of cyclopenol (**7**). This suggested that the *m*-hydroxyl group is introduced into the molecule only after the formation of cyclopenin. The incorporation of $^{18}O_2$ into the hydroxyl group is accompanied by a NIH shift, which indicates that the hydroxylation is catalysed by a mixed function oxygenase (Ref. 6). Similarly, the incorporation of ^{18}O from $^{18}O_2$ into the epoxide ring was demonstrated. By a search for intermediates on the biosynthetic pathway it was found that beside phenylalanine only two N-methylated cyclic peptides, *cyclo*-(anthranoyl-*N*-methylphenylalanyl) (cyclopeptine) (**4**) and 3,10-dehydro-*cyclo*-(anthranoyl-*N*-methylphenylalanyl) (dehydrocyclopeptine) (**5**), were incorporated into cyclopenin without degradation. The non-cyclic peptides, for example anthranoylphenyl-alanyl, were not incorporated. It was found that the cyclopenin (**6**) and cyclopenol (**7**) could be further converted into viridicatin (**8**) and viridicatol (**9**) enzymically (Ref. 7). These findings established the pathway of cyclopenin-cyclopenol biosynthesis shown in Scheme 1. In the first step, the cyclic dipeptide cyclopeptine (**4**) is formed from anthranilic acid (**1**) and L-phenylalanine (**2**). The nitrogen atom of phenylalanine is methylated at some point by the *S*-adenosyl derivative of methionine (**3**). Cyclopeptine (**4**), the first free intermediate, is then transformed into 3,10-dehydrocyclopeptine (**5**). The latter is epoxidized to yield cyclopenin (**6**). This alkaloid is then (at least partially) hydroxylated at the *meta* position of the phenylalanine ring to give cyclopenol (**7**). The

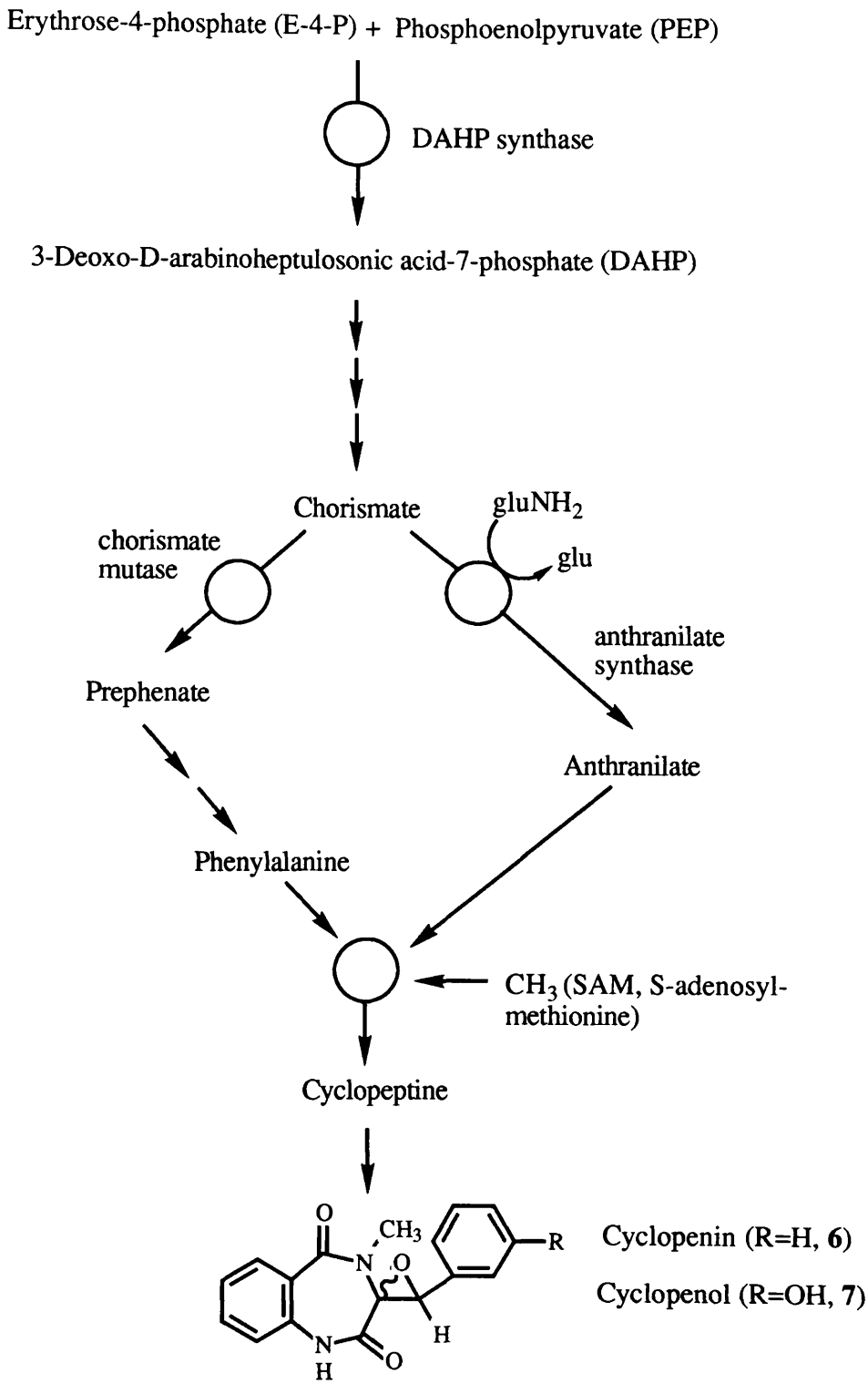
formed cyclopenin (**6**) and cyclopenol (**7**) then are further degraded by bacteria into viridicatin (**8**) and viridicatol (**9**) respectively. Chemical degradation of the cyclopenin (**6**) and cyclopenol (**7**) also led to the formation of viridicatin (**8**) and viridicatol (**9**). Individual steps (reactions I to V) in the biosynthetic pathway in Scheme 1 will now be discussed in more detail.

1.1.1 Biosynthesis of Phenylalanine and Anthranilic acid, and Feedback Activation of their Biosynthesis by Benzodiazepine Alkaloids

The two important precursors, phenylalanine (**1**) and anthranilic acid (**2**), can be biosynthesized from erythrose-4-phosphate and phosphoenolpyruvate *via* the shikimate pathway before the phase of alkaloid production (Scheme 2) in *Penicillium cyclopium.* They are products of two different branches of this sequence which both originate from chorismic acid (Ref. 8). Recently, Ross and Schnauder (Ref. 9) found that cyclopenin and cyclopenol substantially stimulate the activation of DAHP synthase (phospho-2-oxo-3-deoxyheptonate aldolase), anthranilate synthase, and chorismate mutase when added during enzyme incubation and concluded that far-reaching feedback activating effect exerted by the benzodiazepine alkaloids rests on a specific property of the primary metabolic enzymes like DAHP synthase involved in the biosynthesis of benzodiazepine precursor and present during the phase of alkaloid formation. Such a regulatory circuit bears an obvious advantage: it enables the cell to increase alkaloid formation without a prior increase of the steady-state concentration of the precursor amino acid.

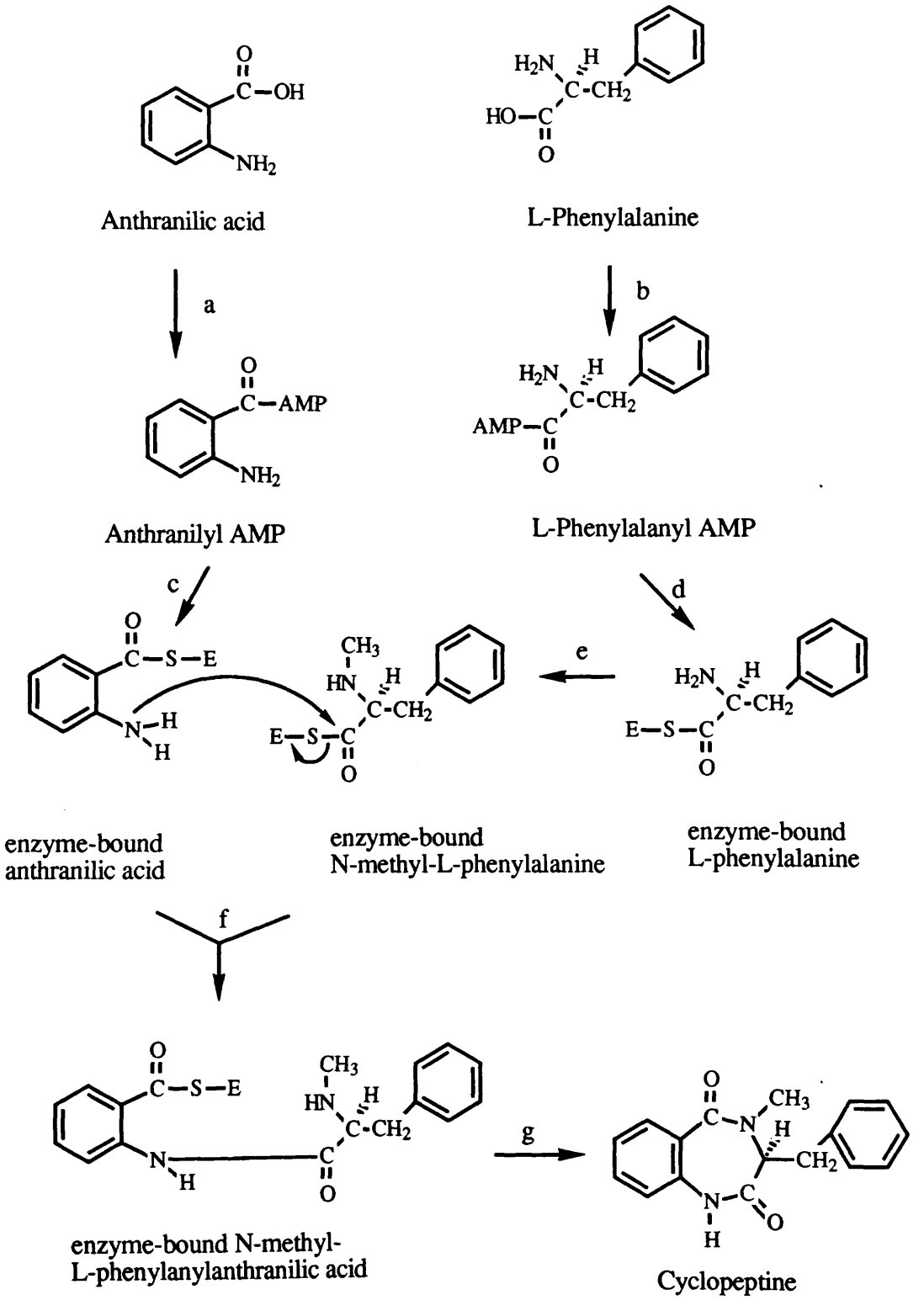
1.1.2 The Formation of Cyclopeptine (reaction I)

The absence of any detectable intermediate between the amino acid precursors and cyclopeptine and the lack of incorporation of potential intermediates led to the conclusion



Scheme 2.

that the first step of cyclopenin biosynthesis is catalyzed by a multienzyme complex, which catalyses the activation of anthranilic acid (**1**) and phenylalanine (**2**), the synthesis


Anthranilic acid
L-Phenylalanine
a
b
Anthranilyl AMP
L-Phenylalanyl AMP
c
d
e
enzyme-bound
anthranilic acid
enzyme-bound
N-methyl-L-phenylalanine
enzyme-bound
L-phenylalanine
f
g
enzyme-bound N-methyl-
L-phenylanylanthranilic acid
Cyclopeptine

Scheme 3.

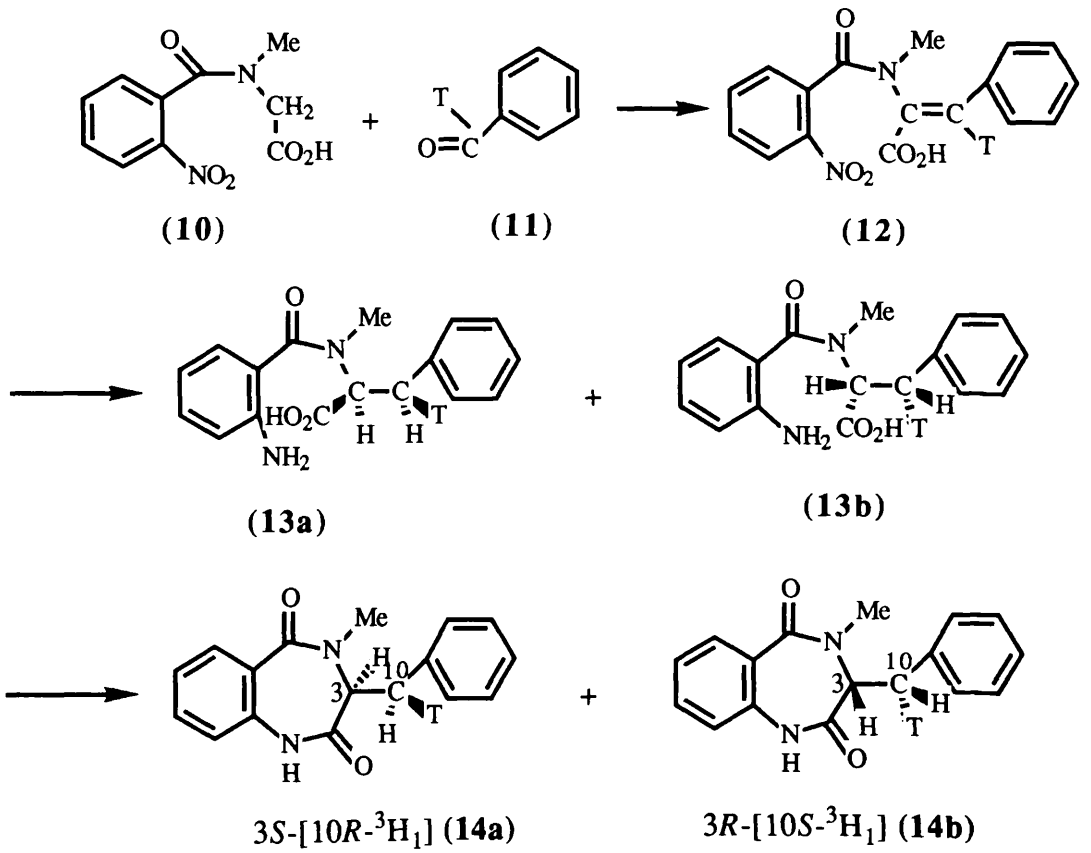
of the two peptide bonds and the methylation. Feeding experiments with labelled compounds *in vivo* indicated that the formation of cyclopeptine (**4**) was indeed catalysed by an enzyme complex (cyclopeptine synthetase) with tightly bound intermediates (Ref.7). In 1978, Voigt *et. al.* (Ref. 10) reported that protein extracts from cyclopeptine-synthesizing cultures of *Penicillium cyclopium* catalysed the activation of anthranilic acid (**2**) by the ATP-dependent formation of anthranoyl-AMP (AnAMP), probably as part of the cyclopeptine (**4**) biosynthesis (Scheme 3) (Refs. 11, 12, 13).

In vitro, experiments established the following partial activities of a cyclopeptine synthetase (Ref.14), which are summarized in Scheme 3: activation of anthranilic acid and L-phenylanine by adenylyl transferase; binding of anthranilic acid and L-phenylanine as thioester to protein; and formation of thioester-bound-*N*-methyl-L-phenylanine and N-methyl-L-phenylalanylanthranilic acid. The activation and binding activities were soluble after centrifugation at 150,000 g and could be attributed to a high molecular weight protein isolated by chromatography on Sepharose 6B. This protein was senstitive to mechanical disruption and protease degradation. However, cyclisation of the enzyme-bound *N*-methyl-L-phenylalanylanthranilic acid to cyclopeptine has not been observed yet, but in chemical experiments derivatives of anthranylyl-*N*-methylphenylanine cyclised easily to cyclopeptine (**4**). There are no indications that compounds of this type are formed by the enzyme preparation of *Penicillium cyclopium*.

1.1.3 The Conversion of Cyclopeptine into Dehydrocyclopeptine (reaction II)

Cyclopeptine (**4**) can be converted into dehydrocyclopeptine (**5**) with cyclopeptine dehydrogenase (Ref. 15). This enzyme is a NAD(P)-dependent flavoprotein which catalyses the reversible transformation of cyclopeptine (**4**) into dehydrocyclopeptine (**5**) by removal of two hydrogen atoms from C-3 and C-10. The activity of this enzyme was assayed in a cell-free preparation with $NAD(P)^+$ as the hydrogen acceptor. X-Press and

acetone treatment proved to be the best methods for cell disintegration. During centrifugation the greater part of the enzyme activity sedimented with the cell wall cytoplasmic membrane fraction. Cyclopeptine dehydrogenase showed a high degree of substrate specificity with respect to functional groups and sterical requirements. During tests with analogues, the enzyme was shown to be inactive towards the 10-hydroxy derivative of cyclopeptine (**4**), indicating the importance of the presence of the -CH-CH_2- group in the molecule. Aboutabl *et al.* (Ref. 16) further investigated the stereochemical aspects of this conversion. To decide which of the two hydrogen atoms of the C-10 methylene group is removed during the dehydrogenation, they prepared specifically tritium labelled cyclopeptines, (**14a**) and (**14b**) from benzaldehyde-[formyl-^{3}H] (**11**) as a tracer by the route outlined in Scheme 4.



Scheme 4.

N-(*o*-Nitrobenzoyl))-*N*-methyl-α-amino-[3'-^{3}H]-cinnamic acid (**12**) was synthesized by condensation of [formyl-^{3}H] benzaldehyde (**11**) with *o*-nitrobenzoyl-sarkosine (**10**). Hydrogenation of this compound (**12**) with platinum catalyst gave, by the usual *cis*-addition of hydrogen (Refs. 17, 18), the two isomers of labelled *N*-(*o*-aminobenzoyl-*N*-methylphenylalanine, (**13a**) and (**13b**), which by treatment with acid, cyclised to a mixture of two isomers of cyclopeptine (**14a**) and (**14b**), labelled at position 10. The compounds (**12**), had the *trans*-configuration, as established by NMR analysis. Of the two isomers present in the radioactive cyclopeptine preparation only the *3S*-[10*R*-^{3}H]-compound (**14a**) was a substrate of cyclopeptine dehydrogenase and the tritium atom remained during the dehydrogenation. Thus cyclopeptine dehydrogenase removed the *pro-S* hydrogen from C-10 to give *Z*-dehydrocyclopeptine (**5**) by a *syn*-periplanar elimination.

1.1.4 The Formation of Cyclopenin (reaction III)

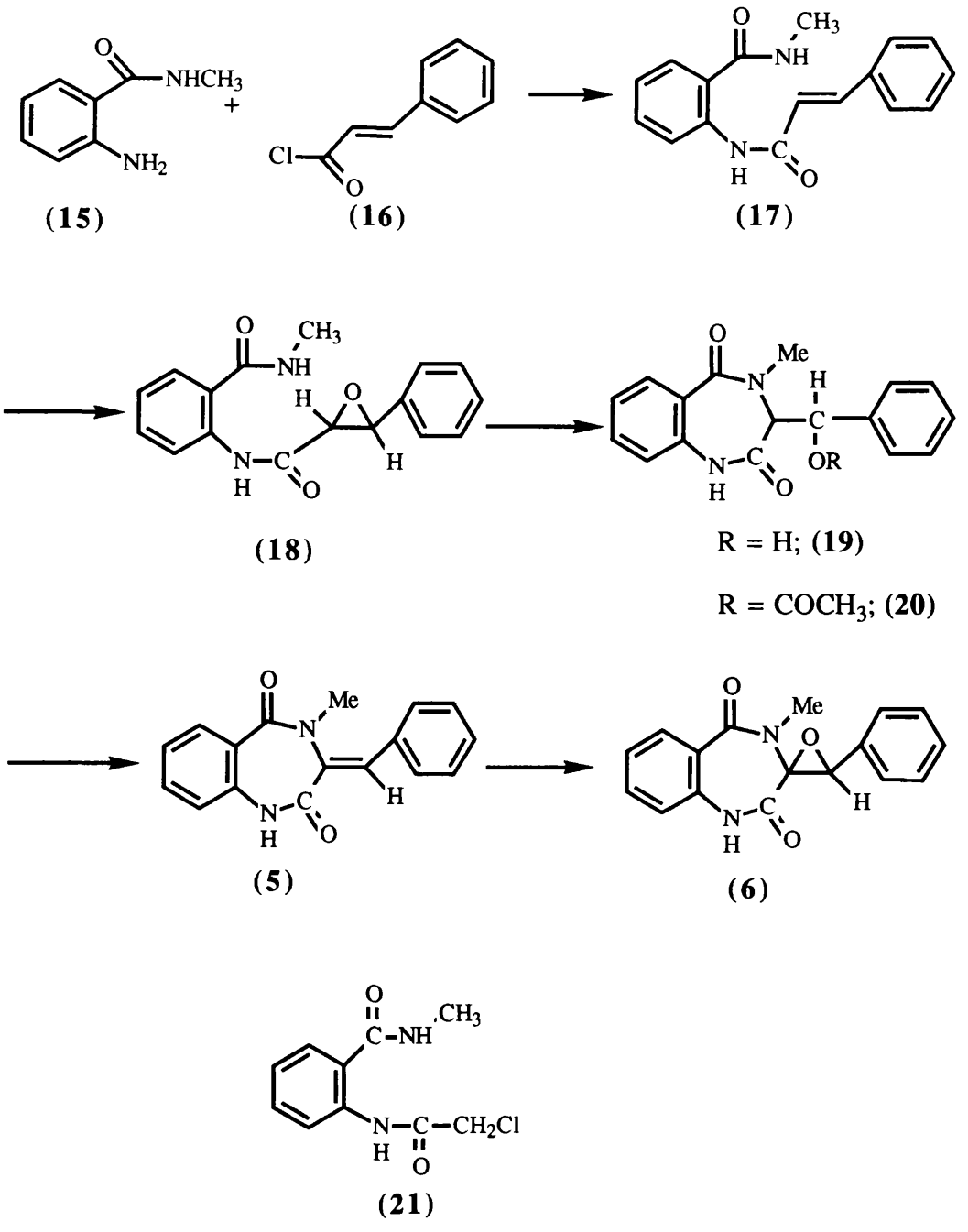
Dehydrocyclopeptine (**5**) can be enzymically converted into cyclopenin (**6**) by dehydrocyclopeptine epoxidase (Ref. 19). Tracer experiments performed in living cells demonstrated that the epoxide oxygen present in the molecule of cyclopenin (**6**) and cyclopenol (**7**) is derived from molecular oxygen, indicating that it works as a mixed function oxygenase (Refs. 5, 20), using NAD(P)H or other reducing compounds such as ascorbic acid and DL-methyl-5,6,7,8-tetrahydropteridine directly as hydrogen donors. The enzyme activity was assayed by following the rate of cyclopenin formation from H-labelled dehydrocyclopeptine (**5**). The radioactivity of this product was determined after converting it into viridicatin, which was chemically oxidized to 2-aminobenzophenone. The highest specific activity of dehydrocyclopeptine epoxidase was measured after acetone treatment of hyphal cells and coridiospores. More than one-third of the measurable activity sedimented with the cell wall plasma membrane fraction, from which

it could be solubilized by a 1% solution of deoxycholate. The soluble enzyme fraction was purified by ammonium sulfate precipitation and gel chromatography, and its molecular weight was estimated to be near 500,000 daltons. Inhibitor experiments indicated that the enzyme is an Fe^{2+}-activated, FAD-containing flavoprotein.

The first total synthesis of cyclopenin (**6**) by Rappoport and colleagues (Scheme 5) (Ref. 21a and b) started with 2-amino-*N*-methylbenzamide (**15**), which was treated with *trans*-cinnamoyl chloride (**16**) to give the cinnamanilide (**17**). The latter was epoxidized with *m*-chloroperbenzoic acid to the β-phenylglycidamide (**18**). Ring closure with potassium *tert*-butoxide in *tert*-butanol yielded the hydroxyl compound (**19**). After this product was acetylated, the *O*-acetyl derivative (**20**) was converted by heating into cyclopeptine (**5**). The final, most complicated step was the epoxidation of the benzylidene compound (**5**) with *m*-chloroperbenzoic acid (at room temperature for 14 days) to yield (±)-cyclopenin (**6**), as proved by spectral comparison with the natural product. During this procedure the relative stereochemistry of the *trans*-cinnamate is retained and hence only one stereoisomer of cyclopenin (**6**) is produced. In this way, the relative stereochemistry for cyclopenin was confirmed. Richter *et. al.* (Ref. 22) synthesized the β-phenylglycidamide (**18**) by reacting *N*-chloroacetyl-*N*-methylanthranoyl amide (**21**) with benzaldehyde.

1.1.5 Cyclopenin *m*-hydroxylation (reaction IV)

In vivo, incorporation of oxygen from $^{18}O_2$ into hydroxyl group of cyclopenin revealed that the hydroxylation is catalysed by a mixed function oxygenase. *In vitro*, a partially purified enzyme (by ammonium sulphate precipitation and calcium phosphate gel treatment) was used to test different substrates. It was found that hydroxylase like cyclopeptine epoxidase it acts as a mixed function oxygenase which reacts with molecular oxygen and requires a hydrogen donor [NAD(P)H, ascorbic acid, tetrahydropteridine] as cosubatrate. Cyclopenin *m*-hydroxylase was not inhibited by CO (Ref. 23). This may



Scheme 5.

indicate that the enzyme does not belong to the group of mixed function oxygenase which contains a cytochrome P-450 moiety. The decrease of enzyme activity after addition of dicoumarol, an inhibitor of flavin protein, indicates that *m*-hydroxylase belongs to this group of proteins, KCN and KCNS inhibit the enzyme activity too. All tested benzodiazepines were transformed into the hydroxyl derivative by cyclopenin *m*-

benzodiazepines were transformed into the hydroxyl derivative by cyclopenin *m*-hydroxylase preparations. Cyclopenin hydroxylation had the highest velocity. The quinoline derivative viridicatin was not attacked.

During aromatic hydroxylation the epoxide intermediates are unstable and rearrange to the hydroxy compounds *via* carbonium ions which are usually stabilized by a 1,2-anionotropy, the NIH shift (Ref. 20). This process results in a translocation of the hydrogen replaced by the hydroxyl group to the *o*-position. To characterize the *m*-hydroxylating enzyme system participating in cyclopenol biosynthesis, experiments with specifically ring-tritiated DL-[carboxyl-^{14}C]phenylalanine were performed. Only with *m*-T-DL-[carboxyl-^{14}C]phenylalanine did a change of the T/^{14}C ratio in cyclopenol occur, though more than half of the T-label is retained, namely *ca.* 70% in two independent experiments. This indicated a partial NIH shift of tritium (*ca.* 30%) after attack at a tritiated *meta* position. In addition to the alkaloids the isolation of L-tyrosine also gave the good evidence for the NIH shift study.

1.1.6 Benzodiazepine-Quinoline Conversion (Reaction V)

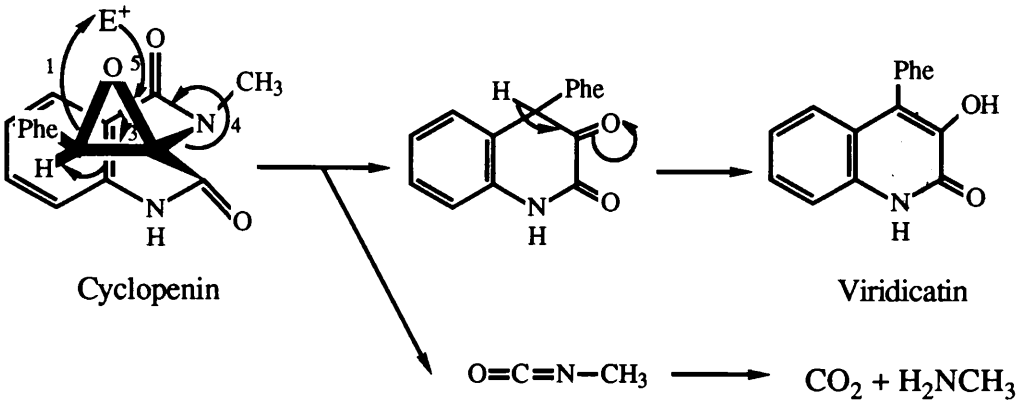
The enzyme, named cyclopenase which was obtained from conidiospores of *Penicillium cyclopium* and *Penicillium viridicatum*, is able to convert the alkaloids cyclopenin (**6**) and cyclopenol (**7**) produced and excreted by these fungi to the quinoline alkaloids viridicatin (**8**) and viridicatol (**9**), respectively (Refs. 24, 25). The enzyme is located at the cytoplasmic side of the conidiospore plasma membrane, therefore significant ammounts of viridicatin (**8**) and viridicatol (**9**) occur solely in the conidia but not in the culture medium (Refs. 26, 27). Nearly all the cyclopenase activity was found in a fraction containing the cell wall together with the cytoplasmic membrane (Refs. 28, 29). The specificity of the enzymatic catalysis is indicated by the very low conversion rate of the artificial substrates *N*-1-methylcyclopenin, *N*-1-ethylcyclopenin and *N,O*-dimethyl-cyclopenol compared with cyclopenin and cyclopenol (Refs. 28). In contrast, the

nonenzymatic, acid-catalyzed conversion of the benzodiazepines to the quinoline proceeds at similar rates in both the natural and alkylated cyclopenin derivatives .

The formation of quinoline alkaloids from cyclopenin (**6**) and cyclopenol (**7**) can be catalysed either by cyclopenase or by treatment with acid or alkali. Enzymatic and acid- or base-catalyzed reactions show close parallels. The conversion involves the liberation of CO_2, which derives from the C-5 carbonyl group, and of methylamine, which contains the N-CH_3 group in position 4 of the benzodiazepine nucleus. This attribution of the reaction products was found by subjecting specifically labelled cyclopenin to cyclopenase- or acid-catalyzed conversions (Ref. 24) and was confirmed by the results of cyclopenin-viridicatin conversion under anhydrous conditions, that is, during thermal degradation, during mass spectrometry, and under the influence of Lewis acids in nonaqueous solvents (Ref. 24, 30, 31). In all the latter cases, the formation of viridicatin (**8**) is accompanied by the liberation of methylisocyanate. This compound, which in aqueous solution immediately yields CO_2 and NH_2CH_3, is therefore suggested to be primarily extruded from the cyclopenin molecule. The reaction involves no incorporation of hydrogen or oxygen from water, as demonstrated in the presence of either ^2H_2O or $H_2{}^{18}O$. This finding demonstrates, that the closure of the heterocycle of the quinoline nucleus occurs between C-5a and C-10 of the benzodiazepine and that the epoxide oxygen of cyclopenin becomes the 3-hydroxyl oxygen of viridicatin.

A mechanism for the transformation of the benzodiazepine to the quinoline structure, which accounts for all data obtained from both cyclopenase- and acid- or base-catalyzed reactions, has been proposed by White and Dimsdale (Ref. 32) (Scheme 6). The conversion is induced by attack of an electron acceptor (*e.g.*, H^+) at the epoxide oxygen or by attack of an electron donor (*e.g.*, OH^-) on the N-1 atom. Bond formation between C-10 and C-5a of cyclopenin is facilitated by the close approach of these two atoms in the boat conformation. Therefore, the formation of a tricyclic intermediate appears likely, although a compound of this type has not been isolated so far. Another

mechanism that would explain the enzymatic and acid-catalyzed conversions was proposed by Luckner and Nover (Ref. 3).



Scheme 6.

In 1980, Luckner (Ref. 33) gave a very detailed review of alkaloid biosynthesis in *Penicillium cyclopium* from the biological point of view and concluded that the formation of the alkaloids of the cyclopenin-viridicatin group exhibits some of the most characteristic features of secondary product formation, *i.e.* restriction of the synthesis of the alkaloids to a limited group of organisms; formation by specific enzymes; strict regulation of the amount and *in vivo* activity of the enzymes; compartmentation of enzymes, precursors, intermediates and products; and lack of significance for the synthesizing cell itself, but possible importance for the producer organism as a whole.

1.2 CHEMISTRY OF ORGANOFLUORINE COMPOUNDS

Almost one hundred years ago in 1896, the synthesis of methyl fluoroacetate by heating methyl iodoacetate with silver fluoride was first described by a Belgian chemist, Swarts (Ref. 34), who heralded the beginnings of modern organofluorine chemistry and laid good foundations for all subsequent organofluorine chemistry. The introduction of fluorine into organic compounds can cause a variety of very significant changes in their chemistry, for instance organofluorine compounds have been widely used as coolants, *e.g.* CF_3Br; aerosol propellants, *e.g.* CCl_2F_2; polymers, *e.g.* polytetrafluoroethylene; anaesthetics, *e.g.* $CF_3CHClBr$; and rather exotically as blood substitutes, *e.g.* perfluorodecalins and polyfluorocyclohexane. In this last role the fluorocarbons act as efficient transporters of both oxygen and carbon dioxide, and can substitute for blood where blood is not readily to hand, as in battlefield situations. The following brief review will illustrate a few of the properties of organofluorine compounds in biological system selection of typical examples.

1.2.1 The Properties of Fluorine

The biological effects of introducing fluorine into organic molecules have been studied widely since 1943 when Marais demonstrated that the toxicity of the South African plant *Dichapetalum cymosum* was due to fluoroacetic acid (Ref. 35). This discovery provided the impetus for studies on the toxicology and pharmacology of organofluorine compounds. The unique physical properties of fluorine that make it particularly useful in this area (Ref. 36, 37, 38) are cited below.

(i) The strength of the carbon fluorine bond exceeds that of the carbon hydrogen bond (Table 1). This often results in increased thermal and oxidative stability of organofluorine compounds (Ref. 39).

Table 1. Bond Energy

X	H	F	Cl	Br	I
bond energy CH_3-X, kcal mol^{-1}	104	109	24	70	56

(ii) Fluorine is the smallest atoms that can replace hydrogen in organic compounds with the formation of a stable covalent bond (Table 2). Consequently it closely mimics hydrogen with respect to steric requirement at enzyme receptor sites (Ref. 39).

Table 2. van der Waals radius and Bond Length

	van der Waals radius (Å)	bond length (Å)
F	1.35	
H	1.10	
C-F		1.26-1.41
C-H		1.08-1.11

(iii) The high electronegativity of fluorine (4.0 on the Pauling scale) frequently alters the electronic properties and thereby the chemical reactivity of the compounds concerned. It may function as H-bond acceptor, and replacement of a hydroxyl group by fluorine often has interesting results. In addition it is a moderately good leaving group and may be displaced by nucleophile at or near to the active site of enzymes, with resultant covalent attachment of an organic molecule to the enzyme.

(iv) The introduction of fluorine into organic molecules usually increases their lipid solubility, thereby enhancing the rates of absorption and transport of drugs *in vivo*.

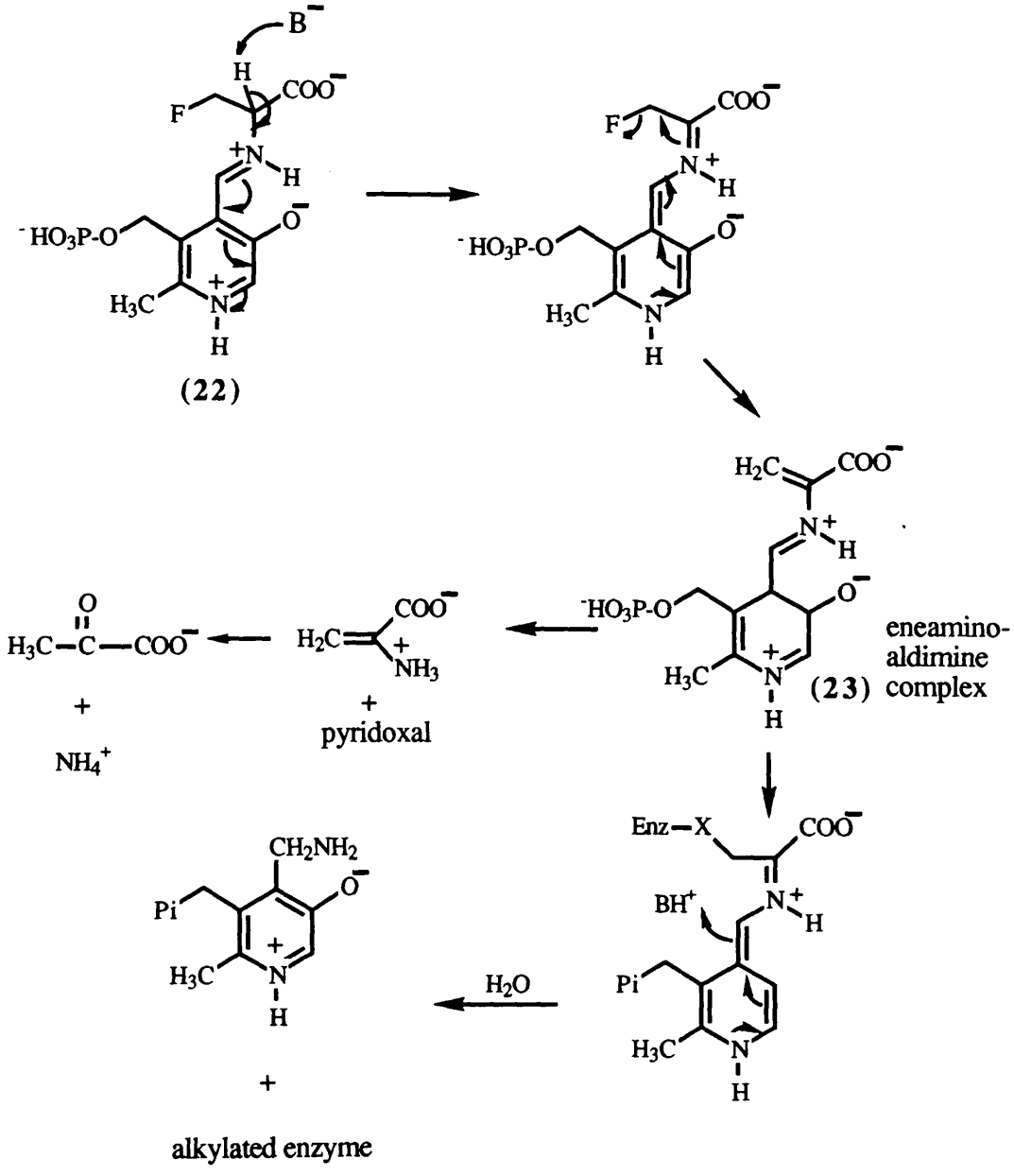
The trifluoromethyl group is one of the most lipophilic group known. This feature is of considerable importance in drug design.

(v) Like hydrogen, ^{19}F has a magnetic spin of 1/2. This property makes fluorine NMR spectroscopy a powerful analytical technique in chemistry. Fluorine NMR spectroscopy has a number of advantages. Fluorine has a detection sensitivity nearly as great as that of hydrogen; chemical shift and substituent effects are frequently an order of magnitude larger than those for hydrogen; and the fluorine NMR resonance can be observed without interference from background protein NMR resonances or resonances from secondary metabolites. Therefore it is widely used in the analysis of biological systems.

1.2.2 Examples

All of these above features, either individually or in combination, will influence the outcome of a reaction (whether enzyme-catalysed or not) in which a fluoro analogue of the natural substrate is employed and it is instructive to consider some compounds which exemplify these effects. The methods of fluorine insertion into organic compounds have been summerized in great detail by Mann in 1987 (Ref. 40).

The introduction of fluorine into organic molecules often results in profound changes in chemical or biological activity. Consequently this technique has been used to develop many classes of drugs with a wide range of biological activities. One method of drug design is to select a crucial intermediate of a key biological reaction which is also of special importance to a 'disease process' (Ref. 41, 42). Having identified a suitable intermediate the next step would be to prepare synthetically an 'anti-metabolite'; that is to prepare a compound that resembled the metabolite very closely, but be different enough to block or inhibit the selected biological process. To accommodate this idea the term 'isogeometric modification' was used to describe the required chemical change. Naturally, the similarity in size between hydrogen and fluorine but their drastically

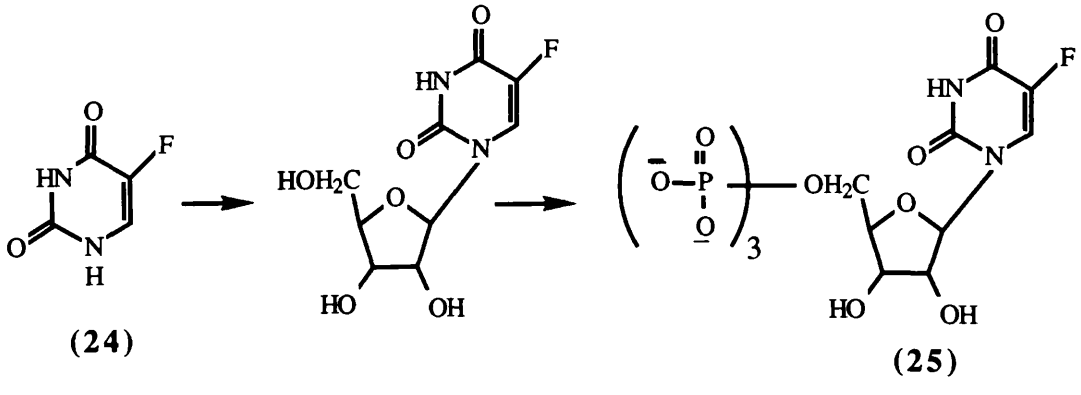


Scheme 7.

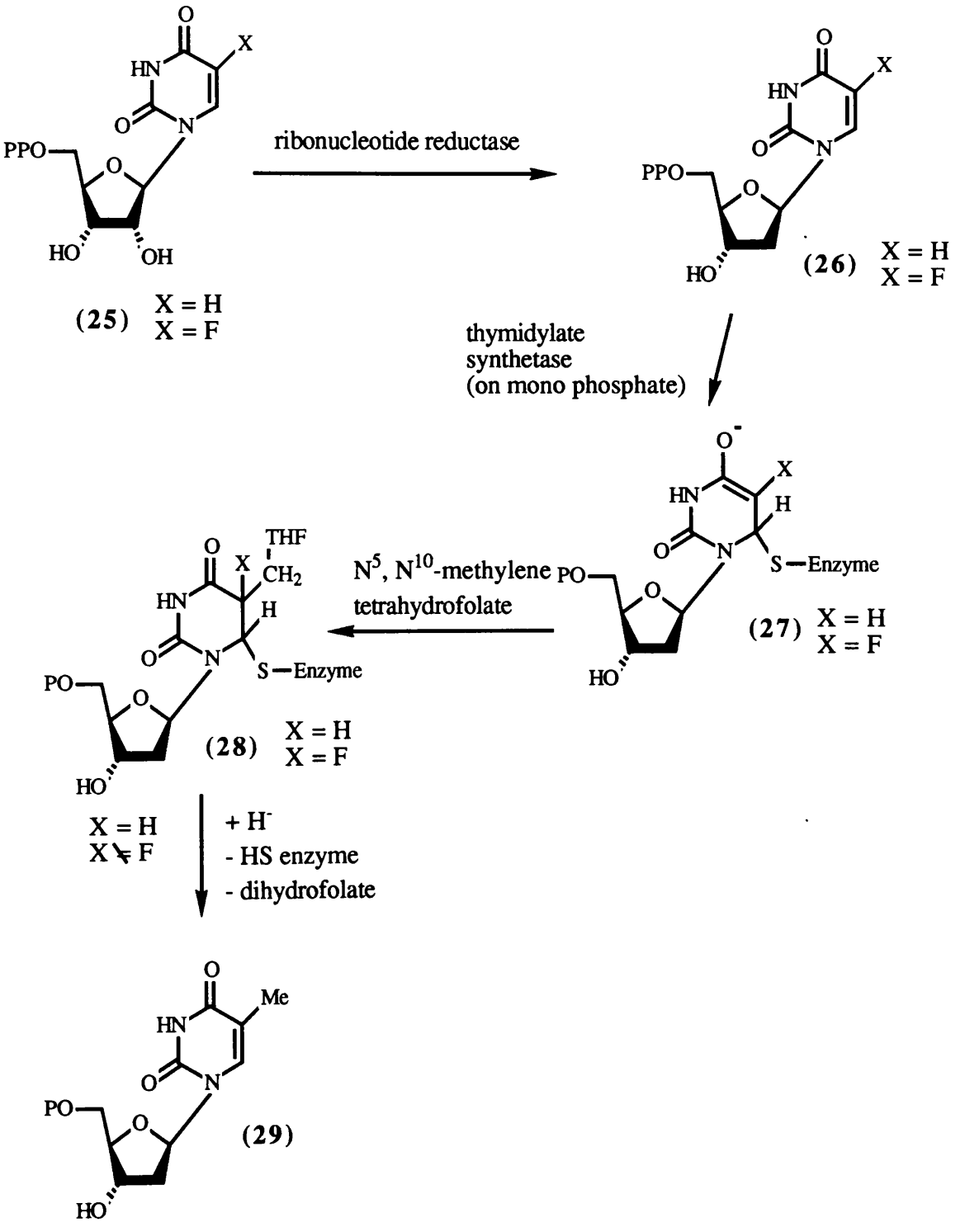
different chemical properties made the use of fluorine an obvious choice for 'isogeometric modification'. These ideas led Kollonitsch and co-workers (Ref. 41) to synthesize 3-fluoro-D-alanine. It is a antimetabolite of D-alanine, which is an important constituent of

bacterial cell wall. 3-Fluoro-D-alanine proved to have a wide spectrum of antibacterial activity both *in vitro* and *in vivo*. Subsequently it was proved that the fluoroalanine did act as an anti-metabolite by being an irreversible inhibitor of the enzyme alanine racemase (Ref. 43). The mechanism for this enzyme inhibition is shown in Scheme 7. The loss of HF from the aldimine (**22**) formed between fluoroalanine and the pyridoxal phosphate (PLP) moiety of the enzyme gives an enamino-PLP complex (**23**). The key property of this complex is that it is electrophilic and thus it is capable of reacting with nucleophile. This complex (**22**) could either be hydrolysed to pyruvate and ammonium ion or be attacked by enzyme bound nucleophile, *e.g.* terminal amino group of a lysine residue to form alkylated and hence irreversibly inhibited enzyme.

Another fascinating example that is frequently cited is the anti-metabolite, 5-fluorouracil, as an anti-cancer drug (Scheme 8) (Ref. 44). There are two different mechanisms for the cytotoxicity of 5-fluorouracil. The size of the fluorine atom allows the fluoro compound to mimic uracil. Consequently, it is enzymatically converted into the nucleotide 5-fluorouridine (**24**) and then into 5-fluorouridine, di- and tri-phosphate (Refs. 45, 46, 47, 48). The triphosphate (**25**) is incorporated into RNA, which is believed to cause transcription errors within the developing tumor cells leading to 'lethal synthesis'.



Scheme 8.


ribonucleotide reductase
(25) X = H X = F
(26) X = H X = F
thymidylate synthetase (on mono phosphate)
(27) X = H X = F
N5, N10-methylene tetrahydrofolate
(28) X = H X = F
X = H X ≠ F
+ H-
- HS enzyme
- dihydrofolate
(29)

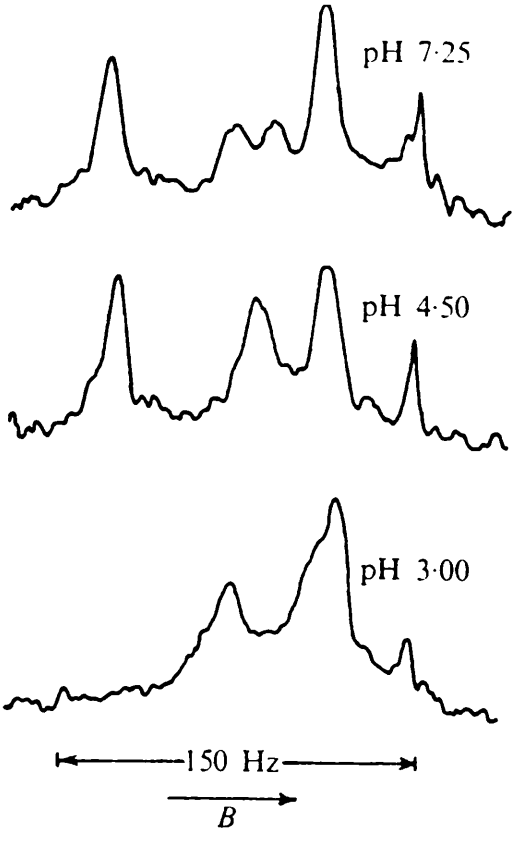
Scheme 9.

More clearly understood, however, is the inhibition of the enzyme thymidylate synthetase (Ref. 46) which catalyses the process shown in Scheme 9, whereby deoxyuridylate (**26**, X=H) is methylated to produce deoxythymidylate (**29**). It wasbelieved that the process proceeds *via* a Michael addition of a thiol group from enzyme to the uracil moiety (**26**), followed by electrophilic addition of N^5, N^{10}-methylene-tetrahydrofolate. The process is completed by elimination. Clearly if 5-fluorouracil has been incorporated into the deoxyuridylate (**26**, X=F), this final step requires abstraction of F^+ which is unlikely. Since formation of deoxythymidylate is essential for DNA biosynthesis, 5-fluorouracil is an effective cytotoxic agent, and is much used in cancer chemotherapy.

The following example highlights the investigation of a biological reaction by fluorine NMR spectroscopy. Lysozyme is a small enzyme (mol. wt. 14,400), which is characterized by its high stability and ability to lyse the cell walls of susceptible bacteria (Ref. 49). Hen egg white lysozyme is the most studied variant of the protein. A nember of mono-, di-, and tri-saccharides are inhibitors of this enzyme. They act competitively with the substrate at one or more of the binding subsites. A lot of experiments using fluorine NMR spectroscopy with fluorine-containing saccharides in the presence of the enzyme have been reported. One example is the binding of the trifluoroacetylated analogue of chitotriose to lysozyme (Ref. 50).

The signal of a one- to one mole ratio of enzyme to trisaccharide, each at 3mM is shown in Scheme 10 at three pH values (Ref. 51). The sharp peak at highest field concentration is due to the trifluoroacetate anion, which was used as an internal reference. Assignments were made on the basis of experiments at higher temperatures(65°C) and at pH4 under conditions of fast exchange with excess trisaccharides present, and also by comparison with the spectra of the pure trisaccharide. In the spectra at pH 4.5, the bound inhibitors resonances are assigned as follows:the resonance at lowest field corresponds to the trifluoroacetyl (F_3Ac) group at the non-reducing end of the trisaccharide; the one next

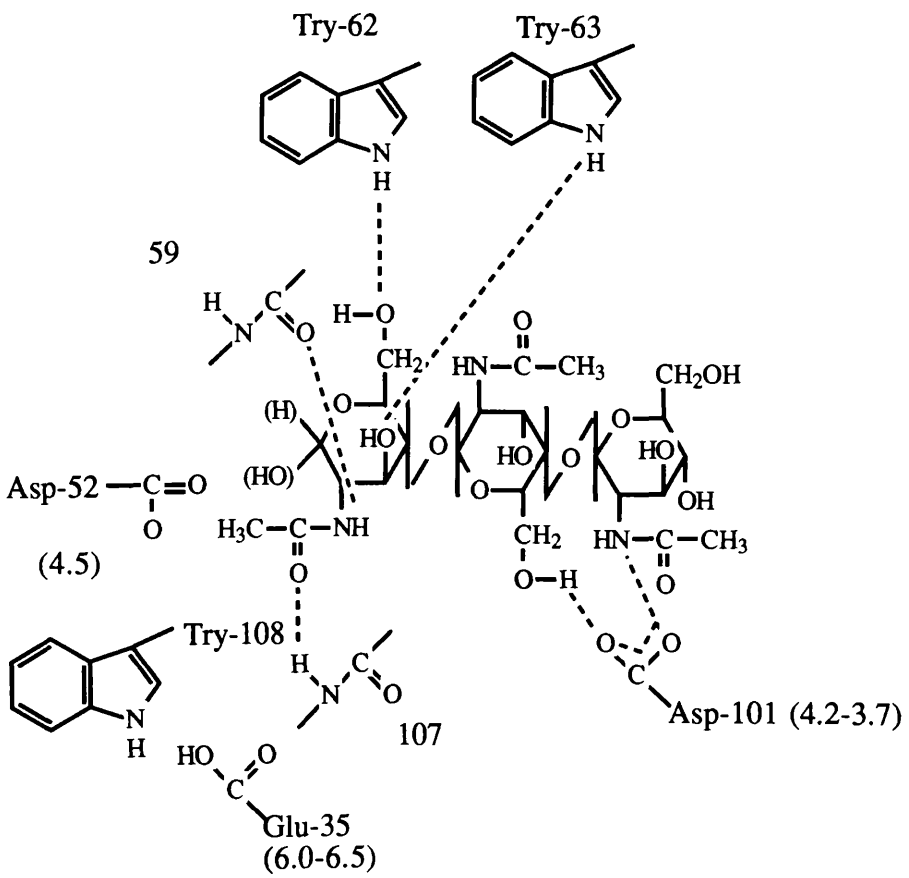
^{19}F NMR Spectra of Solutions of 3mM Trifluoroacetylated Chitotriose and 3mM Lysozyme at 10°C at Three pH Values



Scheme 10.

to high field corresponds to the F3Ac group at the reducing end of trisaccharide (this resonance separates into α- and β-anomeric forms at pH>7.25); the other resonance corresponds to the middle F3Ac group of the trisaccharide. The effects of pH on the position of the resonance was studied and the results showed that the pH depence of the shift of the non-reducing F3Ac end group implies a group with pKa of 3.2. This is identified as Asp-101 in the top of the lysozyme-binding cleft, since the pKa of this group in the enzyme (4.2) changes to 3.6 upon binding of chitotriose and further, a group of pKa 4.2 in the enzyme changes to 3.2 on binding F3Ac-chitotriose.

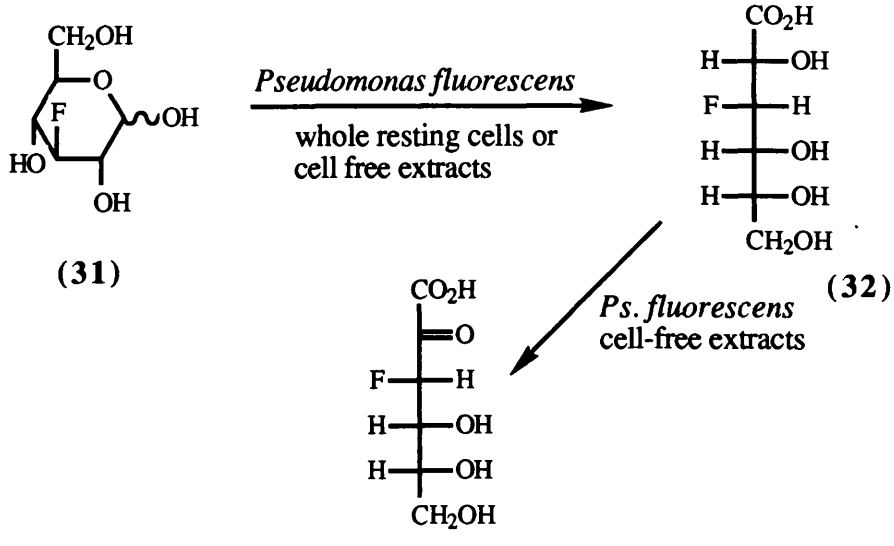
The interaction of chitotriose (**30**) with specific groups on the enzyme, as determined by X-ray analysis are shown in Scheme 11 (Ref. 52). If the fluorinated analogue binds similarly, the formation of a hydrogen bound between Asp-101 and F3Ac group would give a change in the ^{19}F chemical shift of this group. From the model, it is apparent that the F3Ac group at the reducing end of the trisaccharide is the furthest F3Ac group from Asp-101, and yet, unlike the central F3Ac group, it experiences an obvious chemical shift. The origin of the shift is unlikely to be electronic, as is that for the F3Ac group at the non-reducing end. If however the formation of the Asp-101 hydrogen bond causes a conformational change in the complex such that the position of the reducing end of the trifluoroacetamido group is shifted with respect to the aromatic ring of Try-108. The difference in ring-current effects could account for the observed shift.



Scheme 11.

Some attention has also been paid to the microbial metabolism of fluorine substituted compounds. The microbial metabolism of fluoroglucose is a typical example (Scheme 12). In 1972, Taylor *et al.* (Ref. 53) demonstrated that 3-deoxy-fluoro-D-glucose (**31**) was metabolised by whole resting cells of *Pseudomonas fluorescens*, with retention of the C-F bond, to produce 3-deoxy-3-fluoro-D-gluconic acid (**32**). Cell-free extracts of this organism further oxidised compound (**32**) further to 3-deoxy-3-fluoro-2-keto-D-gluconic acid (**33**). It has also been shown that the same enzymes which oxidise D-glucose (glucose oxidase and gluconate dehydrogenase) also oxidise (**31**), and that (**31**) and (**32**) are competitive inhibitors of the enzymes (Scheme 12) (Ref. 54).

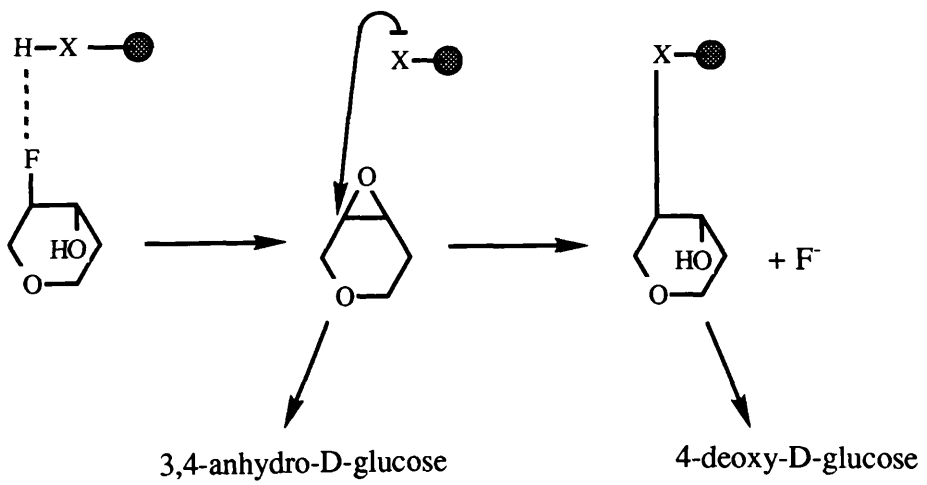
The biochemical effects of the isomeric 4-deoxy-4-fluoro-D-glucose (**34**) on whole resting cells (Scheme 13) and cell-free extracts (Scheme 14) of *Pseudomonas fluorescens* were examined by Taylor *et al.* (Ref. 55). They reported that compound (**34**), unlike (**31**), is not oxidised by whole cells but that there is an immediate release of



(31) **(32)** **(33)**

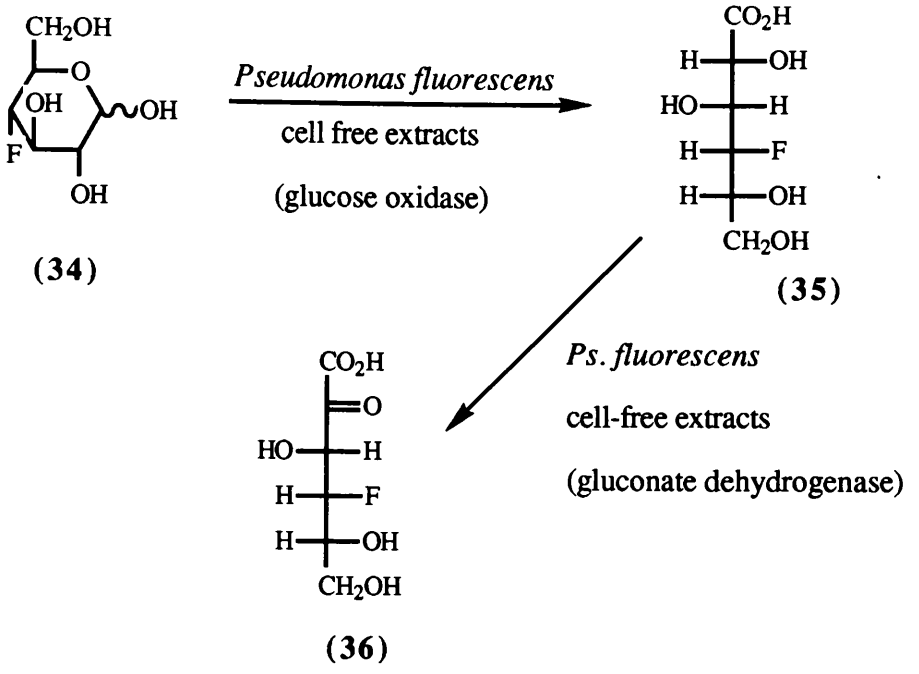
Scheme 12.

a fluoride anion (Scheme 13). The possible defluorinated products of this reaction were 4-deoxy-D-glucose and 3,4-anhydro-D-glucose, which were identified by TLC analysis of the cell supernatants and intracellular contents. When cell-free extracts of *Pseudomonas fluorescens* were incubated with 4-deoxy-4-fluoro-D-glucose (**34**) no significant defluorination occurred. Analogous to 3-deoxy-3-fluoro-D-glucose (**31**), 4-deoxy-4-fluoro-D-glucose (**34**) is converted into 4-deoxy-4-fluoro-D-gluconic acid (**35**) and 4-deoxy-4-fluoro-2-keto-D-gluconic acid (**36**) respectively by glucose oxidase and gluconate dehydrogenase *via* the two-step oxidation (Scheme 14). The extensive defluorination of (**34**) by whole cells of *Pseudomonas fluorescens* and retention of the C-F bond on the treatment of (**35**) with cell-free extracts suggested that C-F cleavage occurs at the cell-wall/membrane level of the organism. A glucose transport system might be present in *Pseudomonas fluorescens* and the failure of the whole cell to oxidise 4-deoxy-



Scheme 13.

4-fluoro-D-glucose (**34**) might be due to a special reaction in which co-valently bonded fluorine is release as fluoride ion.



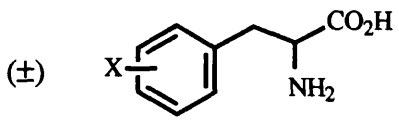
Scheme 14.

Clearly introduction of fluorine into a compound can have a marked effect upon its biological activity. The use of fluorine to probe the mechanism of enzymatic reactions is a promising technique. The examples presented have been chosen in an attempt to highlight these characteristics of fluorine.

1.3 AN OUTLINE OF THE PROJECT

It has been seen from the above review that some aspects of alkaloid metabolism in *Penicillium cyclopium* are now relatively well known, in terms of chemistry, biochemistry and enzymology, whereas some other aspects are still obscure. In particular, the mechanism of *meta* hydroxylation of the phenyl ring of cyclopenin, which results in a partial NIH shift of hydrogen (tritium) is not well understood. The introduction of fluorine into organic compounds can cause dramatic changes in chemical and physical properties, as illustrated above. Therefore, the present project attempted to

investigate the influence of the fluorine atom on the *m*-hydroxylation of cyclopenin. Due to the poor growth of the stock strain of *Penicillium cyclopium* Westling and the limited time, only preliminary experiments were possible. The following four compounds were synthesized to as precursors of the biosynthesis cyclopenin and cyclopenol in *Penicillium cyclopium*.



X = H **(37)**

X = *o*-F **(38)**

X = *m*-F **(39)**

X = *p*-F **(40)**

Scheme 15.

CHAPTER TWO

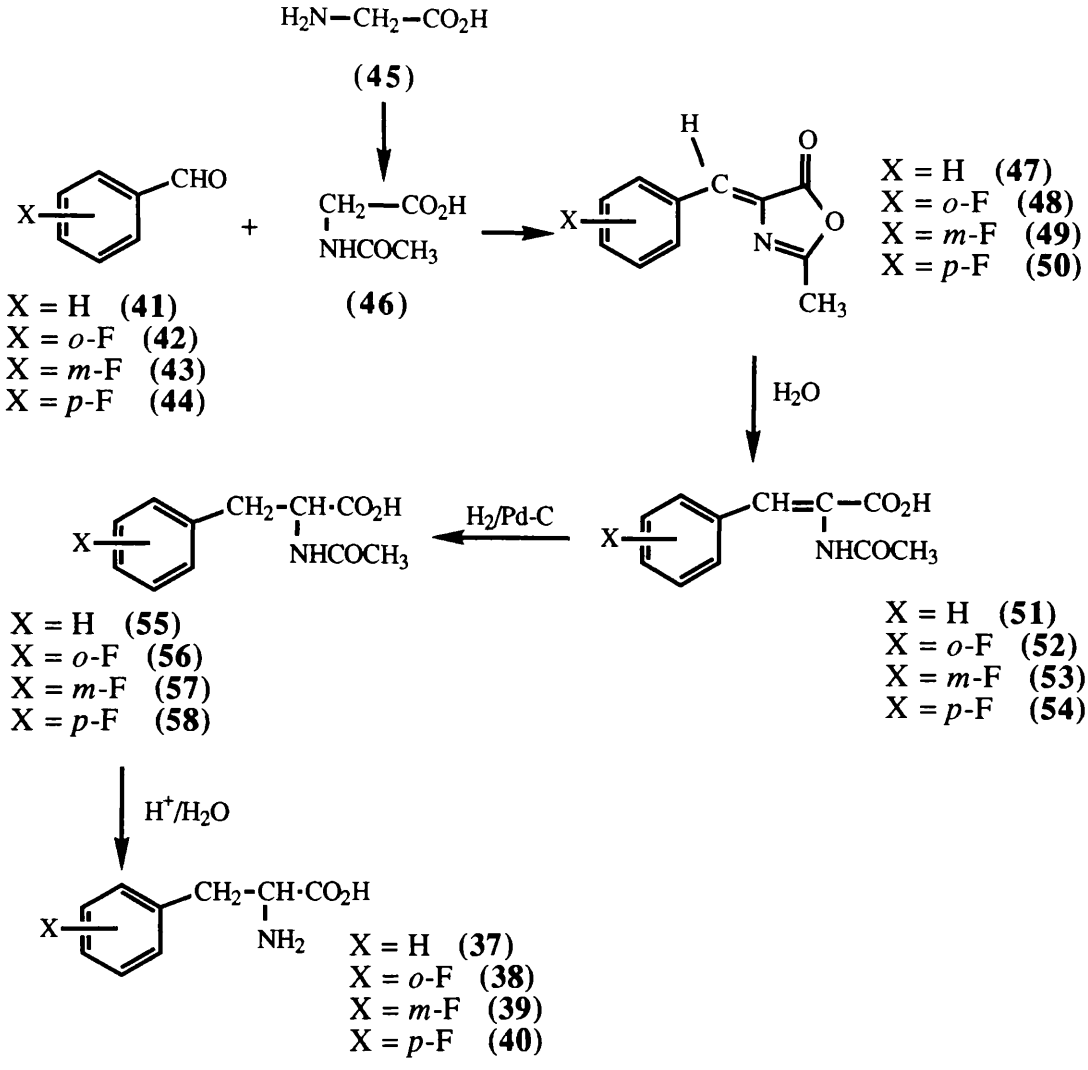
RESULTS AND DISCUSSION

Chapter two begins with a discussion of the synthetic methods for the preparation of fluorophenylalanines in Sec. 2.1. The biotransfromation of fluorophenylalanines into benzodiazepine alkaloids, which is then presented in Sec. 2.2. and finally, some conclusions are drawn in Sec. 2.3.

2.1 CHEMICAL SYNTHESIS OF PHENYLALANINES

Although phenylalanine (**37**) and fluorine-substituted phenylalanines, (**38**), (**39**), and (**40**), are commercially available now, the following route (Scheme 16) was designed for the preparation of radioactive phenylalanines and was used to prepare supplies of the unlabelled amino acids for the feeding experiments.

The reaction of benzaldehyde (**41**) with glycine (**45**) or acetylglycine (**46**) (which was prepared from glycine and acetic anhydride (Ref. 56)), in acetic anhydride in the presence of sodium acetate resulted in the formation of the azlactone of α-acetaminocinnamic acid (**47**) (Ref. 57). It was found that different yields were obtained by using glycine and acetylglycine respectively as starting material. Acetylglycine gave a 20% higher yield of the azlactone than glycine itself. The crude azlactone (**47**) obtained, without purification, was hydrolysed by refluxing with acetone-water to give α-acetamino acid. It was reported that α-acetamino acid (**51**) could be hydrogenated by high pressure hydrogenation in acetic acid with platinum oxide as catalyst to yield N-



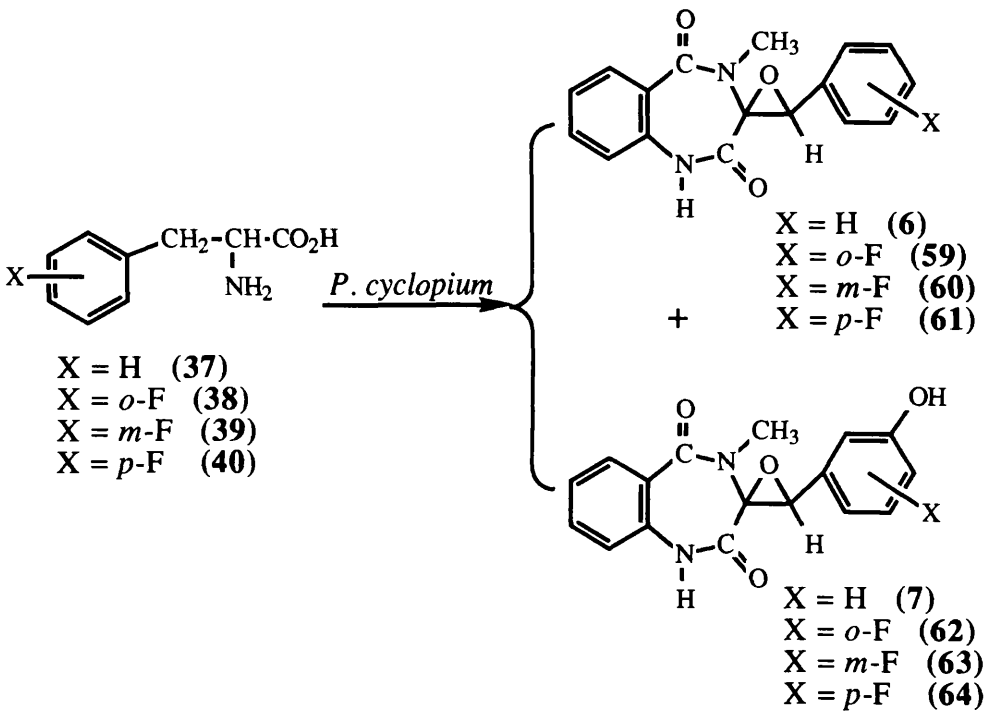
Scheme 16.

acetylphenylalanine (**55**). We prepared*N*-acetylphenylalanine (**55**) in good yield from the acetaminocinnamic acid (**51**) by hydrogenation over 10% palladium-carbon in ethanol at standard pressure and temperature. The *N*-acetylphenylalanine (**55**) was then heated under reflux in dilute hydrochloric acid to effect hydrolysis. The DL-phenylalanine (**37**) was precipitated by carefully adjusting the pH of the aqueous solution with concentrated hydrochloric acid and ammonium hydroxide (Ref. 58).

The corresponding fluoro derivatives, (**38**), (**39**), (**40**), were then prepared similarly.

2.2 BIOSYNTHESIS OF CYCLOPENINS AND CYCLOPENOLS

Penicillium cyclopium (CMI 89374) was grown under the same conditions as Braken *et al.* reported in 1954. Attempts to produce high yields of metabolites failed. The three fluorine substituted phenylalanines were fed in parallel in one portion to 6 days old cultures of *Penicillium cyclopium* with minimum disturbance of the mycelium. Incubation was continued for another 10 days at 25 °C (Scheme 17). The mycelium mat was removed from the top of each culture. The culture medium was then extracted three times with distilled ethyl acetate three times, without prior adjusting of the final pH. The extracts were dried and evaporated to yield an oil, which failed to crystallise, TLC



Scheme 17.

showed weak spots running alongside those of reference samples of cyclopenin (**6**) and cyclopenol (**7**), except with *p*-F phenylalanine substrate, but preparative TLC did not yield any useful quantities of the metabolites. However the mass spectra of the total

extracts showed them to contain the natural metabolites together with small amounts of the expected fluoro metabolites. These results are detailed in Table 3 and 4.

2.3 Conclusions

The present research provided some supplementary results for investigations of the biosynthesis of benzodiazepine alkaloids and microbial metabolism of fluorine substituted compounds. Three fluorophenylalanines were incorporated by *Penicillium cyclopium* into benzodiazepine alkaloids, cyclopenin and cyclopenol, resulting in the formation of corresponding fluorocyclopenin and fluorocyclopenol. Scaling up these experiments and feeding optically pure substrates to obtain enough material for quantitative analysis remains to be done. Separation techniques with high senitivity, such as HPLC, could then be applied. Because the yields of cyclopenin and cyclopenol were much lower than those previously obtained with this organism, deductive conclusions were reached by mass spectra analysis. In addition to normal metabolites, cyclopenin, (**6**) and (**7**), fluorocyclopenin and fluorocyclopenol (possible) were observed with feeding experiment of *o*-F phenylalanine (**38**); fluorocyclopenin but no fluorocyclopenol (might be due to the too low yield) were observed with *m*-F phenylanine (**39**); fluorocyclopenin (possible) but no fluorocyclopenol at all in both two experiments were observed with *p*-F phenylalanine (**40**). There was no evidence which can provide some information of the location of the fluorine.

Due to the limitation of the purity and quantity of metabolic products produced during the present research, ^{19}F NMR spectroscopy could not be used to characterise the metabolite. This can be developed in future research. Further, larger concentrations of the substrates (the concentration of the substrates in the present experiments were less than 5 mM) could be applied to see whether these fluoro-phenylalanines act as inhibitors for the growth of the organism and / or the production of the secondary metabolites.

Table 3. Metabolism of (±)-phenylalanine and (±)-fluorophenylalanines in *P. cyclopium*

Exp. No.	Substrate	Amount of substrate fed[a] (mg)	Yield of the product[b] (mg)	Cyclopenin[c]	Cyclopenol[c]	Fluoro-cyclopenins[c]	Fluoro-cyclopenols[c]
		-		+	+	-	-
1	*o*-F Phe (**38**)[d]	223	112	+	+ (?)	+	+
2	*m*-F Phe(**39**)[d]	223	110	+	+ (?)	+	- (?)
3	*p*-F Phe(**40**)[d]	223,	95 ,	+	+ (?)	+ (?)	-
4		277	160	+	+ (?)	+ (?)	-

a. Fed to 1L of culture.

b. Weight of crude ethyl acetate extract.

c. The presence (+) of each metabolite was deduced from the mass spectra of each extract.

d. Experiments in parallel.

(?). not certain.

Table 4. Mass Data of Metabolites in *P. cyclopium*

Exp. No	Substrate	Low resolution (m/e)	Deduced assignment*	high resolution (m/e)	Deduced assignment
1	*o*-F Phe (**38**)	312(2.0%), 294 (10.0%), 255 (1.7%), 253 (1.2%), 237 (13.9%)	FN, N, FVN, VL (?**),VN	328.0844 (0.6%), 312.0900 (10.2%), 310.0987 (4.8%), 294.1013 (57.2%)	FL (?) requires 328.0866, FN requires 321.0899, L (?) requires 310.0940, N requires 294.1097
2	*m*-F Phe (**39**)	312 (0.2), 294 (1.2), 252 (0.2), 237 (0.8)	FN, N, VL-1 (?), VN	312.0902 (1.4), 294.0993 (11.1),	FN requires 312.0893, N requires 294.0982
3	*p*-F Phe (**40**)	294 (2.5), 253 (0.4), 237 (1.3)	N, VL (?), N	312.0907 (1.2), 310. 0979 (0.5), 294.1044 (31.2)	FN requires 312.0895, L(?), N(?)

* FN- fluorocyclopenin; FL- fluorocyclopenol; N-cyclopenin; L- cyclopenol; FVN-fluoroviridicatin; FVL-fluoroviridicatol; VN- viridicatin; VL-viridicatol;

** not certain.

CHAPTER THREE

EXPERIMENTAL

3.1 CHEMICAL SYNTHESIS OF PHENYLALANINES

Acetylglycine (46).- In a 1 L conical flask were placed glycine (75g, 1 mol) and water (300 ml). The mixture was stirred vigorously unitil the glycine had almost completely dissolved, then acetic anhydride (98%, 215 g, 2 mol) was added in one portion. Vigorous stirring was continued for another 30 min, during which the solution became hot and acetylglycine (**46**) began to precipitate out. The reaction mixture was left overnight at room temperature to effect complete precipitation. The white precipitate of acetylglycine was collected, washed with ice-cold water and dried over phosphorous pentaoxide, m.p. 207-208.5 °C (lit. 207-208 °C). The product was used directly without further purification. The combined filtrate and washings were evaporated to dryness under reduced pressureto yield additional product, the total yield was 90%.

α-Acetaminocinnamic acids.- General method: A mixture of acetylglycine (5.85g, 0.05 mol), anhydrous sodium acetate (3.01 g, 0.037 mol), freshly distilled benzaldehyde (7.9 g, 0.074 mol) and acetic anhydride (134 g, 0.125 mol) in a round-bottomed flask fitted with a condenser and a drying tube was warmed on the steam bath with occasional stirring until solution was complete, *ca.*, 15 min. The resulting solution was boiled for one hour under reflux, cooled and placed in a refrigerator overnight. The resulting solid mass of yellow crystals was treated with 50ml of cold water and broken up with a stirring

rod. The crystals were then collected and washed thoroughly with sufficient cold water and dried to give the crude azlactone. The azlactone was used for further hydrolysis without further purification.

The crude azlactone of α-acetateminocinnamic acid (4.7 g, 0.025 mol) was dissolved by boiling in a mixture of acetone and water (2.5/1, v/v). Hydrolysis was completed after refluxing for 4 h. Most of the acetone was then removed under reduced pressure. The residual solution was diluted with water (40 ml), heated to reflux for 10 min to ensure complete solution of the acetamino acid, and filtered. The undissolved material which remained on the filter paper was washed with boiling water. Any crystals which separated from the filtrate were redissolved by heating, after that the solution was boiled for with charcoal for 5 min and then filtered with the aid of gentle suction while still at the boiling point. The charcoal was washed thoroughly on the funnel with boiling water to remove the crystals which separated during the filtration and the washings were added to the main filtrate. After standing in a refrigerator overnight the light coloured crystalline needles were collected and dried under vaccum to yield the target product, α-Acetaminocinnamic acid.

The characterization data for the four analogues of α-acetaminocinnamic acid prepared, (**51**), (**52**), (**53**), and (**54**) are listed in Table 5.

Phenylalanines. - General method: A solution of α-acetateminocinnamic acid (2.05g, 10 mmol) in ethanol (150 ml) was place in a round-bottomed flask and stirred under a hydrogen atmosphere with 10% palladium-carbon (205 mg, 10% by weight) until the calculated amount of hydrogen had been taken up (about 230 ml). The catalyst, palladium-carbon was removed by filtration and washed with a sufficient amount of ethanol. The combined filtrate and washings were evaporated to dryness under reduced pressure on a water bath and the residue was hydrolysis directly without further

purification. The *N*-acetophenylalanines, (**55**), (**56**), (**57**), and (**58**), were obtained in good yield (Table 6).

The residue was taken up in 1N hydrochloric acid (40 ml) and refluxed overnight. The resulting solution was evaporated to dryness and the residue was dissolved in boiling water (40 ml). The pH of the solution was adjusted until it was basic to Congo red,but still acid to litmus, by careful addition of concentrated ammonia and acetic acid. Then two volumes of absolute ethanol was added to aid the separation of phenylalanine. The mixture was placed in the refrigerator overnight to complete the cryatallisation. The solid was collected and washed with ice-cold water and absolute ethanol. Pure phenylalanine was obtained.

In total four phenylalanines, (**37**), (**38**), (**39**), and (**40**), were prepared by the methods described above and listed in Table 7.

3.2 BIOSYNTHESIS OF CYCLOPENINS AND CYCLOPENOLS IN *PENICILLIUM CYCLOPIUM*

Chemicals: Three precursors, *o*-F phenylalanine, *m*-F phenylalanine, and *p*-F phenylalanine were prepared by the author as described in Sec. 3.1. and the other chemicals were obtained from commercial purchase at the analytical grade.

Maintenance: The strain *Penicillium cyclopium* westling (CMI 89374) was maintained on 2% malt-agar slants at 25 °C.

Culture conditions: (Raulin-Thom solution)

Glucose	50 g
Tartaric acid	2.66 g
Ammonium tartrate	2.66 g

Ammonium hydrogen phosphate	0.4 g
Ammonium sulphate	0.17 g
Potassium carbonate	0.4 g
Magnesium carbonate	0.27 g
Ferrous sulphate ($FeSO_4 * 7H_2O$)	0.05 g
Zinc sulphate ($ZnSO_4 * 7H_2O$)	0.05 g
Deionised water	1000 ml

General procedure: 200 ml of culture medium was put into 1L Roux bottle flasks which were plugged with cotton wool and sterilised (Ref. 59). Each flask was incubated with a 2 ml portion of a spore suspension, in sterile deionised water, of *Penicillium cyclopium* westling strain (CMI 89374) which had been cultivated on 12 malt-agar slopes at 24 °C for 10 days. The mould was allowed to grow on the surface. After 4 days, the surface of the medium in most of the flasks was practically covered with a thin, white growth showing occasional green patches of sporing growth. After 6 days the medium in almost all flasks was covered with a somewhat fragile bright parrot-green felt which had an orange or pinkish-orange reverse. The culture solution was now bright orange in colour. Precursors were dissolved in sterile deionised water and fed to 6 days old cultures of *Penicillium cyclopium*, and the concentration of the precursor was 5 mM. The top layer of the mycelium was kept to the minium disturbance in case of low incorporation. The fed flasks were incubated at 25 °C for 10 days more, and then the mycelium was washed with water and discarded and the culture media and washings were combined. The combined aqueous solution was extracted with distilled ethyl acetate at the end pH. Evaporation of ethyl acetate extracts gave the products as oils which failed to be recrystallised.

Feeding of *o*-F phenylalanine (38).- *o*-F Phenylalanine (223 mg) in sterile deionised water (18 ml) was fed in two batches to a 6 days old culture of *Penicillium cyclopium* as described in the general procedure. In the first batch the phenylalanine was fed in two portions to the culture medium, *i.e.* the concentration of the substrate was 2.5 mM each feeding. An oily product was obtained which was identified by its mass spectrum (see Table 3 & 4).

Feeding of *m*-F phenylalanine (39). - *m*-F Phenylalanine (223 mg) in sterile deionised water (18 ml) was fed to a 7 days old culture of *Penicillium cyclopium* . The product was identified by its mass spectrum (see Table 3 & 4).

Feeding of *p*-F phenylalanine (40). - *p*-F Phenylalanine (223 mg) was fed to a 6 days old culture of *Penicillium cyclopium*, and the product was identfied by its mass spectrum (see Table 3 & 4).

Table 5. α-Acetaminocinnamic Acid and its Derivatives

Reactants		Products	Yield	M.p.	NMR Spectrum*
benzaldehyde (**41**)	glycine (**45**) acetylglycine (**46**)	α-acetamino- cinnamic acid (**51**)	31%, 50%	193-194 °C 193-195 °C	1.92(3H,s,COMe),7.10-7.75 (6H,m,Ar-H and -CH-),8.30-8.95(1H,brs) and 9.35 (1H,s) exchange with D_2O.
o-F benzaldehyde (**42**)	glycine (**45**) acetylglycine (**46**)	*o*-F-α-acetamino- cinnamic acid (**52**)	30%, 50%	213-215°C, 214-216°C	1.99(3H,s,COMe),7.25 (3H,m,2',6'-H and -CH=),7.70(2H,m,3'5'-H), 8.5(1H, brs),and 9.50(1H,s) exch. with D_2O
m-F benzaldehyde (**43**)	glycine (**45**)	*m*-F-α-acetamino- cinnamic acid (**53**)	32%	196-199 °C	1.95(3H,s,COMe),7.20-7.65 (6H,m,Ar-H and -CH-, 1H exch. with D_2O),and 9.45 (1H,s) exchange with D_2O.
p-F benzaldehyde (**44**)	glycine (**45**) acetyglycine (**46**)	*p*-F-α-acetamino- cinnamic acid (**54**)	35%, 58%	219-220°C, 218-220°C	1.94(3H,s,COMe),7.25(3H,m,3',5'H), 7.72 (2H,m,2',4'-H),8.30-8.95(1H,brs), and 9.38 (1H,s) exchange with D_2O.

* ^1H NMR spectra in D_6-DMSO, TMS as standard.

Table 6. *N*-Acetophenylalanine and its Derivatives

Reactants	Products	Yield	M.p.*
α-acetamino-cinnamic acid (**51**)	*N*-acetophenylalanine (**55**)	98%	150-152 °C (Ref. 60d)
o-F-α-acetamino-cinnamic acid (**52**)	*N*-aceto-*o*-F phenylalanine (**56**)	90%	146-149 °C (Ref. 60a)
m-F-α-acetamino-cinnamic acid(**53**)	*N*-aceto-*m*-F phenylalanine (**57**)	96%	154-157 °C (Ref. 60b)
p-F-α-acetamino-cinnamic acid (**54**)	*N*-aceto-*p*-F phenylalanine (**58**)	94%	150-152 °C (Ref. 60c)

*Crude product

Table 7. Phenylalanine and Its Derivatives

Reactants	Products	Yield	M.p.*
α-acetamino-cinnamic acid (**51**)	phenylalanine (**37**)	85%	264-267 °C (Ref. 60d)
o-F-α-acetamino-cinnamic acid (**52**)	*o*-F phenylalanine (**38**)	80%	241-244 °C (Ref. 60a)
m-F-α-acetamino-cinnamic acid (**53**)	*m*-F phenylalanine (**39**)	88%	239-245 °C (Ref. 60b)
p-F-α-acetamino-cinnamic acid (**54**)	*p*-F phenylalanine (**40**)	84%	251-255 °C (Ref. 60c)

*. From ethanol-water

REFERENCES

1. Y. S. Mohammed and M. Luckner, *Tetrahedron Lett.*, 1963, 1953.
2. M. Luckner and K. Mothes, *Tetrahedron Lett.*, 1962, 1035.
3. M. Luckner and K. Mothes, *Archiv. der Pharmazia*, 1963, **296**, 18.
4. M. Luckner and L. Nover, *Abh. Dtsch. Akad. Wiss. Berlin,* 1971, 525.
5. L. Nover and M. Luckner, *Eur. J. Biochem.*, 1969, **10**, 268.
6. L. Nover and M. Luckner, *Abh. Dtsch. Akad. Wiss. Berlin*, 1971, 535.
7. J. Framm, L. Nover, A. El. Azzonny, H. Richer, K. Winter, S. Werner and M. Luckner, *Eur. J. Biochem.* , 1973, **37**, 78.
8. M. Luckner, W. Lerbs and W. Werner, in "Regulation of Secondary Metabolite Formation", Eds., H. Kleinkauf H. v. Dohren and G. Nesemann, *Workshok Conf. Hoechst,* **16**, VCH Weinheim, 133.
9. W. Roos and H. P. Schnauder, *FEMS Microbiol. Lett.*, 1989, **59**, 27
10. S. Viogt, S. El. Kousy, N. Schwelle, L. Nover, and M. Luckner, *Phytochemistry,* 1978, **17**, 1705.
11. S. G. Laland and T. L. Zimmer, *Essays Biochem.,* 1963, **9**, 31.
12. H. Kleinkauf, H. Koischwitz, *Prog. Mol. Subcell. Biol.*, 1978, **6**, 59.
13. H. Kleinkauf, H. von. Dohren, *Curr. Topics. Microbiol. Immunobiol,* 1981, **91**, 129.
14. M. Gerlach, N. Schwelle, W. Lerbs and M. Luckner, *Phytochemistry,* 1985, **24**, 1935.
15. El. S. Al. Aboutabl, and M. Luckner, *Phytochemistry*, 1975, **14**, 2573.

16. El. S. Al. Aboutabl, A. El. Azzonny, K. Winter, and M. Luckner, *Phytochemistry*, 1976, **15**, 1925.

17. P. N. Rylander, "Catalytic Hydrogenation over Platinium Metals", Academic Press, New York,1967.

18. G. W. Kirby, J. Michael, and S. Narayanaswami, *J. Chem. Soc. Perkin. Trans. I*, 1972, 203.

19. S. Viogt and M. Luckner, *Phytochemistry*, 1977, **16**, 1651.

20. L. Nover and M. Luckner, *FEBS Letters*, 1969, **3**, 292.

21. (a) J. F. Martin, H. Rappoport, H. W. Smith, J. L. Wong, *J. Org. Chem.*, 1969, **34**, 1359.

    (b) H. W. Smith, P. Wegfarth and H. Rappoport, *J. Am. Chem. Soc.*, 1968, **90**, 1668.

22. I. Richter , K, Winter, S. El-Kousy and M. Luckner, *Pharmazie*, 1974, **29**, 506.

23. I. Richter and M. Luckner, *Phytochemistry*, 1976, **15**, 64.

24. M. Luckner and K. Winter, *Eur. J. Biochem.* , 1969, **7**, 380.

25. M. Luckner, *Eur. J. Biochem.*, 1967, **2**, 74.

26. W. Roos, in "Biochemistry of Alkaloids" ed. K. Mothes, H. R. Schutte and M. Luckner, VEB Deutscher Verlag der Wissenschaften, Berlin, 1985, 42.

27. W. Roos and M. Luckner, *Biochem. Physiol. Pflanz.* 1977, **171**, 127.

28. S. Wilson, I. Schmidt, W. Roos, W. Furst and M. Luckner, *Z. Allg. Mikrobiol.*, 1974, **14**, 515.

29. S. Wilson and M. Luckner, *Z. Allg. Mikrobiol.*, 1975, **15**, 45.

30. M. Luckner, K. Winter, L. Nover and J. Reisch, *Tetrahedron*, 1969, **25**, 2575.

31. M. McCamish and J. D. White, *Org. Mass Spectrum,* 1970, **4**, 24.

32. J. D. White and M. J. Dimsdale, *Chem. Commun.*, 1969, 1285.

33. M. Luckner, *J. Nat. Prod.*, 1980, **43(1)**, 21.

34. F. Swarts, *Bull. Soc. Chem. Belg.*, 1896, **15**, 1134.

35. J. S. C. Marais, *Onderstepoort J. Vet. Sci. Anim. Ind.*, 1943, **18**, 203.

36. P. Goldman, *Science*, 1969, **164**, 1123.

37. R. Filler, Y. Kobayashi, in "Fluorine in Biomedicinal Chemistry", Kodansha, Tokyo, Japan, 1982.

38. I. Collier, Ph. D. Thesis, 1988, 17.

39. "Handbook of Chemistry and Physics", 55th Ed., 1975.

40. J. Mann, *Chem. Soc. Rev.*, 1987, **16**, 381.

41. J. Kollonitsch, L. Barash, F. M. Kahan and H. Kropp, *Nature*, 1973, **24** , 346.

42. J. K. Kollonitsch, in "Biomedical Aspects of Fluorine Chemistry", Eds. R. Filler and Y. Kobayashi, Kodansha, Tokyo, Japan, 1982, 93.

43. E. Wang and C. Walsh, *Biochemistry*, 1978, **17**, 1313.

44. D. V. Santi, in "Biomedical Aspects of Fluorine Chemistry", Eds. R. Filler and Y. Kobayashi, Kodansha, Tokyo, Japan, 1982, 124.

45. D. V. Santi and C. S. McHenry, *Proc. Nat. Acad. Sci.*, 1972, **69**, 1855.

46. P. V. Danenberg, R. J. Langenbach, and C. Heidelberger, *Biochemistry*, 1974, **13**, 926.

47. A. L. Pogolotti and D. V. Santi, *Biochemistry*, 1974, **13**, 456.

48. A. L. Pogolotti, K. M. Ivanetich, H. Sommer, and D.V. Santi, *Biochim. Biophys. Res. Commun.*, 1976, **70**, 972.

49. M. A. Raftery and R. W. Dahlquist, *Prog. Chem. Org. Nat. Prod.*, 1969, **27**, 341.

50. F. Millet and M. A. Raftery, *Biochemistry*, 1972, **11**, 1639.

51. "Nuclear Magnetic Resonance (NMR) in Biochemistry", eds., R. A. Dwek. Clarendon Press. Oxford, 1973, 164.

52. C. C. F. Blake, L. N. Johnson, G. A. Mair, A.C.T. North, D. C.Philips and V. A. Sarma, *Proc. Roy. Soc. (London)*, 1967, **B167**, 378.

53. N. F. Taylor, F. H. White and R. E. Eisenthal, *Biochem. Pharmacol.*, 1972, **21**, 347.

54. N. F. Taylor, L. Hill and R. E. Eisenthal, *Canad. J. Biochem.*, 1975, **53**, 57.

55. N. F. Taylor, A. Romaschin and D. Smith, in "Biochemistry Involving Carbon-Fluorine Bonds". eds, R. Filler, Am. Chem. Soc. Washington D. C. 1976, 99.

56. *Org. Syn. Coll.* Vol. II, 11.

57. *Org. Syn. Coll.* Vol. II, 1.

58. *Org. Syn. Coll.* Vol. II, 491.

59. A. Bracken, A. Pocker and H. Raistrick, *Biochem. J.*, 1954, **57**, 587.

60. a. *Beil.*, 14 (3), 1671.

b. *Beil.*, 14 (3), 1672.

c. *Beil.*, 14 (3), 1673.

d. *Beil.*, 14 (1), 498.