# MICROBIAL SYNTHESIS OF 4-ALKYLMUCONOLACTONES

A thesis in part fulfilment of the
requirement for the Degree of
Doctor of Philosophy.

by

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To my mum, dad and family for their constant love, support and encouragement.

Finally, I dedicate this Thesis to Willie, with love

# SUMMARY

The work described in this thesis concerns the microbial degradation of 4-alkylpyrocatechols by the bacterium *Pseudomonas putida* and a mutant of the fungus *Aspergillus niger*. The aim was to extend current studies on the methylmuconate pathways by exploring the size and shape of the active sites in the dioxygenase and cycloisomerase enzymes in *P. putida* and *A. niger*, and the methylmuconolactone methyl-isomerase enzyme in *Rhodococcus rhodocrous* N75.

(1) A range of 4-alkylpyrocatechols was synthesised and tested as substrates for the enzymes in *P. putida*. Each pyrocatechol was fed to a culture of the organism, with 4-methylpyrocatechol being fed in parallel as a control. The % yields of 4-alkylmuconolacatones depended upon the alkyl group as follows: Me, 46; Et, 46; Pr$^i$, 31; Pr$^n$, 14; allyl, 10; Bu$^n$, trace; Bu$^t$, 0%. Thus, the ethyl and isopropyl groups, having chains of two carbons, clearly fit well the active sites of both the dioxygenase and cycloisomerase enzymes, even though the latter group is much wider. However, the bulkier *tert*-butyl group is not accommodated by the dioxygenase. The three-carbon chains of the propyl and allyl groups are less well accommodated than the shorter chains, although useful yields of the 4-alkylmuconolactones were obtainable. However, 4-butylpyrocatechol was metabolised to only a small extent. When the methyl group was replaced by the electron-withdrawing 4-

trifluoromethyl group, no products of dioxygenase cleavage were observed.

(2) It is known that the dioxygenase and cycloisomerase enzymes present in *A. niger* are not as effective at dissimilating 4-methylpyrocatechol as *P. putida*. This was exaggerated with bulkier side chains, exemplified by 4-isopropylpyrocatechol being converted to 3-isopropylmuconolactone in trace quantities and 4-*n*-propylpyrocatechol failing to produce any enzymic metabolites.

(3) The enzymic 4-alkylmuconolactones prepared with cultures of *P. putida* were tested as substrates for the methylmuconolactone isomerase in *R. rhodocrous* N75. The rate of isomerisation to the 3-alkylmuconolactones decreased with increasing size of the alkyl group as follows: methyl > ethyl > isopropyl > propyl > allyl.

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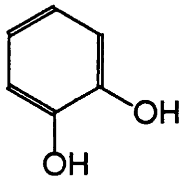
# CHAPTER 1 INTRODUCTION

Aromatic compounds are degraded by certain species of bacteria and fungi. Naturally-occurring benzene derivatives are metabolised in the first instance to either pyrocatechol (1) or protocatechuic acid (2). Unnatural benzenoid compounds that have been passed into the environment as pollutants present micro-organisms with a challenge. In order to utilize these benzene derivatives as a source of carbon and energy, bacteria and fungi must either adapt their existing pathways for the assimilation of simple substrates, or evolve new enzymic routes to cope with the new compounds. Compounds whose biodegradation pathways have been studied include benzene-derivatives with a variety of different substituents - alkyl, acyl, carboxyl, halo, amino, hydroxy, alkoxy, nitro and sulfonyl.



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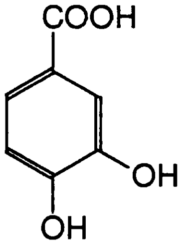

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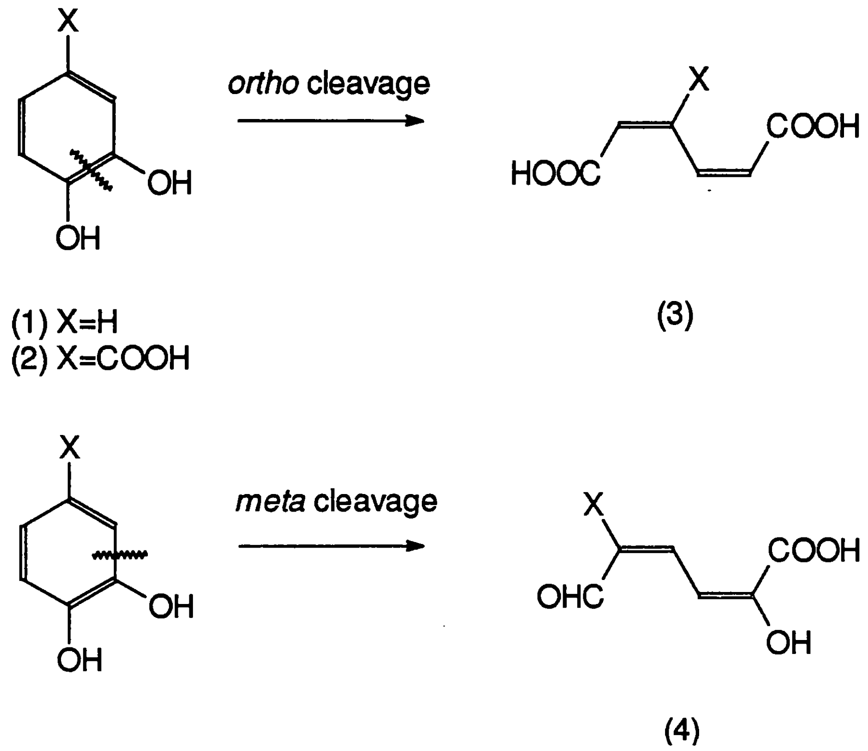
## 1.1 Background

A multitude of aromatic substances is initially metabolised by micro-organisms to pyrocatechol (1) and its derivatives, which become substrates for reactions which cleave the aromatic ring. The non-aromatic products of this cleavage are then metabolised *via* a variety of pathways.

Pyrocatechol (1), protocatechuic acid (2) and a variety of other pyrocatechol derivatives are a point of secondary metabolic divergence during the microbial assimilation of aromatic compounds. Two main pathways exist for the assimilation of these compounds by cleavage of the benzene ring: *ortho* cleavage (cleavage of the carbon-carbon bond between the vicinal hydroxy groups) and *meta* cleavage (cleavage of a carbon-carbon bond adjacent to the vicinal hydroxy groups) (Scheme 1). *Ortho* fission leads to the formation of muconic acids (3), and then to further metabolism in a branch of the 3-oxoadipate pathway. *Meta* fission of the aromatic nucleus gives a 2-hydroxymuconic semialdehyde derivative (4) which is assimilated in a separate pathway.[1] In general, a single species or strain will entertain only one pathway or the other. In *Pseudomonas acidovorans* and *testosteroni*, for example, protocatechuic acid (2) and its biochemical precursors are metabolised via the *meta*-cleavage pathway,[2] while the *ortho*-cleavage route is employed by all other *Pseudomonas* species for this compound.[3] However, in certain bacteria, both pathways can occur for assimilation of different primary substrates. The metabolically tangential nature of these pathways is maintained due to the specific induction of each set of enzymes in each process.[4-7]

**Scheme 1**



The ring cleavages are oxidative and are mediated by oxygenases.[8] These enzymes utilize oxygen as a substrate, and therefore the assimilation of aromatic substrates is an aerobic process, and cannot operate anaerobically.[9,10] Also, despite the reduction of oxygen in the initial reactions of aromatic substrate degradation, this does not lead directly to the generation of ATP and the production of energy. This appears to be limited to the subsequent breakdown of the aliphatic metabolites in the tricarboxylic acid cycle.[1]

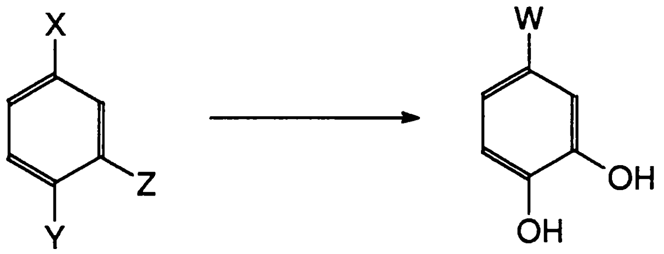
As Stanier found with the bacterium *Pseudomonas putida*,[11] the enzymes of the 3-oxoadipate pathway are inducible, that is their synthesis in microorganisms is controlled by the presence of particular metabolites from the pathway. The nature of this regulation has been studied in many microbial genera, at both the physiological and genetic levels.

### 1.1.1 Formation of Pyrocatechols

In 1947, Evans[12] identified pyrocatechol (1) as having accumulated during the growth of the bacterium *Acinetobacter calcoaceticus*[13] on phenol (5), and benzoic (6) and *p*-hydroxybenzoic (7) acids (Scheme 2). With the bacterium *Pseudomonas putida*, Stanier and Sleeper[14] established that pyrocatechol was an intermediate from benzoic acid oxidation.

Fungi also produced pyrocatechol from aromatic precursors. *Trichosporon cutaneum* fungi converted phenol (5) into pyrocatechol (1) and resorcinol (8) into 1,2,4-benzenetriol (9) (Scheme 2).[15]

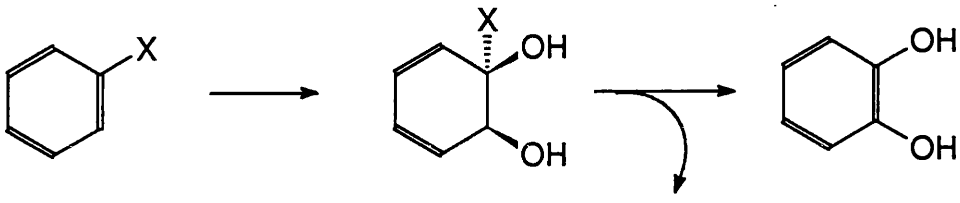
**Scheme 2**



| | X | Y | Z | | |
|---|---|---|---|---|---|
| (5) | H | OH | H | (1) | W = H |
| (6) | $CO_2H$ | H | H | (9) | W = OH |
| (7) | $CO_2H$ | OH | H | | |
| (8) | OH | H | OH | | |

The formation of pyrocatechols occurs by dioxygenation of the benzene (10) nucleus followed by a dehydrogenation. In the bacterium *Moraxella B*, isotope experiments proved that the oxygenase enzyme incorporated both oxygen atoms from dioxygen. NADH was generated during the course of the dehydrogenation of the isolable intermediate *cis*-1,2-dihydroxydihydrobenzene (11) in cell-free extracts (Scheme 3).[16] Similarly, the degradation of benzoic acid (6) by *Arthrobacter globiformis* was by dioxygenation to *cis*-1,2-dihydroxy-1,2-dihydrobenzoic acid (12), followed by dehydrogenation and decarboxylation to pyrocatechol (1) (Scheme 3).[17]

**Scheme 3**



(10) X = H

(6) X = $CO_2H$

(11) X = H

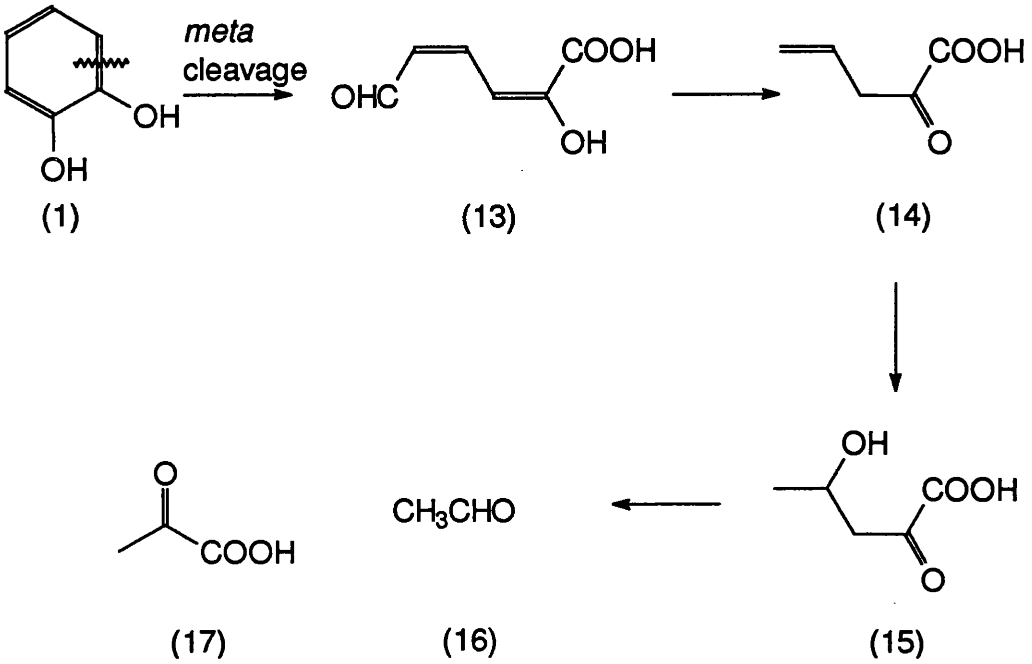
(12) X = $CO_2H$

(1)

## 1.2 *Meta* Cleavage

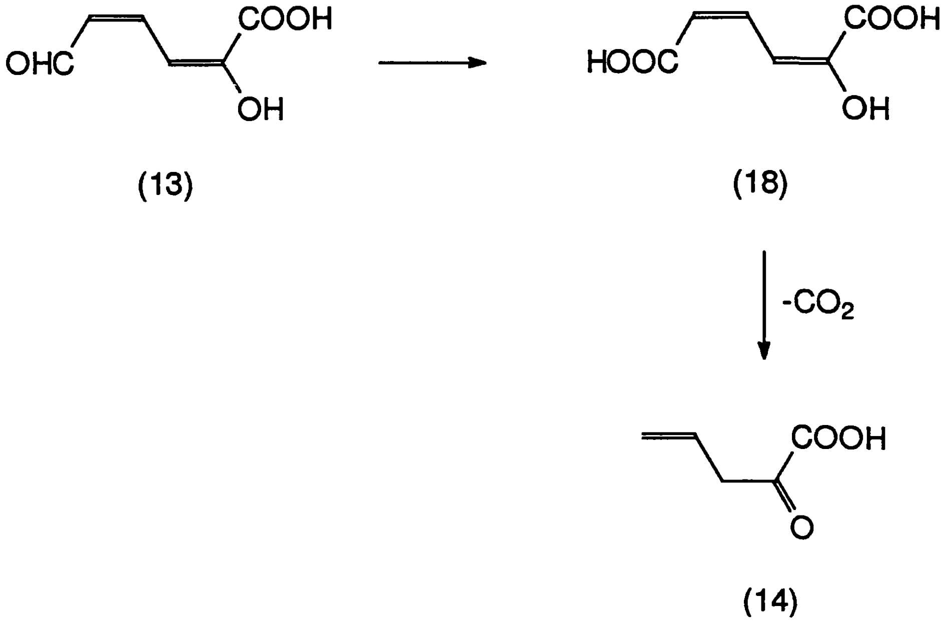
*Meta*-cleavage of pyrocatechol (1) was discovered in 1959 by Dagley and Stopher.[18] A species of *Pseudomonas* was isolated containing a pyrocatechol-2,3-oxygenase enzyme,[19] which cleaved the aromatic ring between carbons 2 and 3, giving 2-hydroxymuconic semialdehyde (13). A similar enzyme was observed in another pseudomonad, and termed *meta*-oxygenase.[20] Steps in the *meta*-cleavage pathway were elucidated by Dagley and Gibson[21] and further modified by Bayly and Dagley.[22] Following initial conversion of pyrocatechol (1) into 2-hydroxymuconic semialdehyde (13), a hydrolytic cleavage of formate occurs giving 2-oxopent-4-enoic acid (14) (Scheme 4). This is hydrated to 4-hydroxy-2-oxopentanoic acid (15), prior to cleavage to acetaldehyde (16) and pyruvic acid (17).[23] A similar sequence degrades protocatechuic acid (2), to formic acid and two molecules of pyruvic acid (17), and also various alkylpyrocatechols. However, the enzymes of the pathway are different for the conversion of pyrocatechol (1) and protocatechuic acid (2).

**Scheme 4**



A variation of the sequence involving an oxidation by an NAD-dependent dehydrogenase was reported by Nishizuka in 1962.[24] 2-Hydroxymuconic semialdehyde (13) was oxidised to 2-hydroxy-*cis,cis*-muconic acid (18) (Scheme 5), with carbon dioxide being expelled from this molecule instead of formic acid to give the corresponding 2-oxopent-4-enoic acid (14) as was formed in the formate-expulsion route. This is then degraded as before. The dehydrogenase and a tautomerase which effect the decarboxylation were found to be induced at the same time as the 2,3-oxygenase enzyme in *Azotobacter* and *Pseudomonas* genera.[25,26] The hydrolase in the formate-producing step of the previous pathway was not induced, suggesting that it is non-specific. Since the pathway in Scheme 5 requires a specific tautomerase whereas that in Scheme 4 a non-specific hydrolase, it may be that the latter pathway is more common in bacteria.

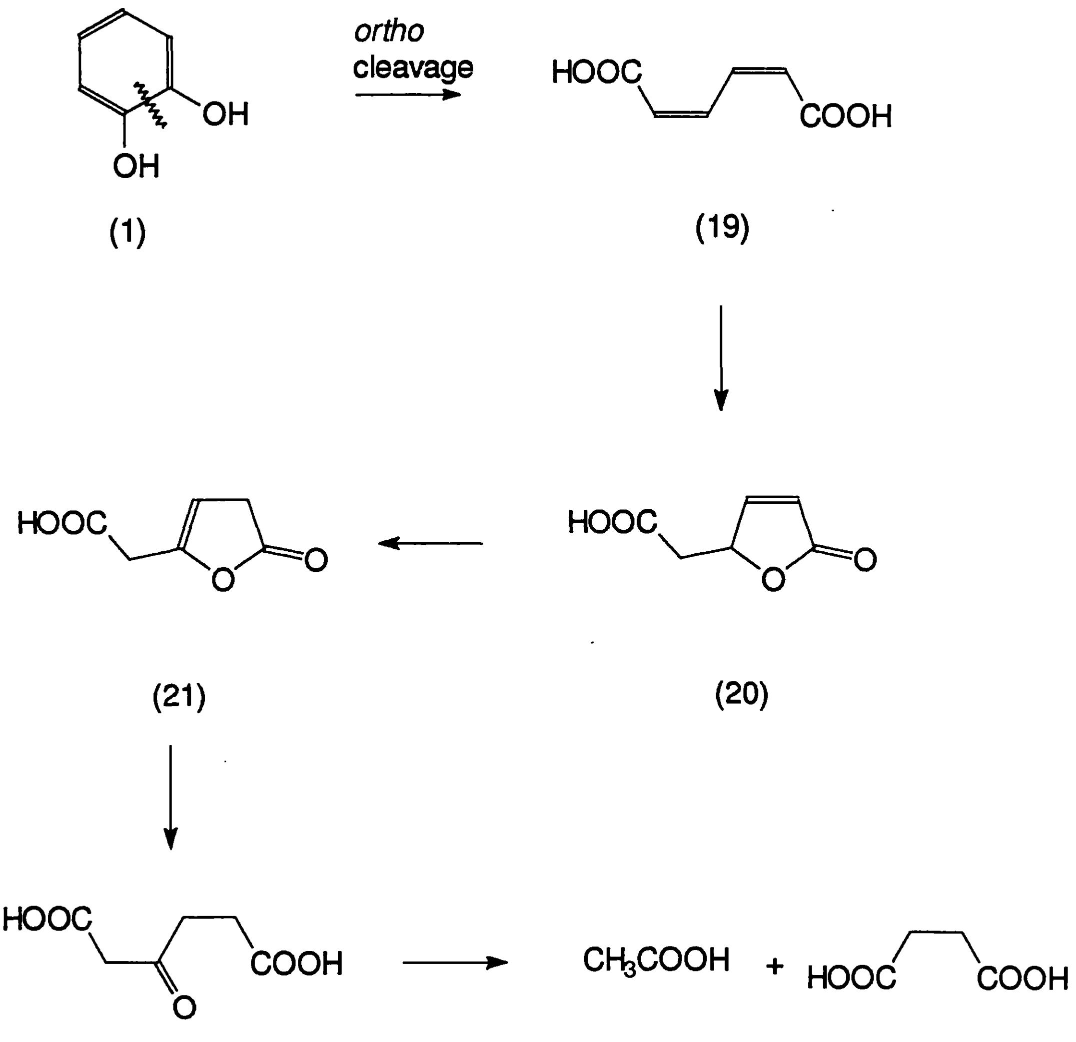
**Scheme 5**



## 1.3 Ortho Cleavage

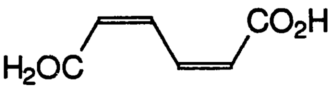
*Ortho*-cleavage of pyrocatechol (1) involves initial conversion of pyrocatechol (1) into *cis,cis*-muconic acid (19). Cyclisation occurs giving muconolactone (20) (Scheme 6). This lactone undergoes isomerisation to 3-oxoadipic acid enol lactone (21), prior to hydrolysis to 3-oxoadipic acid (22). This molecule then enters the tricarboxylic acid cycle and is converted into acetic acid (23) and succinic acid (24). Details of these transformations are discussed in the subsequent sections of this chapter.

Scheme 6



### 1.3.1 Formation of *cis,cis*-Muconic Acids

The initial *ortho*-cleavage of pyrocatechol by a partially purified enzyme from a *Pseudomonas* species gave muconic acid (19),[27] identified as the *cis,cis-* isomer following synthesis of *cis,cis-* (19), *cis,trans-* (25), and *trans,trans*-muconic acids (26).[28,29] Oxidation of pyrocatechol (1) to *cis,cis*-muconic acid (19) (Scheme 7) in bacteria has been observed in whole cells of a *Pseudomonas*,[30] *Acinetobacter lwoffi*,[31] *Corynebacterium*[32] and cell-free extracts of *Klebsiella aerogenes* NCTC 418.[33] Muconic acid was also formed in fungi, such as whole cells of the yeast strains *Rhodotorula rubra* IFO 0892 and 1101.[34,35]



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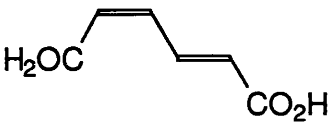

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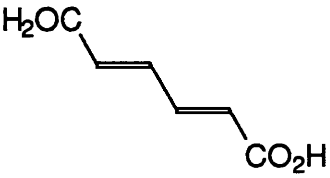

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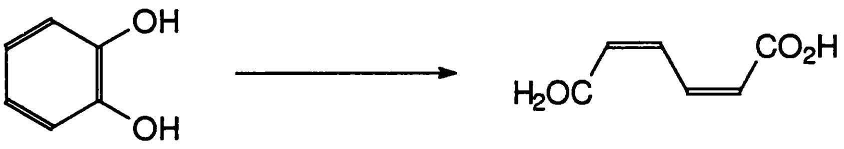
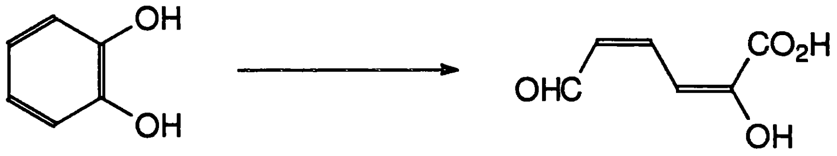
Scheme 7



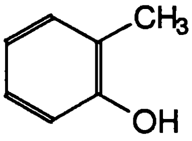
(1) (19)

*Meta* fission has also been observed (see Scheme 4). For instance, whole cells and cell-free extracts of an *Acinetobacter* oxidized pyrocatechol (1) to 2-hydroxymuconic semialdehyde (13) (Scheme 8).[30] The occurrence of the two types of cleavage appears to be mutually exclusive, and tends to depend on the substrate. In strains of *Pseudomonas putida*, pyrocatechol from benzoic acid underwent *ortho* cleavage while pyrocatechols produced from *o*- (27) and *p*-cresol (28) underwent *meta* cleavage.[4] By use of both nonmetabolizable inducers and blocked mutants, it was found that the enzymes of the *meta* pathway were induced by the primary substrate, cresol or phenol, or analogues.

**Scheme 8**



(1) (13)



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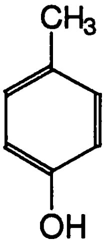
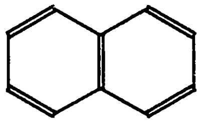


(28)

The control and regulation of the enzymes has been widely studied. For example, Bird and Cain[36] found that *cis,cis*-muconic acid (19) was the product inducer of catechol 1,2-oxygenase in *Pseudomonas aeruginosa*. Both *cis,cis*-muconic acid and pyrocatechol induced the enzyme under aerobic conditions, but it was postulated that this was probably due to the endogenous formation of *cis,cis*-muconic acid.

Several other compounds are degraded *via* pyrocatechols, for example naphthalene (29) by *Pseudomonas putida*,[37] benzoic, *p*-toluic (30), and *m*-toluic (31) acids by *Gordona rubra* N657,[38] and benzene, benzoic acid, *o*-cresol, guaiacol (32), phenol, protocatechuic acid (2), and salicylic acid (33) by a *Rhizobium* species.[39] Catechol 1,2-dioxygenase was present in the crude extracts and *cis,cis*-muconic acid was identified in the spent cultures.



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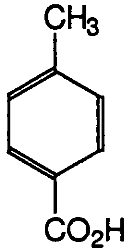

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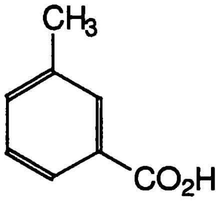

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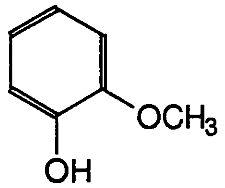

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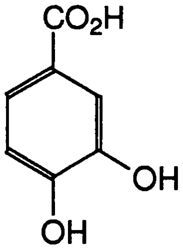

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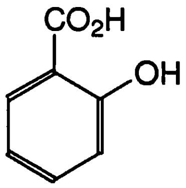


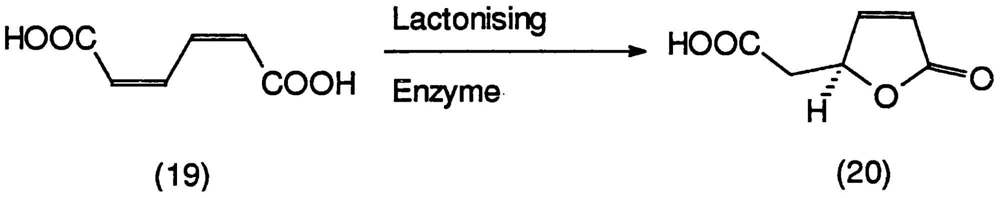
(33)

### 1.3.2 Formation of Muconolactones

(+)-Muconolactone (20), formed by the cyclisation of *cis,cis*-muconic acid (19) (Scheme 9), has been observed several times in bacteria, such as in cell-free extracts of *Klebsiella aerogenes* NCTC 418[33] and a strain of *Moraxella B*,[16] and whole cells of *Acinetobacter* *lwoffi*,[31] a strain of *Pseudomonas*,[30,37] a *Corynebacterium* species[32] and *Arthrobacter globiformis*.[17]

In fungi, formation of muconolactone (20) was studied using whole cells of the yeast strains of *Rhodotorula rubra* IFO 0892 and 1101,[34,35] *Rhodotorula glutinis*,[40] and recently in the deuteromycetous fungus *Penicillium frequentans* Bi 7/2.[41]

**Scheme 9**



Muconolactone (20) is not an intermediate in the *meta* pathway. For example, whole cells and cell-free extracts of an *Acinetobacter* which degraded pyrocatechol by *meta* fission did not degrade *cis,cis*-muconic acid (19).[30] (+)-Muconolactone (20) was shown to be the product of the effect of a lactonizing enzyme (muconate cycloisomerase) on *cis,cis*-muconic acid.[42] Ornston[43] investigated the isolation, purification, optimum activity pH range and molecular weight of the muconate lactonizing enzyme from *Pseudomonas putida* A. 3.12. The X-ray crystal structure of muconate cycloisomerase from *Pseudomonas putida* has been reported.[44] The manganese (II)-dependent enzyme has also been reported in other bacteria.[31,39]

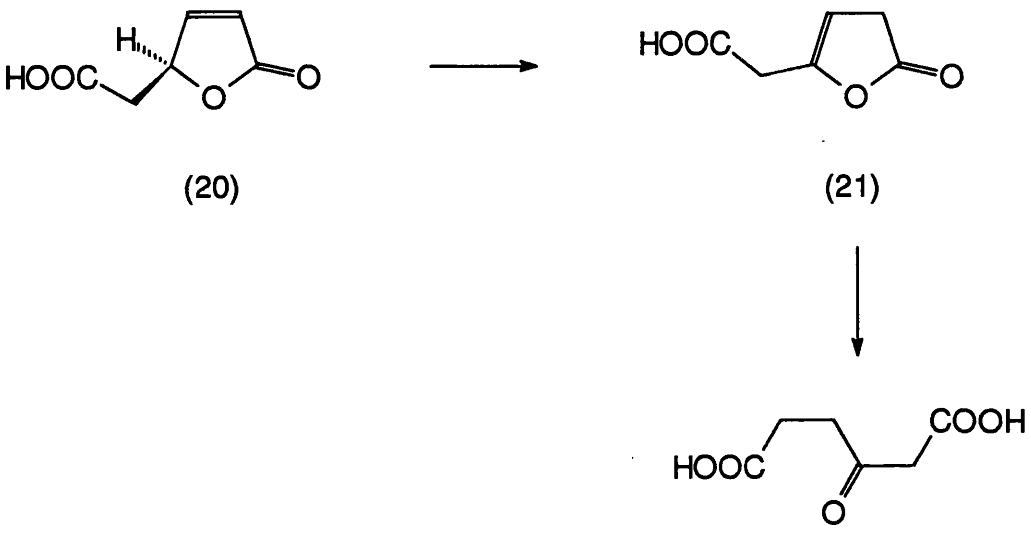
The stereoselectivity of the enzyme has been studied in bacteria and fungi. Muconate-lactonizing enzyme from *Trichosporon cutaneum* was purified 50-fold.[15] Both this enzyme and chloromuconate cycloisomerase (Section 1.6) from *Pseudomonas* species B13 converted *cis,cis*-muconic acid to (4*S*)-(+)-muconolactone (20) by a *syn* lactonization, determined from $^1$H NMR measurements (Scheme 9).[45] The stereochemistry of the transformation was identical to that observed with muconate lactonizing enzyme from *Pseudomonas putida*.[46] The amino acid sequence determined for the *cis,cis*-muconate lactonizing enzyme from *Trichosporon cutaneum* fungi did not exhibit any similarity to that of the muconate lactonizing enzyme from *Pseudomonas putida*.[45] It did, however, exhibit moderate sequence similarity with 3-carboxy-*cis,cis*-muconate lactonizing enzyme from the fungus *Neurospora crassa*, which catalyzed a stereochemically-identical lactonization reaction with 3-carboxymuconic acid (Section 1.4). *Syn* cyclization of *cis,cis*-muconic acid has also been shown to form the (4*S*)-(+)-muconolactone in *Aspergillus niger*.[47]

The catalytic mechanism of muconate cycloisomerase was investigated by computer modelling. Manganese (II) and a lysine residue were proposed to provide electrophilic stabilization of the enol/enolate intermediate that forms during the course of the reaction.[44] In *Pseudomonas aeruginosa* the enzyme was induced by *cis,cis*-muconic acid.[36]

### 1.3.3 Formation of 3-Oxoadipic Acid

In both bacteria and fungi, (4*S*)-(+)-muconolactone (20) is isomerised to 3-oxoadipic acid enol lactone (21), and this lactone is hydrolysed enzymically to 3-oxoadipic acid (22) (Scheme 10).

**Scheme 10**



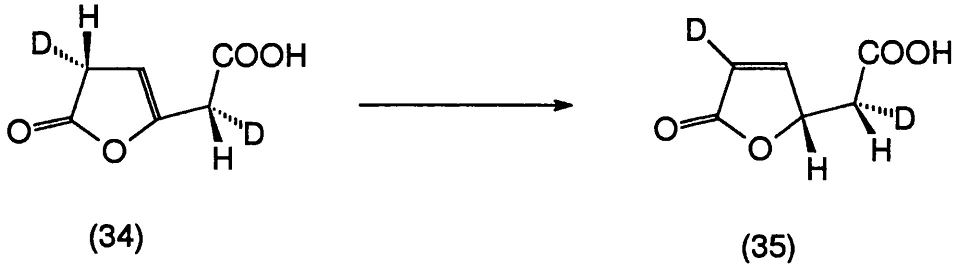
(20) (21) (22)

The conversion of (4*S*)-(+)-muconolactone (20) into 3-oxoadipic acid enol lactone (21) has been observed from the action of an isomerase enzyme in cell-free microbial extracts,[5] and in the degradation of benzoic acid by *Arthrobacter globiformis*.[17] However the transformation did not occur when the *meta* pathway was induced in an *Acinetobacter*.[30] The enzyme responsible for enol lactone formation, (4*S*)-(+)-muconolactone isomerase, was induced by *cis,cis*-muconic acid in *Pseudomonas aeruginosa*.[36,4] 3-Oxoadipic acid enol lactone (21) was also identified as a product of fungal degradation of phenol, by the yeast strains of *Rhodotorula rubra* IFO 0892 and 1102.[34,35]

Muconolactone isomerase has been isolated and characterised, in terms of molecular weight and optimum activity pH range, from *Pseudomonas putida* A.3.12.[43] The crystal structure of the *Pseudomonas putida* enzyme showed a large central cavity,[48, 49] and it was similar in structure to the same enzyme from *Alcaligenes eutrophus* JMP134.[50] This enzyme showed high activity with (4*S*)-(+)-muconolactone (20) as substrate.

The stereochemical course of the enzymic reaction was determined, by performing the reverse reaction in deuterium oxide. A 2,5-dideuterio-3-oxoadipic acid enol lactone (34) of defined stereochemistry was converted into 2,5-dideuteriomuconolactone (35) (Scheme 11). This could only happen by a 1,3-shift of hydrogen across one side of the enol lactone (34).[69]

**Scheme 11**



Since Evans[12] identified a keto acid (later identified as 3-oxoadipic acid (22) [51]) as having accumulated during the growth of the bacterium *Acinetobacter calcoaceticus*[13] on phenol and benzoic and *p*-

hydroxybenzoic acids, 3-oxoadipic acid has also been identified several times from the breakdown of pyrocatechols.

A hydrolase enzyme was also identified, converting the enol lactone (21) into 3-oxoadipic acid (22).[3] In bacterial cell-free extracts only the *cis,cis*-isomer of muconic acid was converted into 3-oxoadipic acid,[52, 53] as was (4*S*)-(+)-muconolactone (20).[54] Conversion of (4*S*)-(+)-muconolactone into 3-oxoadipic acid has been also been found in *Pseudomonas*[30,37] and *Acinetobacter lwoffi*.[31] *Klebsiella aerogenes* NCTC 418 adapted to benzoic acid and *p*-hydroxybenzoic acid oxidized pyrocatechol to 3-oxoadipic acid.[33] 3-Oxoadipic acid enol lactone and 3-oxoadipic acid were formed in the degradation of benzoic acid by *Arthrobacter globiformis*.[17] Neither substance is formed by the *meta* pathway, exemplified by the fact that whole cells and cell-free extracts of an *Acinetobacter* did not degrade (4*S*)-(+)-muconolactone or 3-oxoadipic acid while the *meta* pathway was induced.[30] (4*S*)-(+)-Muconolactone isomerase and 3-oxoadipic acid enol lactone hydrolase in *Pseudomonas aeruginosa* were induced by *cis,cis*-muconic acid.[36,4]

In fungi, formation of 3-oxoadipic acid (22) from phenol was studied using whole cells in the yeast strains of *Rhodotorula rubra* IFO 0892 and 1101.[34,35] 3-Oxoadipic acid was produced from muconolactone (20) by the crude cell free extract[34] and whole cells[55] of *Rhodotorula rubra* IFO 0892 and 1101.

The purification, optimum activity pH range and molecular weights of 3-oxoadipic acid enol-lactone hydrolase from *Pseudomonas putida* A. 3.12 have been studied.[43] 3-Oxoadipic acid enol lactone hydrolase from *Pseudomonas cepacia* has also been purified to apparent homogeneity and characterized.[56]

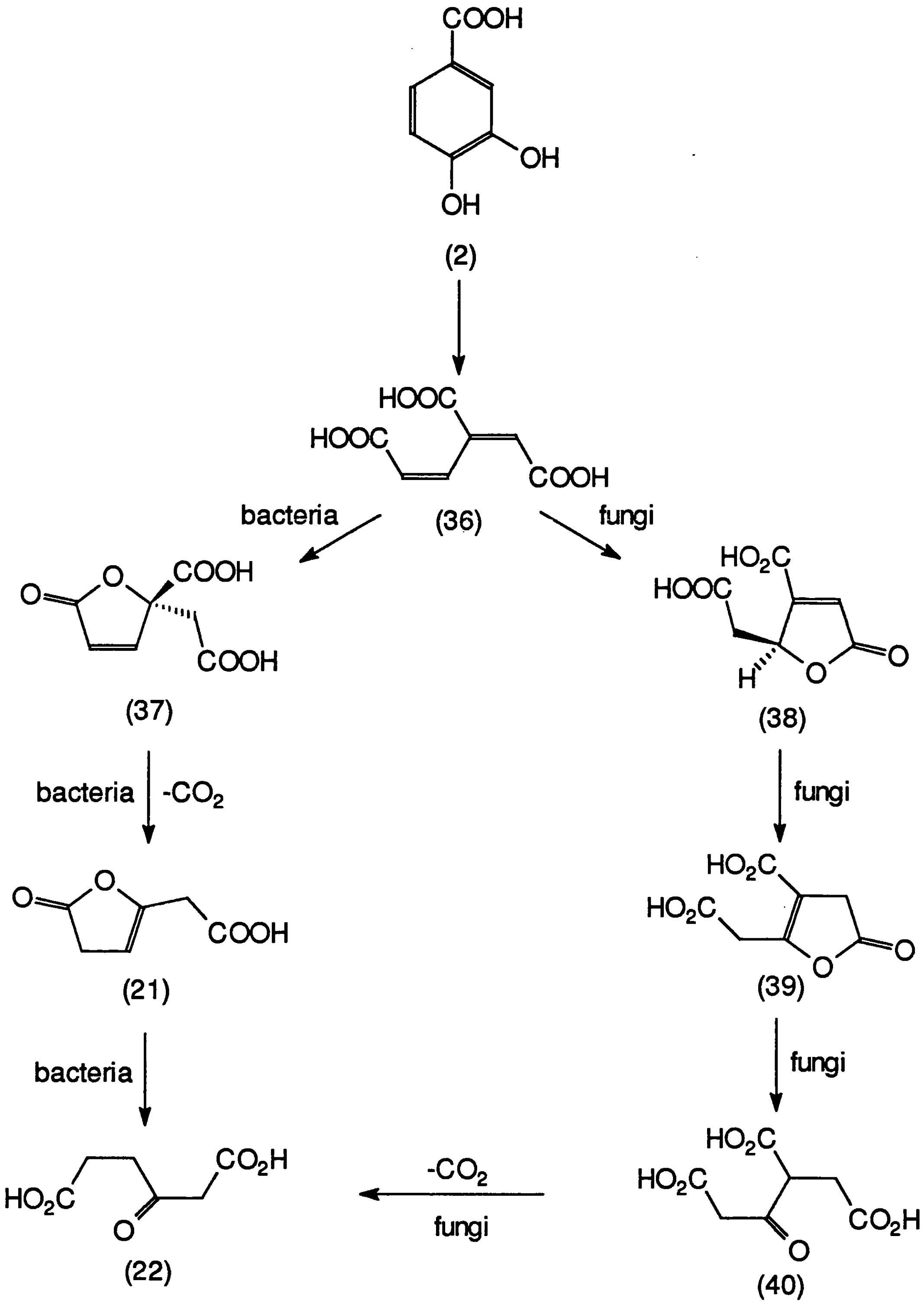
Katagiri and Hayaishi[57] established that 3-oxoadipic acid was converted into the corresponding coenzyme-A ester prior to its cleavage to give the co-A thioesters of succinic acid (24) and acetic acid (23).

## 1.4 Degradation of Protocatechuic Acid

As indicated in the previous section, both fungi and bacteria assimilate pyrocatechol via the same intermediates, whether by the *ortho* or *meta* pathways. 4-Substituted pyrocatechols which undergo *ortho* cleavage, by analogy, might be expected to be assimilated through 3-substituted muconic acids. These unsymmetrical muconic acids give rise to two possible modes of cyclisation to muconolactones. The pathways of degradation of protocatechuic acid are found to be different in bacteria and fungi.

*Ortho*-cleavage of protocatechuic acid (2) involves initial conversion of protocatechuic acid (2) into 3-carboxy-*cis,cis*-muconic acid (36). In bacteria, cyclisation occurs giving 4-carboxymuconolactone (37) (Scheme 12). This lactone undergoes isomerisation and decarboxylation to 3-oxoadipic acid enol lactone (21), prior to hydrolysis to 3-oxoadipic acid (22). However, in fungi cyclisation occurs giving 3-carboxymuconolactone (38) (Scheme 12). This lactone undergoes isomerisation to 4-carboxy-3-oxoadipic acid enol lactone (39). The enol lactone (39) is then hydrolysed to 4-carboxy-3-oxoadipic acid (40), which is decarboxylated to give 3-oxoadipic acid (22). Details of these differences in bacteria and fungi are outlined in this section.

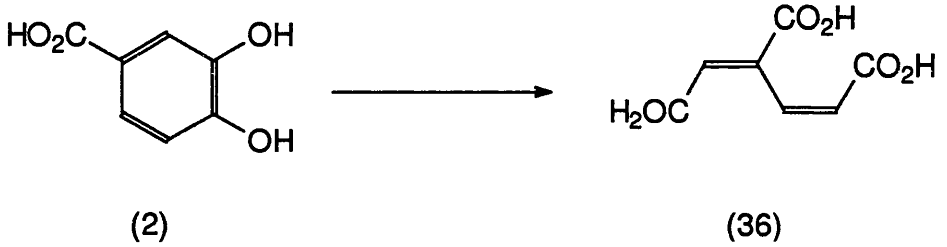
**Scheme 12**


(2)
bacteria
(36)
fungi
(37)
(38)
bacteria
-CO2
fungi
(21)
(39)
bacteria
fungi
-CO2
fungi
(22)
(40)

### 1.4.1 Formation of 3-Carboxy-*cis,cis*-muconic Acid (36)

Protocatechuic acid (2) accumulated from the oxidation of phenol, and benzoic and *p*-hydroxybenzoic acids[12] by *Acinetobacter calcoaceticus,*[13] and the oxidation of *p*-hydroxybenzoic acid by *Pseudomonas putida.*[14] The immediate product of *ortho*-cleavage of protocatechuic acid (2) by a partially purified oxygenase (Scheme 13) was identified as 3-carboxy-*cis,cis*-muconic acid (36) in 1954.[58]

**Scheme 13**



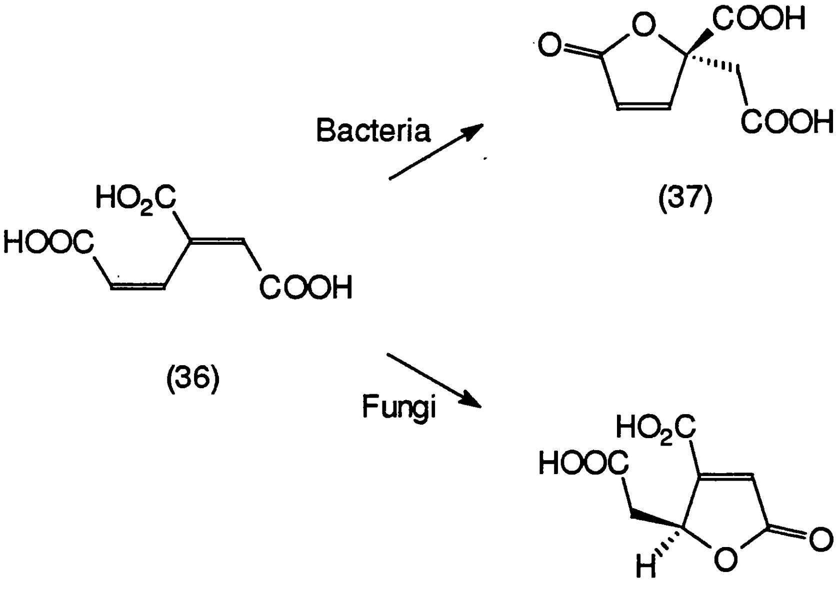
Cain *et al*[59] found that certain fungi oxidised *p*-hydroxybenzoic acid and protocatechuic acid but not pyrocatechol. These fungi possessed a protocatechuate-3,4-oxygenase enzyme and accumulated 3-carboxymuconic acid (36) as the product of protocatechuic acid oxidation. The same transformation was observed in *Neurospora species.*[60] In *Rhodotorula,* the enzyme was found to be competitively inhibited by pyrocatechol.[59]

### 1.4.2 Formation of 3-Carboxymuconolactone (38) and 4-Carboxymuconolactone (37)

Bacteria cyclise 3-substituted muconic acids to 4-substituted muconolactones.[1] In particular, 3-carboxy-*cis,cis*-muconic acid (36) was converted enzymically into 4-carboxymuconolactone (37) (Scheme 14) by enzymes isolated from *Pseudomonas*.[3] Ornston studied the isolation, purification, optimum activity pH range and molecular weight of 3-carboxymuconic acid lactonizing enzyme from *P. putida* A. 3.12.[43]

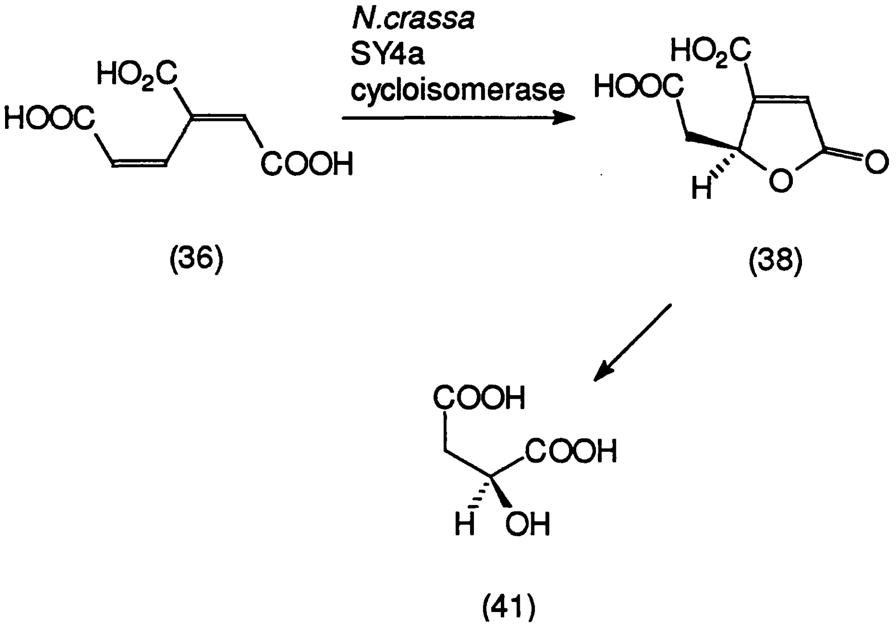
However, 3-carboxymuconic acid (36) was converted by fungi (for example *Neurospora* species[60]) into 3-carboxymuconolactone (38) (Scheme 14), which was isolated and characterized. In contrast, none of the fungi produced the corresponding bacterial intermediate 4-carboxymuconolactone (37). The lactonizing enzymes of *Rhodotorula* and *Neurospora crassa* were characterised.[59]

**Scheme 14**



In 1975, Kirby *et al*[61] showed that the reaction catalysed by this enzyme from the fungus *Neurospora crassa* proceeded by *syn*-addition to the C-4-C-5 double bond[61] to give (*S*)-(-)-3-carboxymuconolactone (38) (Scheme 15). The absolute configuration of the lactone was determined by ozonolysis to give (*S*)-malic (*L*-malic) acid (41).[62] 3-Carboxy-*cis,cis*-muconic acid lactonizing enzyme from *Neurospora crassa* exhibited moderate sequence similarity with the *cis,cis*-muconic acid lactonizing enzyme from the fungus *Trichosporon cutaneum*,[45] which catalyzed a stereochemically-identical lactonization reaction with muconic acid (Section 1.3).

**Scheme 15**

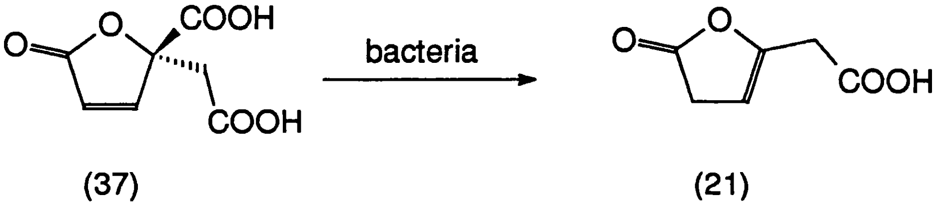


Non-enzymic, oxidative ring scission of protocatechuic acid (2) with 15-20% sulphuric acid-free peracetic acid gave only 3-carboxymuconolactone (38).[63] 3-Carboxy-*cis,cis*-muconic acid (36) was not isolated from the reaction.

### 1.4.3 Assimilation of 3-Carboxymuconolactone and 4-Carboxymuconolactone

In bacteria, 4-carboxymuconolactone (37) is decarboxylated with a concommitant double bond isomerisation, giving 3-oxoadipic acid enol-lactone (21) (Scheme 16). This is then hydrolysed as in the degradation of muconolactone. 4-Carboxymuconolactone (37) was transformed into this enol-lactone (21) by enzymes isolated from *Pseudomonas* species.[3] Ornston studied the isolation, purification, optimum activity pH range and molecular weight of 4-carboxymuconolactone decarboxylase from *Pseudomonas putida* A. 3.12.[43]

Scheme 16



3-Oxoadipic acid enol lactone (21), therefore, was found to be common to both pyrocatechol and protocatechuic acid metabolism in bacteria in general, with all other genera studied exhibiting no diversity from the pathways observed in *Pseudomonas*.[64-66]

However, fungi operate a different pathway involving instead 3-carboxymuconolactone (38). Fungi did not degrade 4-carboxymuconolactone (37) or 3-oxoadipic acid enol lactone (21) at significant rates, and thus they differed radically from bacteria. In bacteria, 3-oxoadipic acid enol lactone was the precursor of 3-oxoadipic acid in all strains examined.[59] Nevertheless, 3-oxoadipic acid was found in the metabolites from protocatechuic acid by crude cell-free extracts of the yeast *Rhodotorula rubra* IFO 0892 and 1101.[35,67] Furthermore, 3-carboxymuconolactone (38) was converted into 3-oxoadipic acid by *Neurospora* species.[60] However, both chemically and enzymically prepared 3-carboxymuconolactone (38) could not be converted into 3-oxoadipic acid by bacterial extracts.[58, 60] Again, this stark contrast between the *Neurospora* fungi and bacteria was found to be a characteristic of all fungal genera studied.[59]

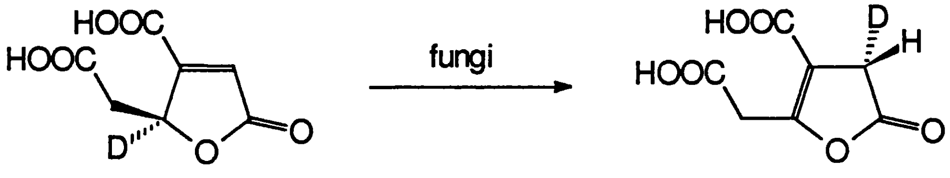
3-Carboxy-4-carboxymethyl-4-hydroxycrotonolactone hydrolase, responsible for the conversion of 3-carboxymuconolactone (38) to 3-oxoadipic acid in *Aspergillus niger*, was purified 250-fold.[68] 4-Carboxymuconolactone (37) did not act as substrate, but 3-oxoadipate enol lactone (21) and muconolactone (20) were hydrolysed slowly. All the lactones acted as competitive inhibitors. Evidence suggested an active-site thiol group in the enzyme and the presence of a tightly bound divalent metal ion.

Thus the product of 3-carboxymuconolactone metabolism by fungal extracts was 3-oxoadipic acid, and evidence for a CoA-bound derivative of this metabolite was found during further metabolism of the keto acid.[59]

A strain of *Penicillium* formed pyruvic acid (17) from protocatechuic acid (2), but not via 3-oxoadipic acid (22), suggesting the existence also of a *meta*-type ring cleavage among fungi.[59]

In 1979, a paper by Hill *et al*[68] showed with deuterium-labelling studies that the conversion of 3-carboxymuconolactone to 3-oxoadipic acid, catalyzed by a cell-free preparation from *Neurospora crassa*, involved an intramolecular 1,3-suprafacial hydrogen-shift consistent with an initial, allylic isomerization of the substrate (Scheme 17). The stereochemical course of the reaction catalyzed by 4-carboxymuconolactone decarboxylase was also determined.[69]

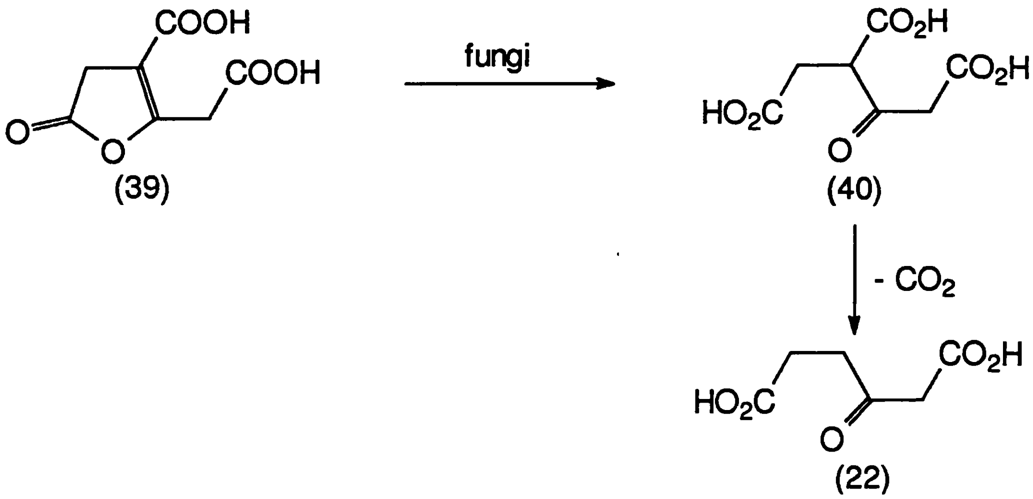
Scheme 17



(42) (43)

This was confirmed when the degradation of 3-carboxymuconolactone (38) to 3-oxoadipic acid by the multifunctional enzyme complex of *Neurospora* was studied. The overall transformation was found from deuterium labelling studies to involve an intramolecular, suprafacial 1,3-shift of hydrogen from C-4 in the lactone to C-5 in the oxoadipic acid.[62] Thus, it was demonstrated that the pathway in fungi involves the formation of 4-carboxy-3-oxoadipic acid enol lactone (39), by the 1,3-hydrogen migration, and subsequent hydrolysis of this lactone gives the 4-carboxy-3-oxoadipic acid (40). It is at this point in the fungal pathway that decarboxylation occurs, yielding 3-oxoadipic acid (22) (Scheme 18).

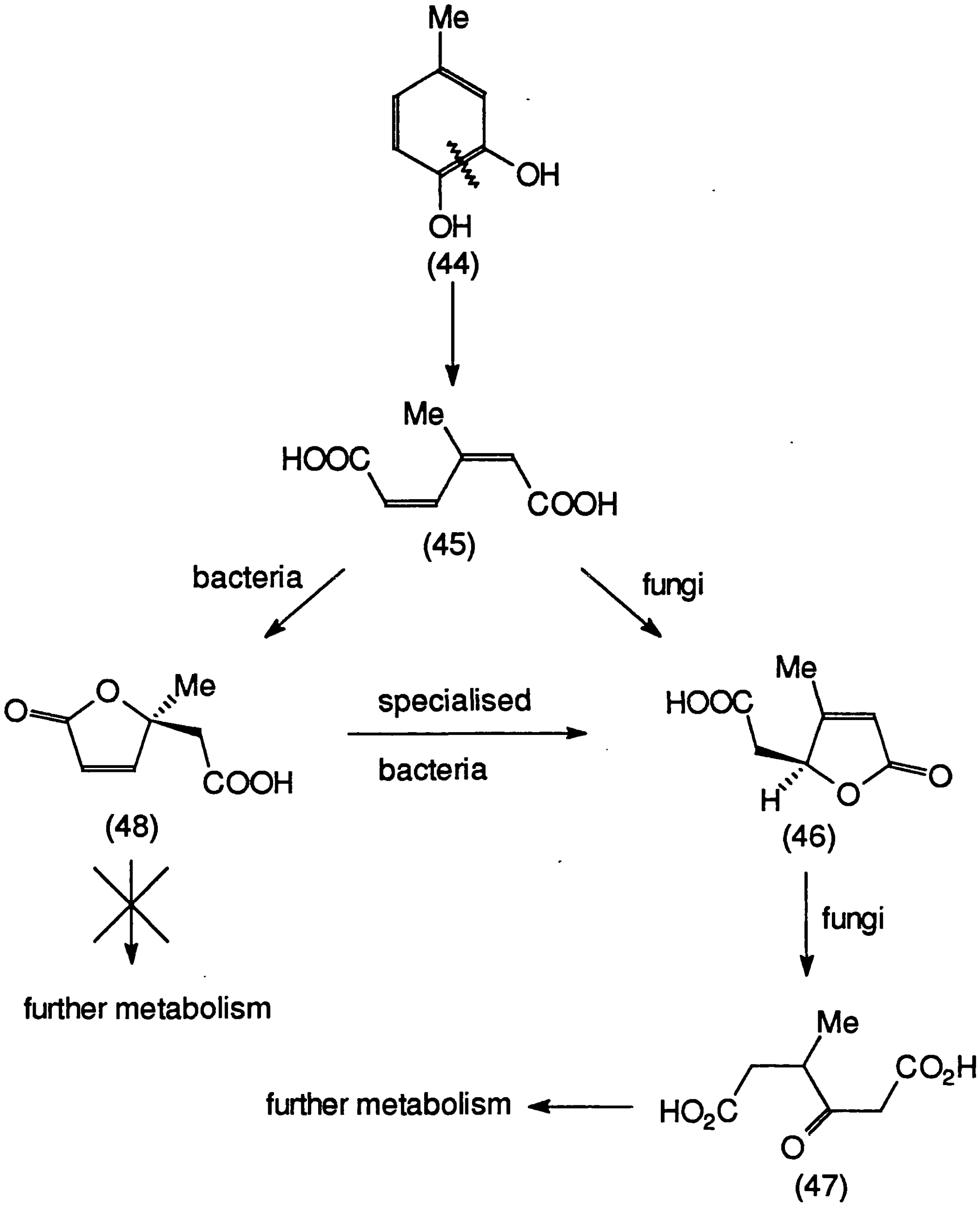
Scheme 18



## 1.5 *Ortho* Cleavage Of Methylpyrocatechols

*Ortho*-cleavage of 4-methylpyrocatechol (44) involves initial conversion of 4-methylpyrocatechol (44) into 3-methyl-*cis,cis*-muconic acid (45). In fungi, cyclisation occurs giving 3-methylmuconolactone (46) (Scheme 19). This lactone undergoes isomerisation and hydrolysis to 4-methyl-3-oxoadipic acid (47), which is metabolised further. In bacteria, cyclisation of (45) occurs giving 4-methylmuconolactone (48) (Scheme 19). This lactone cannot undergo isomerisation like that of muconolactone (20) or 4-carboxymuconolactone (37). Thus, it is resistant to further metabolism. However, certain specialised bacteria have acquired the capability to convert the bacterial lactone (48) into the fungal lactone (46), thereby overcoming the block to further metabolism. Details of these different pathways in bacteria and fungi are outlined in this section.

Scheme 19

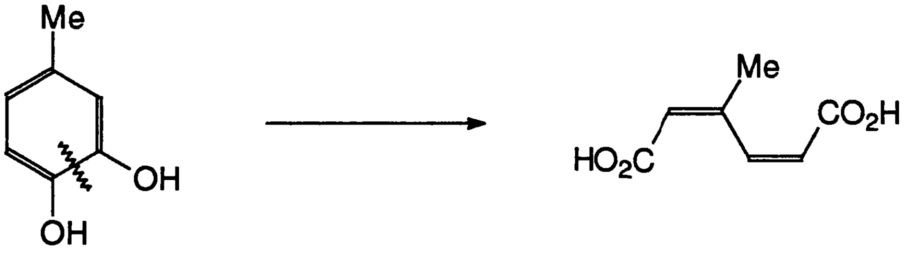


## 1.5.1 Formation of Methylpyrocatechols and Methylmuconic Acids

Substituted muconic acids were prepared from the corresponding pyrocatechols using pyrocatechase II from *Pseudomonas* species B13.[70]

Pyrocatechol-1,2-oxygenase was isolated from *Gordona rubra* N657 and incubated with 4-methylpyrocatechol (44) to form 3-methyl-*cis,cis*-muconic acid (45) (Scheme 20).[38]

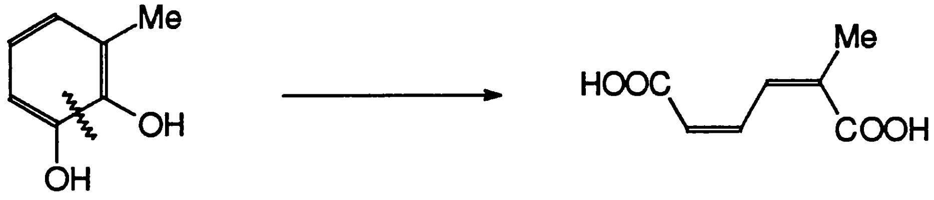
**Scheme 20**



(44) (45)

4-Methylpyrocatechol was detected as a metabolite from the metabolism of cresols by *Trichosporon cutaneum* WY 2-2, suggesting that an additional hydroxy group was introduced into the aromatic ring without oxidation of the methyl group itself.[71] WY 2-2 possessed a dioxygenase enzyme which had the same substrate specificity irrespective of the growth substrate used, and which converted 4-methylpyrocatechol (44) into 3-methylmuconic acid (45) (Scheme 20) and 3-methylpyrocatechol (49) into 2-methylmuconic acid (50) (Scheme 21).[72] Several other *Trichosporon* and *Candida* strains possessed a pyrocatechol-1,2-dioxygenase which in some cases showed higher specificity for 4-methylpyrocatechol than for pyrocatechol.

Scheme 21



(49) (50)

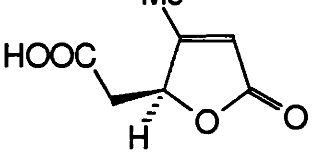
Enzymes in *Nocardia rubra* N75 also metabolized 4-methylpyrocatechol and 3-methyl-*cis,cis*-muconic acid.[38]

The deuteromycetous fungus *Penicillium frequentans* Bi 7/2 produced 3-methylmuconic acid from substrates such as phenol, *p*-cresol, anisole and benzoic acid, but the oxidation appeared unspecific.[41]

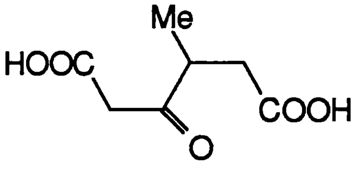
## 1.5.2 Formation of Methylmuconolactones

Similar to the regiochemical difference in cyclisation of 3-carboxy-*cis,cis*-muconic acid, fungi and bacteria produce regioisomeric lactones from methyl-substituted muconic acids.

In 1966, Erlebach[73] noted that biological decomposition of 3-methylmuconic acid at pH 5 gave a variety of organic acids. Further to this, various microbial catabolites were isolated from *Trichosporon cutaneum* including (4*S*)-(-)-3-methylmuconolactone (46) and (-)-3-oxo-4-methyladipic acid (47).[74]



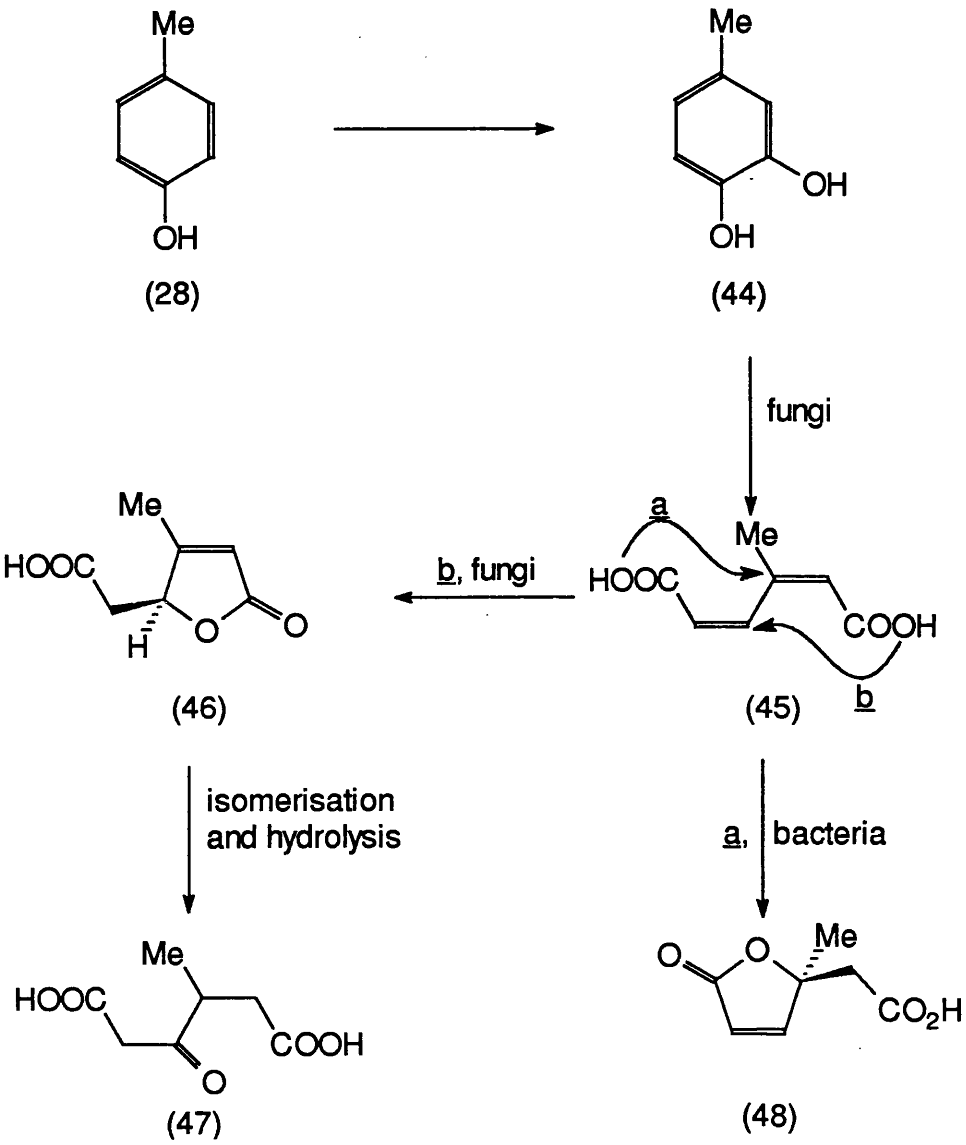
(46)



(47)

A notable feature of this sequence is the formation of 3-methylmuconolactone (46), which was readily metabolized. This was in contrast to the analogous reaction in bacteria giving the dead-end compound 4-methylmuconolactone (48), which could not be enzymically degraded and at the time was thought to render the 3-oxoadipate pathway unavailable for methyl-substituted bacterial sources of carbon that are catabolized by way of 4-methylpyrocatechol (Scheme 22). For example, *ortho* cleavage of the intermediate 4-methylpyrocatechol led to 4-methylmuconolactone as dead-end product in cells of *Bacillus pumilus*. This bacterium was also able to cooxidize *m*- as well as *o*-cresol to 3-methylpyrocatechol, which underwent *ortho* cleavage to the dead-end product 2-methylmuconolactone. After adaptation of the cells to *m*- and *o*-cresol, 3-methylpyrocatechol underwent *meta* instead of *ortho* cleavage.[75] Utilisation of *p*-cresol as sole carbon source occurred by the oxidation of the methyl substituent and *ortho* cleavage of the resulting protocatechuic acid (Section 1.4).

**Scheme 22**



In some cases, bacteria are able to produce both 3- and 4-methylmuconolactones. Thus, the product formed from 4-methylpyrocatechol by enzymes in *Nocardia rubra* N75 was a mixture of these isomeric lactones.[38] In 1985, Pieper *et al*[76] showed that *Alcaligenes eutrophus* JMP134 produced both 4-methylmuconolactone (48) and 3-methylmuconolactone (46) as products of the cyclisation of

3-methylmuconic acid (45). 4-Methyl-3-oxoadipic acid (47) was also identified, as the hydrolysis product of 3-methylmuconolactone. 3-Methyl-*cis,cis*-muconic acid from *Pseudomonas* species B13 was also found to cycloisomerize non-enzymically in slightly acidic solutions.[70]

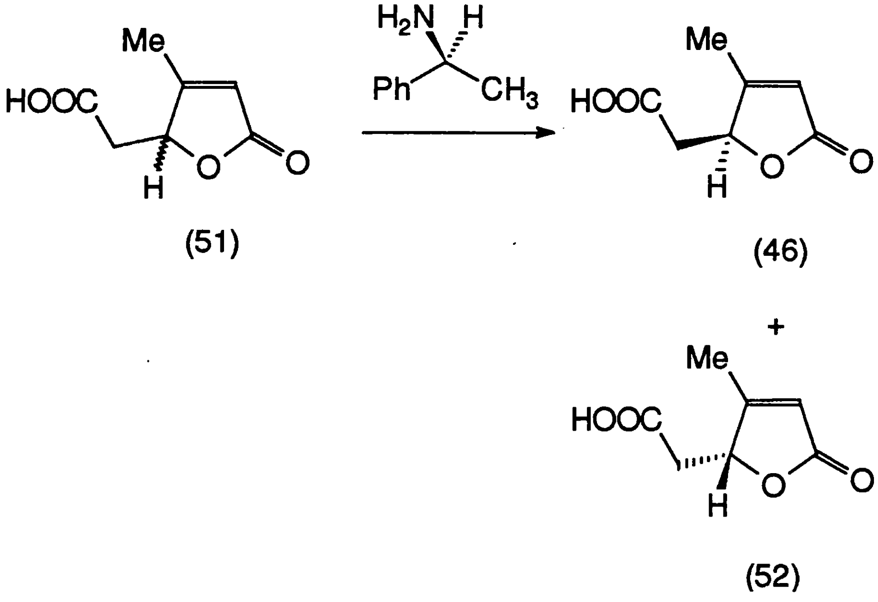
The formation of 4-methyl-3-oxoadipic acid (47) from 4-methylpyrocatechol (44) was investigated by Bruce and Cain[77] in *Rhodococcus ruber* N75, *Rhodococcus corallinus* N657, *Rhodococcus rhodochrous* N5, and *Rhodococcus* strains BCN1, BCN2,and 4PH1.

### 1.5.3 Stereochemistry of the 3-Methyl-*cis,cis*-muconic Acid Cyclisation

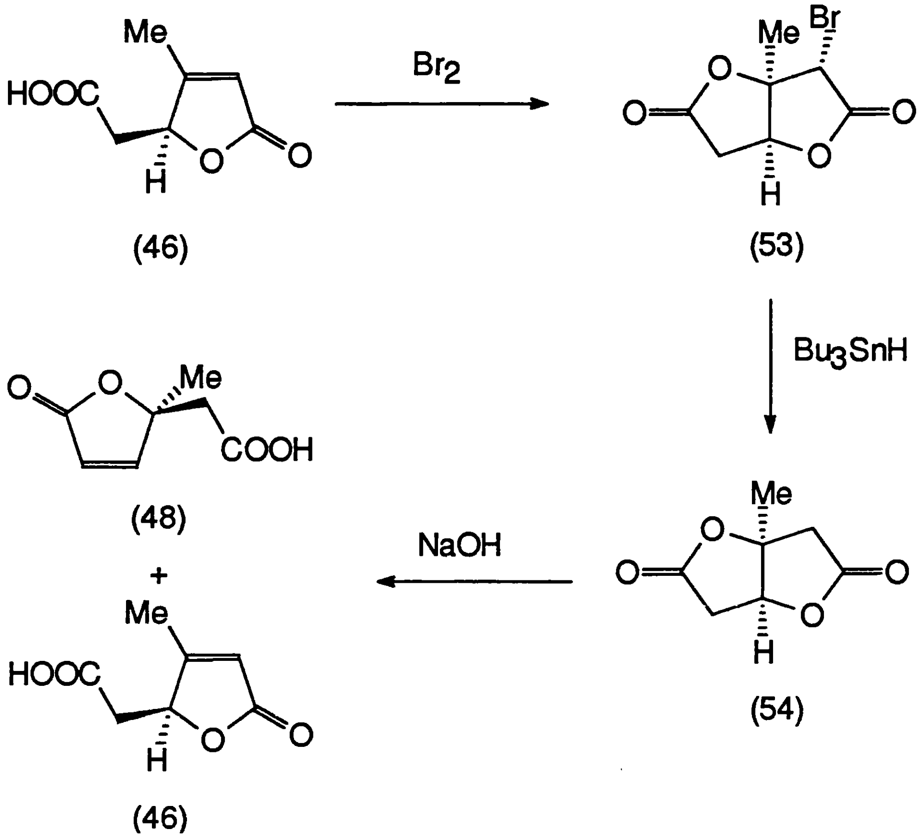
In 1989 Kirby and co-workers[78] demonstrated that the enzyme-catalyzed cyclization of 3-methyl-*cis,cis*-muconic acid (45) proceeded by *syn* addition of carboxyl groups to double bonds to form (4*S*)-3-methylmuconolactone (46) in the fungus *Aspergillus niger* and (4*S*)-4-methylmuconolactone (48) in the bacterium *Pseudomonas putida* (Scheme 22).

To establish the absolute configuration of each lactone, this group first resolved synthetic (±)-3-methylmuconolactone (51) via fractional crystallisation of the (*S*)-(-)-1-phenylethylammonium salts (Scheme 23).[79, 80] X-Ray crystallographic analysis of these salts established the absolute configuration of each enantiomeric 3-methylmuconolactone. The (*S*)-(-)-3-methylmuconolactone (46) was then shown to be identical to the lactone from the fungus *Aspergillus niger*. To establish the absolute configuration of the bacterial 4-methyl lactone (48), the (*S*)-(-)-3-methylmuconolactone (46) was converted with bromine into the bromo dilactone (53). This was reduced with tributyltin hydride to give the (-)-1-methylmuconodilactone (54) (Scheme 24). With aqueous sodium hydroxide, the dilactone (54) gave (*S*)-(+)-4-methylmuconolactone (48), which was found to be identical to the lactone from bacteria, together with (*S*)-(-)-3-methylmuconolactone. The absolute configuration of the bromo dilactone (53) was determined by X-ray diffraction, exploiting the bromine as a heavy atom, thereby confirming the absolute configurations of both the fungal (46) and bacterial (48) muconolactones.[80]

**Scheme 23**

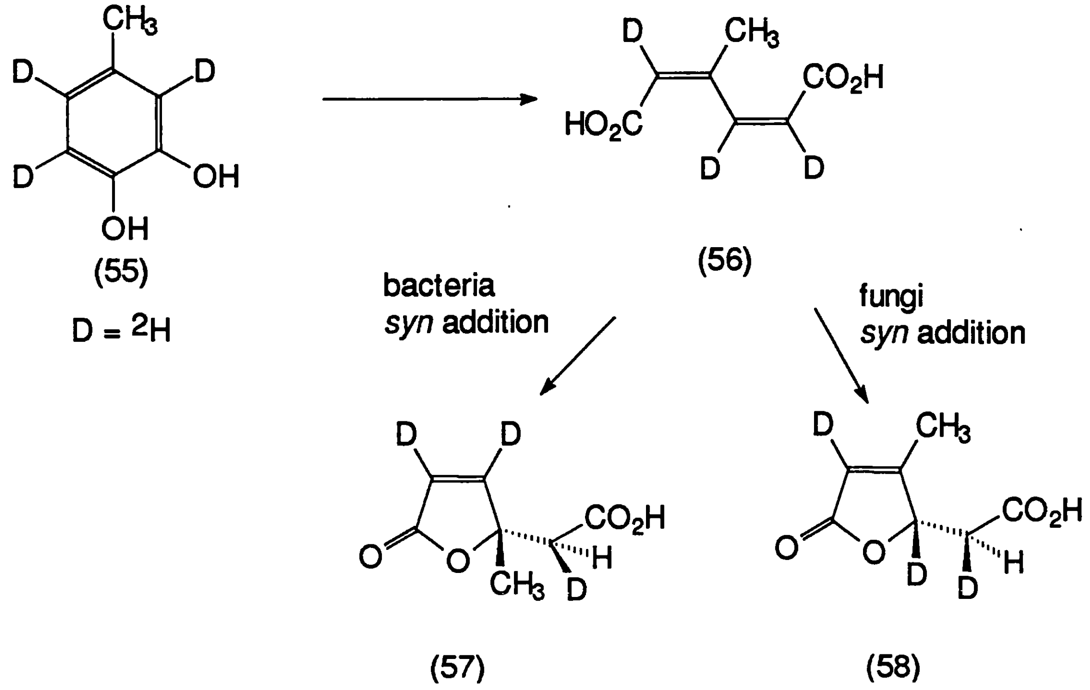


**Scheme 24**



Kirby and co-workers [47] demonstrated in experiments with the deuteriated 4-methylpyrocatechol (55) that the product of its enzymic oxygenation, deuteriated 3-methyl-*cis,cis*-muconic acid (56), underwent enzymic cyclization by *syn* addition of carboxy groups to distal double bonds to form the (*S*)-4-methylmuconolactone (57) in the bacterium *Pseudomonas putida* and the (*S*)-3-methylmuconolactone (58) in the fungus *Aspergillus niger* (Scheme 25). Similarly, *syn* cyclization of the unsubstituted *cis,cis*-muconic acid was shown to occur in *Aspergillus niger* to form the (*S*)-muconolactone (Section 1.3).

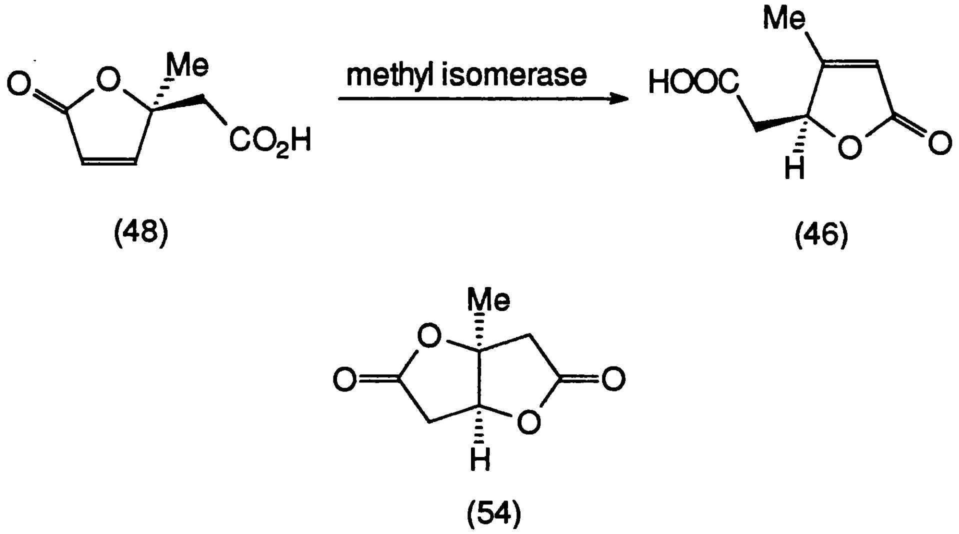
Scheme 25



### 1.5.4 Isomerisation of 4-Methylmuconolactone to 3-Methylmuconolactone

The conversion of 4-methylmuconolactone (48) to 3-methylmuconolactone (46) by a new enzyme from *Rhodococcus* species was described (Scheme 26).[77] The novel enzyme 4-methylbut-2-enelactone methyl-isomerase was detected in, and purified to electrophoretic homogeneity from, *Rhodococcus rhodocrous* N75, a nocardioform actinomycete, by Bruce *et al.*[81] The new isomerase was highly specific with respect to its lactone substrate. Of the methyl-substituted lactones, (4*S*)-(+)-4-methylmuconolactone (48) and a possible isomerization reaction intermediate (-)-1-methyl-3,7-dioxo-2,6-dioxabicyclo[3.3.0]octane (54) were accepted as substrates. Only the (4*S*)-(-)-3-methylmuconolactone (46) was produced. Some other lactone analogs acted as competitive inhibitors. Kinetic data showed that dilactone (54) was isomerised faster than the lactone (48). It was suggested that the isomerization did not involve actual methyl migration, but proceeded via the 1-methyldilactone (54).

Scheme 26



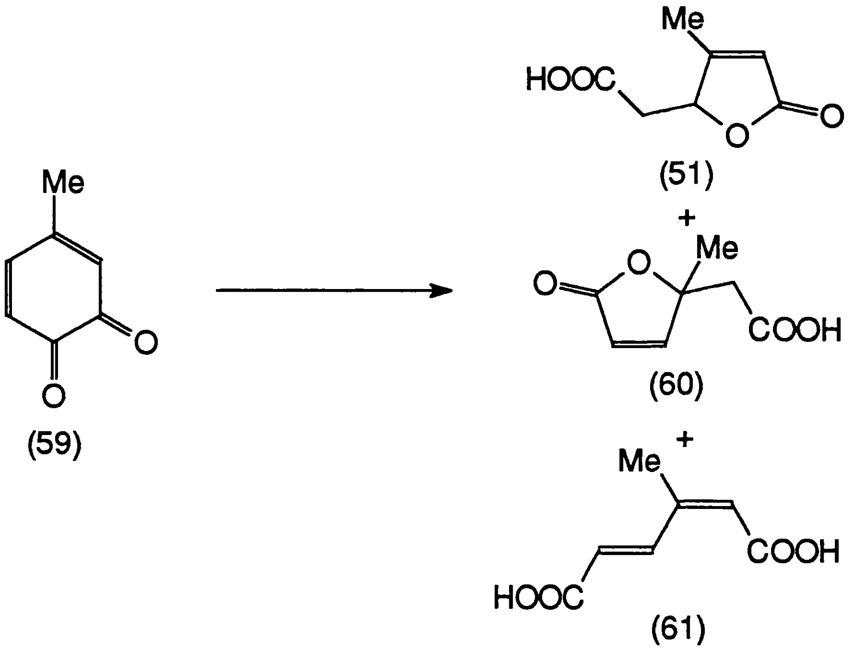
In an independent study, a similar lactone isomerase, transforming the lactone (48) into the lactone (46), was purified from *Pseudomonas* species B13 derivative FR1, carrying the plasmid-encoded isomerase gene cloned from *Alcaligenes eutrophus* JMP 134.[82] Again, in addition to 4-methylmuconolactone, the dilactone (54), was a substrate for the enzyme, but this time kinetic data showed that it could not be a free reaction intermediate. Thus, the monolactone (48) was transformed faster than the dilactone (54) into the product (46). Isomeric, methyl-substituted muconolactones were neither substrates nor inhibitors.[82]

### 1.5.5 Non-Enzymic Formation of 4-Methylpyrocatechol Metabolites

Several of the previously-mentioned metabolites have been isolated and their properties studied in chemical oxidations of methyl-substituted phenols, catechols, quinones and derivatives.[80, 83-86.]

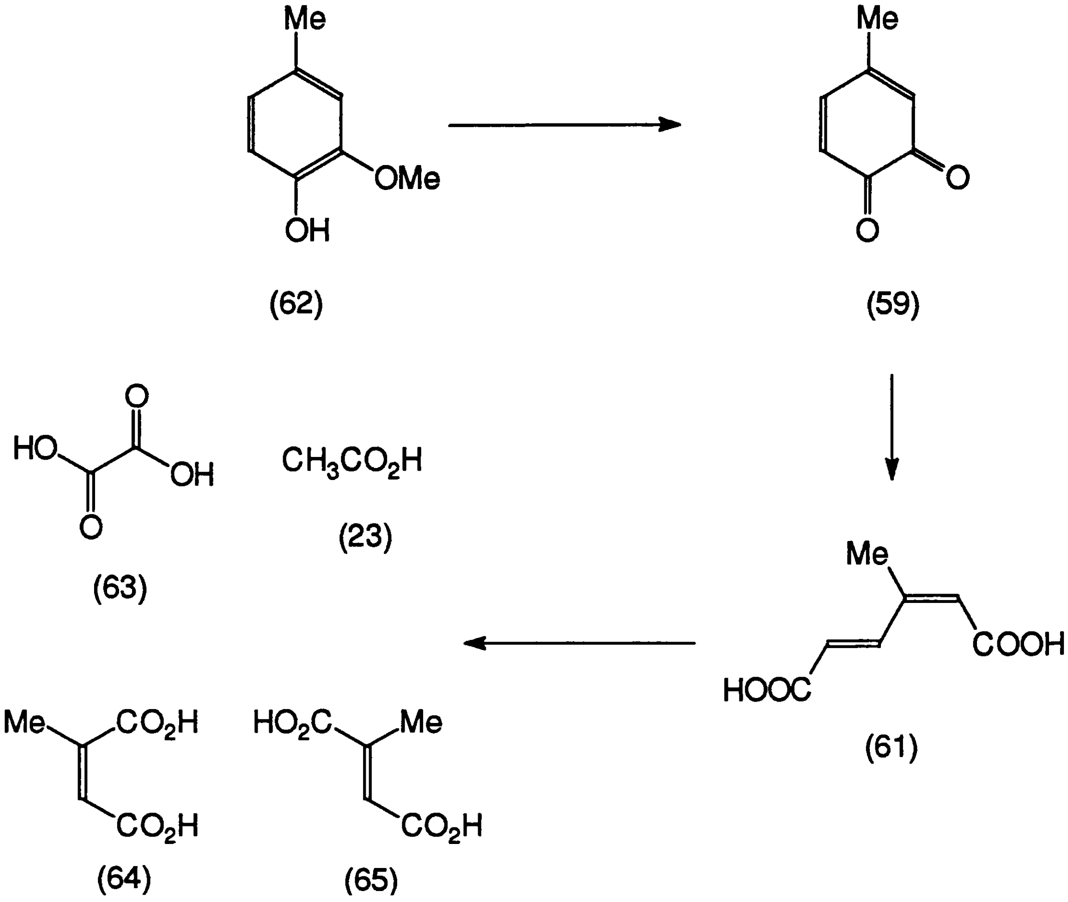
In addition to the syntheses already cited in Section 1.5.3,[80] 3-methylmuconolactone (51) and 4-methylmuconolactone (60) were formed from the peroxyacetic acid-mediated oxidation of *p*-cresol, 4-methylpyrocatechol and 4-methyl-*o*-benzoquinone (59) (Scheme 27).[85] 3-Methyl-2-*cis*-4-*trans*-muconic acid (61) was also identified as a product of these reactions.

**Scheme 27**



2-Methoxy-4-methylphenol (62) was converted into 4-methyl-*o*-benzoquinone (59) by treatment with alkaline hydrogen peroxide.[83] The quinone (59) was further oxidised to 3-methyl-2-*cis*-4-*trans*-muconic acid (61) and eventually converted into oxalic (63), acetic (23), methylmaleic (64) and methylfumaric (65) acids (Scheme 28).

**Scheme 28**

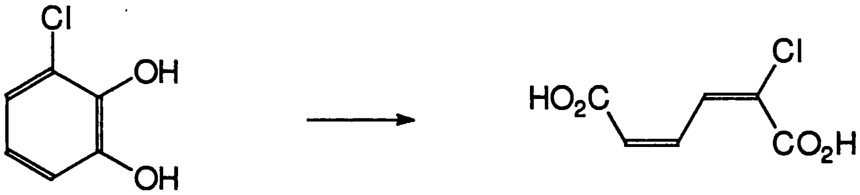


## 1.6 Ortho Cleavage of Halopyrocatechols

### 1.6.1 Formation of Halopyrocatechols and Halomuconic Acids

Several aromatic compounds are converted microbially in the first instance into halogenated derivatives of pyrocatechol. In the study of the degradation of chloropyrocatechols, it was found that the cleavage of the benzene ring of some derivatives was mediated by 1,2-dioxygenases from bacteria.[87-89] These enzymes were specific for mono- and dichloropyrocatechols. *Ortho*-cleavage of 3-chloropyrocatechol (66) gave 2-chloro-*cis,cis*-muconic acid (67) (Scheme 29), while 4-chloropyrocatechol (68) gave 3-chloro-*cis,cis*-muconic acid (69) (Scheme 30).[70] Enzymes purified from *Arthrobacter* species also converted 4-chloropyrocatechol into 3-chloro-*cis,cis*-muconate.[90]

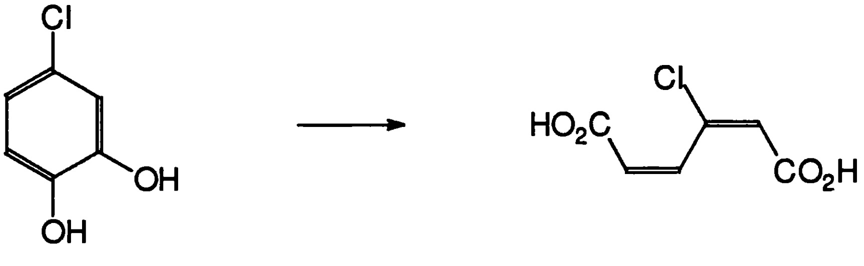
Scheme 29



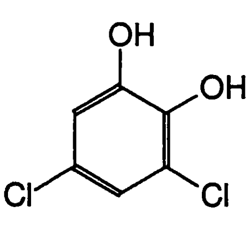
(66) (67)

3-Chlorophenol and 4-chlorophenol were degraded by a yeast strain, *Rhodotorula glutinis*, with a stoichiometric release of chloride ions. 4-Chloropyrocatechol was observed as a product of the degradation.[40] 2-Chlorophenol was degraded less.

**Scheme 30**

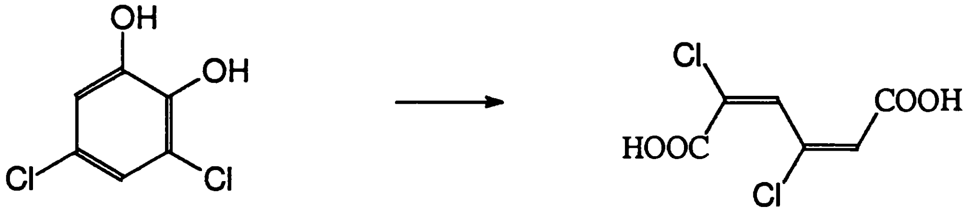

(68) (69)

In *Pseudomonas* species B13, two separate dioxygenase enzymes appeared to be induced for the assimilation of pyrocatechols and chloropyrocatechols. Pyrocatechase I was specific for the cleavage of pyrocatechol, while pyrocatechase II cleaved chloropyrocatechols.[88, 89] Substituted muconic acids were prepared from the corresponding pyrocatechols by pyrocatechase II from *Pseudomonas* species B13 for comparison of their stabilities under different pH conditions.[70] The enzyme was more active for 4-chloropyrocatechol than 3-chloro- and 3,5-dichloro-pyrocatechol (70). Both enzymes were found in *Pseudomonas* derivatives and in *Alcaligenes* species.[91, 92]


(70)

Cell-free extracts from *Pseudomonas* species strain B13 were found to completely degrade 3,5-dichlorocatechol (70). The proposed route of assimilation involved cleavage to 2,4-dichloro-*cis,cis*-muconic acid (71) (Scheme 31).[93] Enzymes from cell-free extracts of *Pseudomonas* species strain NCIB 9340[94] effected the transformation of (70) to (71), as did enzymes from *Arthrobacter*.[95]

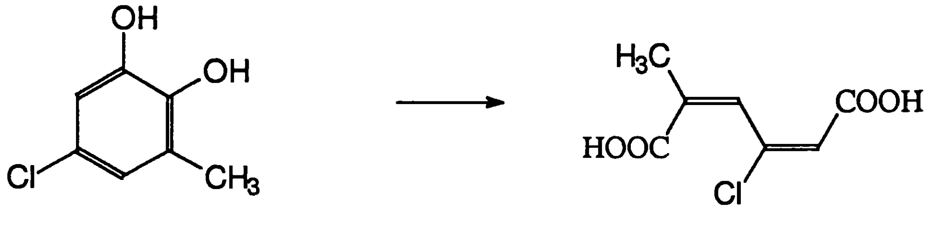
**Scheme 31**



(70) (71)

Similarly, cell-free extracts of *Pseudomonas* species strain NCIB 9340 gave the 2-methyl-4-chloromuconic acid (73) from 3-methyl-5-chloropyrocatechol (72) (Scheme 32).[94]

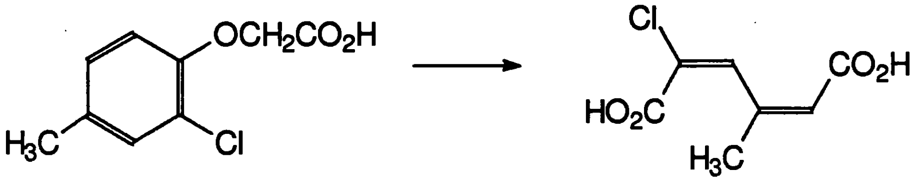
**Scheme 32**



(72) (73)

2-Chloro-4-methylphenoxyacetic acid (74) was not a growth substrate for *Alcaligenes eutrophus* JMP 134 and JMP 134-1, but it was transformed enzymically into 2-chloro-4-methyl-*cis,cis*-muconic acid (75) (Scheme 33).[96]

**Scheme 33**



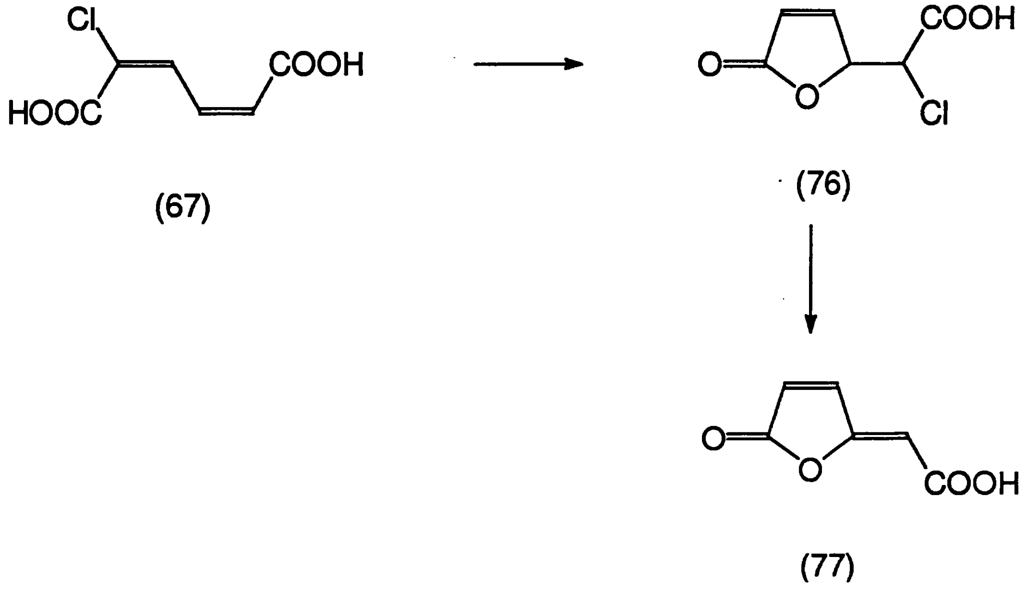
(74) (75)

Halophenols have also been co-metabolically degraded and partly dehalogenated by the deuteromycetous fungus *Penicillium frequentans* Bi 7/2.[41]

### 1.6.2 Formation of Halomuconolactones

3-Substituted *cis,cis*-muconic acids cycloisomerized readily in slightly acidic solutions to give the corresponding 3-substituted muconolactones.[70] However, enzymic cyclisation of the two regioisomers of chloromuconic acid gave geometrical isomers of 4-carboxymethylene-but-2-en-4-olide. With *Pseudomonas* species strain B13, 2-chloro-*cis,cis*-muconic acid (56) yielded *trans*-4-carboxymethylenebut-2-en-4-olide (77), a process thought to occur by *anti*-elimination from 5-chloromuconolactone (76) (Scheme 34).

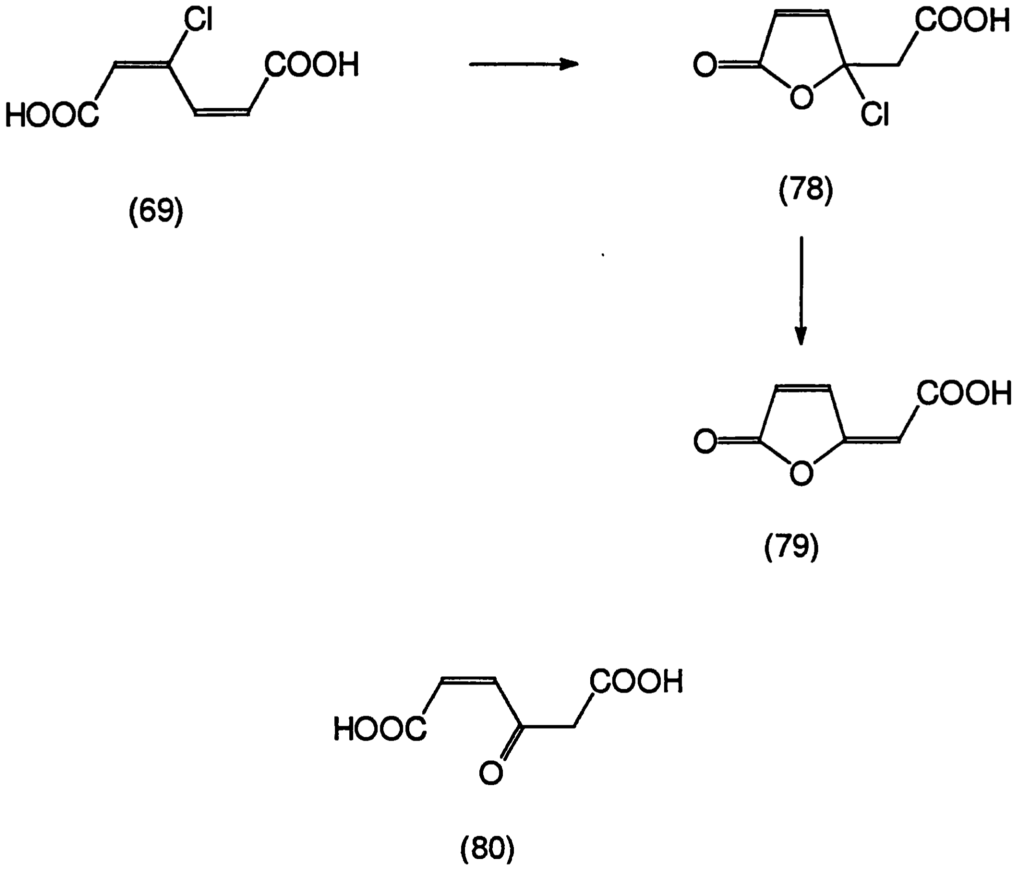
**Scheme 34**



Earlier, 4-chloropyrocatechol had been found to be degraded by bacterial enzymes to 4-carboxymethylenebut-2-en-4-olide.[97] 3-Chloro-*cis,cis*-muconic acid (69) gave *cis*-4-carboxymethylenebut-2-en-4-olide (79), postulated to arise from *anti*-elimination from the initially-formed 4-chloromuconolactone (78) (Scheme 35).

Both *cis*- and *trans*-isomers of the diene lactone (79) were substrates for a hydrolase enzyme which converted them into maleylacetic acid (80).[98] Maleylacetic acid is not a direct entry point into the 3-oxoadipate pathway, but in some cases can be converted into 3-oxoadipic acid or its subsequent metabolites.[93]

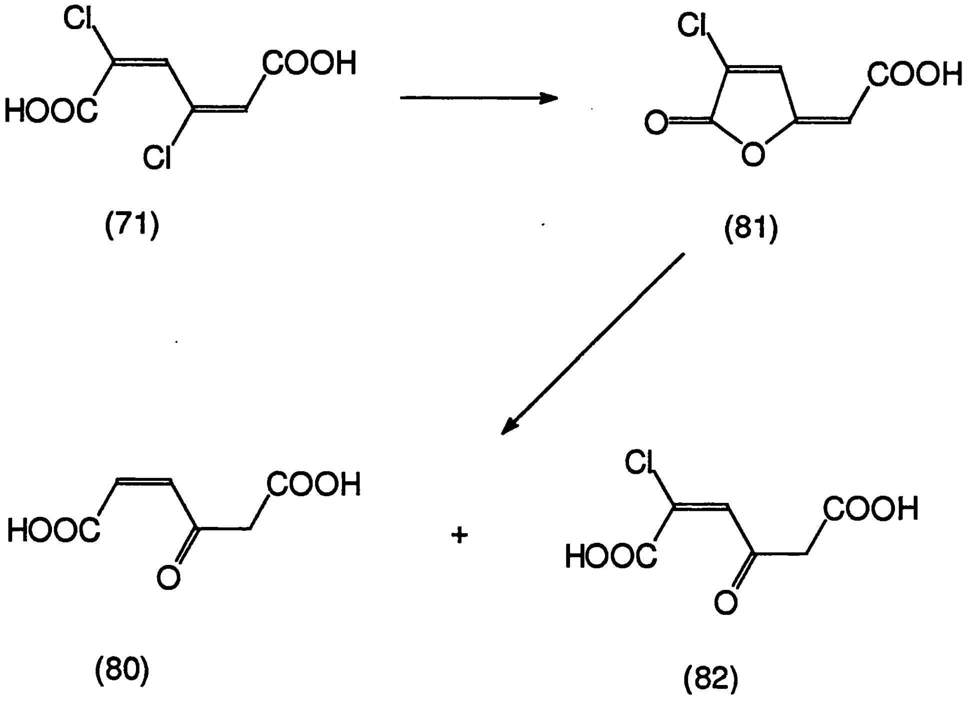
**Scheme 35**



The cyclisation of muconic and chloromuconic acids was also found to be effected by two different enzyme types. Cycloisomerase type I was induced during the degradation of benzoic acid (the precursor of pyrocatechol) and was specific for *cis,cis*-muconic acid itself. Cycloisomerase type II was induced in response to the assimilation of 3-chlorobenzoic acid, and had relaxed specificity, utilising *cis,cis*-muconic acid, 2-chloro-*cis,cis*-muconic acid (67) and 3-chloro-*cis,cis*-muconic acid (69) as substrates. Unlike the pyrocatechases, only one enzyme was present in each bacterial strain.[98] 3-Carboxy-*cis,cis*-muconate cyclase was inhibited by the *cis,cis*-, *cis,trans*- and *trans,trans*- isomers of 3-chloromuconic acid, none of which being a substrate of the enzyme.[99]

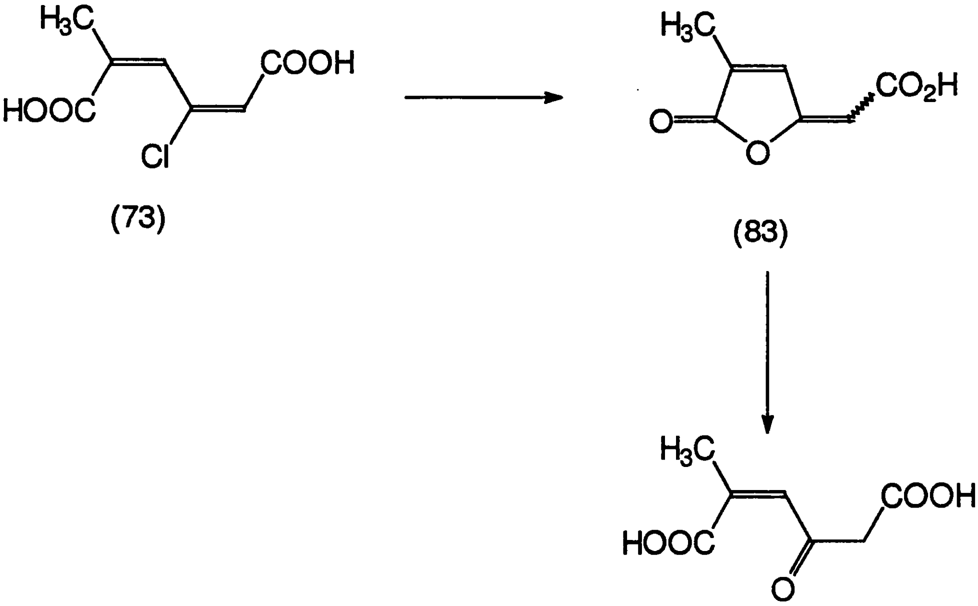
Cell-free extracts from *Pseudomonas* species strain B13 were found to effect cyclisation of 2,4-dichloromuconic acid (71) to *cis*-2-chloro-4-carboxymethylenebut-2-en-4-olide (81),[93] as were cell-free extracts of *Pseudomonas* species strain NCIB 9340[94] (Scheme 36) and a lactonizing enzyme from *Arthrobacter*.[95] Conversion of this product into maleylacetic acid (80) and 2-chloromaleylacetic acid (82) was also observed.[93, 94]

**Scheme 36**



Dichloromuconate cycloisomerase from *Alcaligenes eutrophus* JMP 134 was purified and characterised.[100] The specificity of the enzyme was demonstrated by the observed low activities with *cis,cis*-muconic and monochloro-*cis,cis*-muconic acids, whereas it exhibited high activity with 2,4-dichloro-*cis,cis*-muconic acid (71). All of these chlorinated compounds were found to be biological precursors of both 2-chloromaleylacetic acid (82) and maleylacetic acid (80). Maleylacetic acid was formed by the degradation of 4-chloropyrocatechol by *Pseudomonas* species.[101] Similarly, cell-free extracts of *Pseudomonas* species strain NCIB 9340 converted 2-methyl-4-chloromuconic acid (73) into 2-methyl-4-carboxymethylenebut-2-en-4-olide (83) and 2-methylmaleylacetic acid (84) (Scheme 37).[94]

**Scheme 37**



(73)

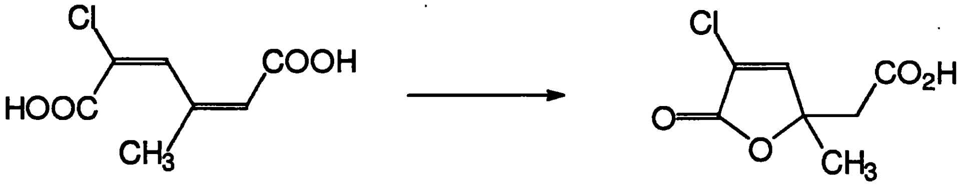
(83)

(84)

2-Chloro-4-methyl-*cis,cis* muconic acid (75) was converted by enzymic cycloisomerization in *Alcaligenes eutrophus* JMP 134 and JMP

134-1 into 2-chloro-4-methylmuconolactone (85), which was a dead-end metabolite (Scheme 38).[96]

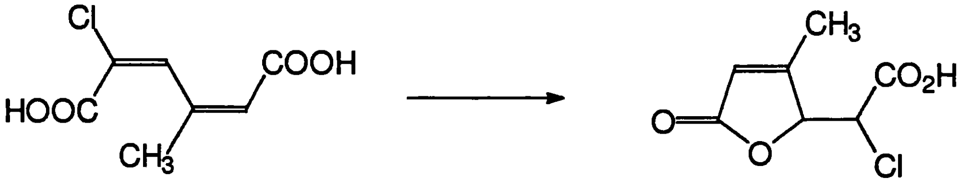
**Scheme 38**



(75) (85)

Non-enzymic cycloisomerization of 2-chloro-4-methyl-*cis,cis*-muconic acid (75) gave both diastereomers of 5-chloro-3-methylmuconolactone (86) (Scheme 39). Lactones with a chloro substituent on the 4-carboxymethyl side chain were stable under both physiological and acidic conditions.[96]

**Scheme 39**

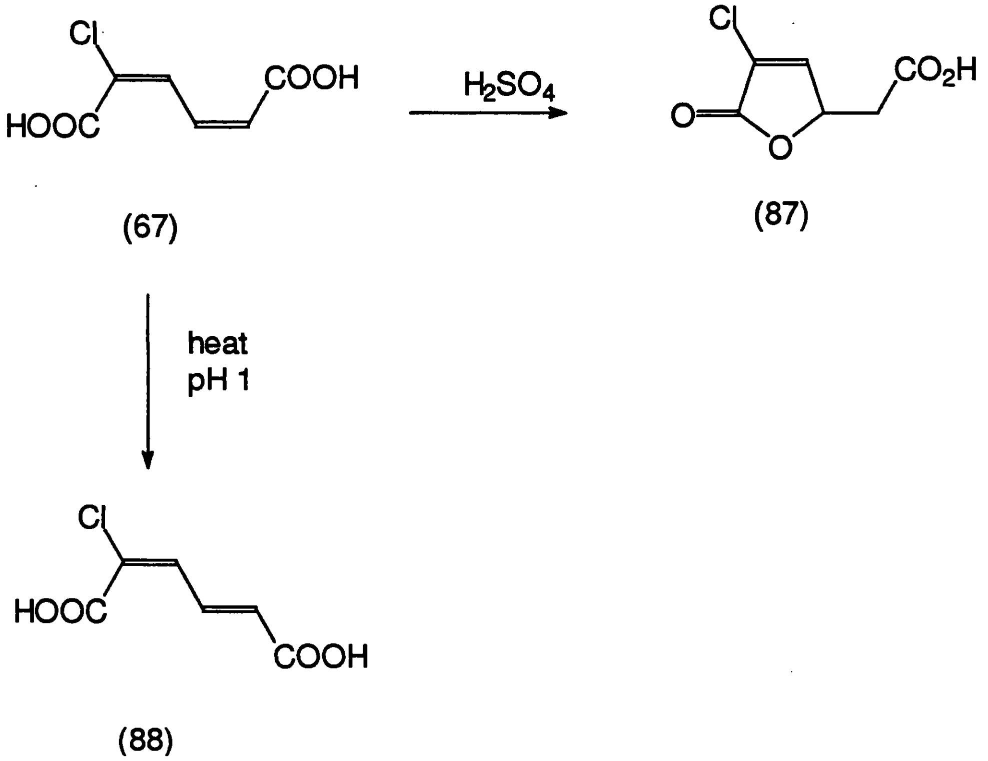


(75) (86)

2-Chloro-*cis,cis*-muconic acid (67) was stable under slightly acidic conditions and was isolated as a crystalline compound. However cycloisomerization did occur in 75% sulphuric acid to yield 2-

chloromuconolactone (87), while isomerisation to the *cis,trans* form (88) occurred with heating at pH 1 (Scheme 40).[70]

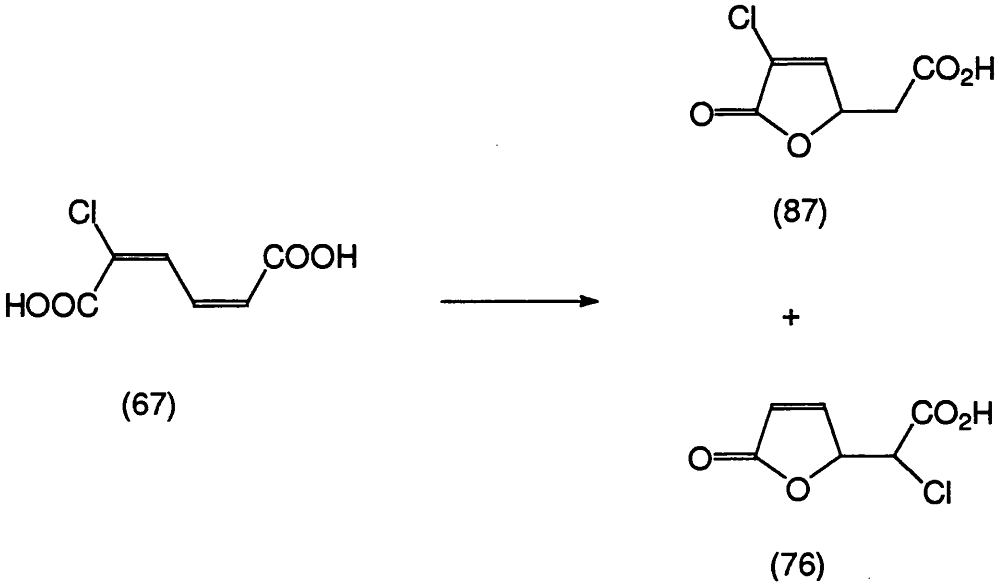
**Scheme 40**



The conversion of 2-chloro-*cis,cis*-muconic acid (67) by muconate cycloisomerase from *Pseudomonas putida* PRS2000, *Acinetobacter calcoaceticus* ADP1 and *Pseudomonas* species strain B13 yielded two products, identified as 2-chloro- (87) and 5-chloro-muconolactone (76) (Scheme 41).[102] Cyclisation by the enzyme from *Pseudomonas putida* gave initially 2-chloromuconolactone (87) as the major product, while 5-chloromuconolactone (76) dominated after prolonged incubation. Both chloromuconolactones were stable at physiological pH.

It was postulated that the chloromuconate cycloisomerases must have evolved the capability to cleave the carbon-chlorine bond during their divergence from normal muconate cycloisomerases, since the former enzymes of *Pseudomonas* species strain B13 and *Alcaligenes eutrophus* JMP 134 had been shown previously to produce the *trans*-dienelactone (77) from 2-chloro-*cis,cis*-muconic acid.[102]

**Scheme 41**

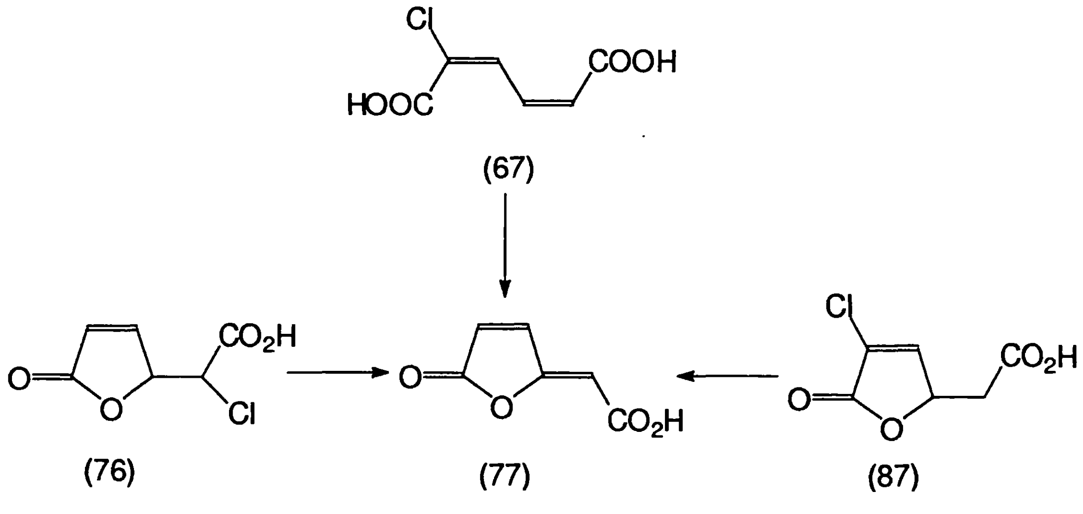


Muconate cycloisomerase and chloromuconate cycloisomerase from *Rhodococcus erythropolis* 1CP cells were purified and characterized.[103] Both enzymes discriminated between the two possible directions of 2-chloro-*cis,cis*-muconic acid (67) cycloisomerization and converted this substrate into 5-chloromuconolactone (76) only (Scheme 41). Unlike the chloromuconate cycloisomerases of gram-negative bacteria, the corresponding *Rhodococcus erythropolis* enzyme was unable to catalyze elimination of chloride from (+)-5-

chloromuconolactone (76). Moreover, in being unable to produce (+)-2-chloromuconolactone (87), the two cycloisomerases of *Rhodococcus erythropolis* 1CP differed significantly from the known muconate and chloromuconate cycloisomerases of gram-negative strains.

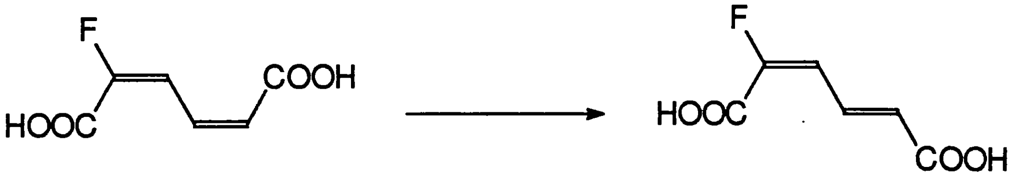
In 1995, Vollmer and Schloemann[104] studied the conversion of 2-chloro-*cis,cis*-muconic acid (67) into the *trans*-diene lactone (*trans*-4-carboxymethylenebut-2-en-4-olide) (77) by purified preparations of the pJP4-, and pAC27-encoded chloromuconate cycloisomerases from *Alcaligenes eutrophus* and *Escherichia coli* DH1, respectively (Scheme 42). (+)-2-Chloro- (87) and (+)-5-chloro-muconolactone (76) also gave *trans*-4-carboxymethylenebut-2-en-4-olide (77) with these enzyme preparations. Thus, in common with other chloromuconate cycloisomerases, the pJP4- and pAC27-encoded versions were able to catalyze chloride elimination from (+)-5-chloromuconolactone. However the ability to eliminate chloride from (+)-2-chloromuconolactone differentiated these enzymes from other groups of cycloisomerases.[104]

**Scheme 42**



2-Fluoro-*cis,cis*-muconic acid (89) was stable under slightly acidic conditions, but was isomerized to the *cis,trans* form (90) by heating at pH 1 (Scheme 43).[70] Also, it was not cycloisomerized by crude extracts or cycloisomerase II preparations from *Pseudomonas*.[70]

**Scheme 43**

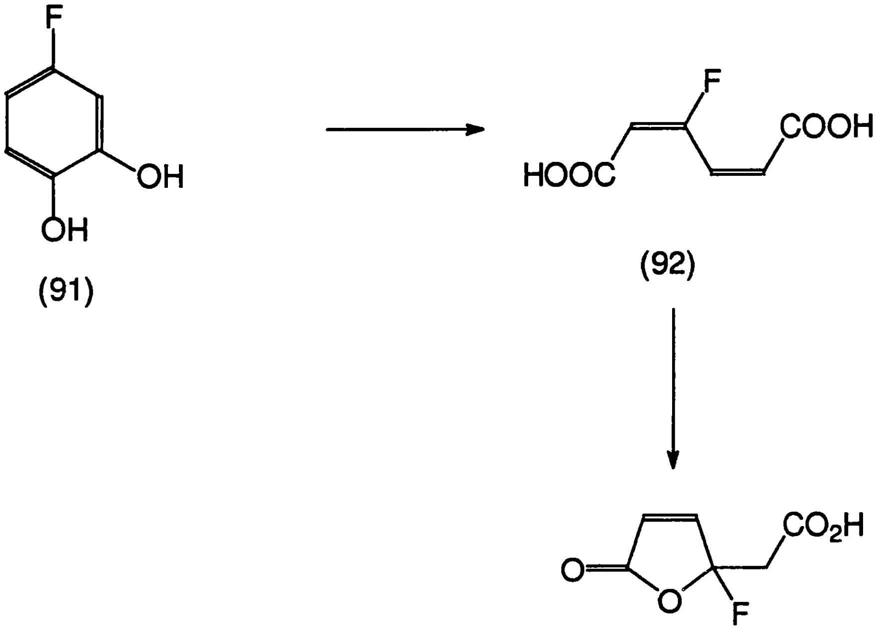


(89)

(90)

However, the simultaneous presence of partially purified preparations of pyrocatechol 1,2-dioxygenase from *Pseudomonas cepacia* and muconate cycloisomerase from *Alcaligenes eutrophus* 335 converted 4-fluoropyrocatechol (91) into (+)-4-fluoromuconolactone (93) (Scheme 44).[105] (+)-4-Fluoromuconolactone (93) was also shown to be the only major product formed from 3-fluoro-*cis,cis*-muconic acid (92) by the action of muconate cycloisomerases from *Alcaligenes eutrophus* 335, *Alcaligenes eutrophus* JMP134, and *Pseudomonas cepacia* as well as by the action of dichloromuconate cycloisomerase from *Alcaligenes eutrophus* JMP134 (Scheme 44). This suggested that dichloromuconate cycloisomerase, like the muconate cycloisomerases, catalyzed primarily a cycloisomerization reaction. Only in the case of chloro- and bromo-substituted substrates was a dehalogenation observed at this stage.[105]

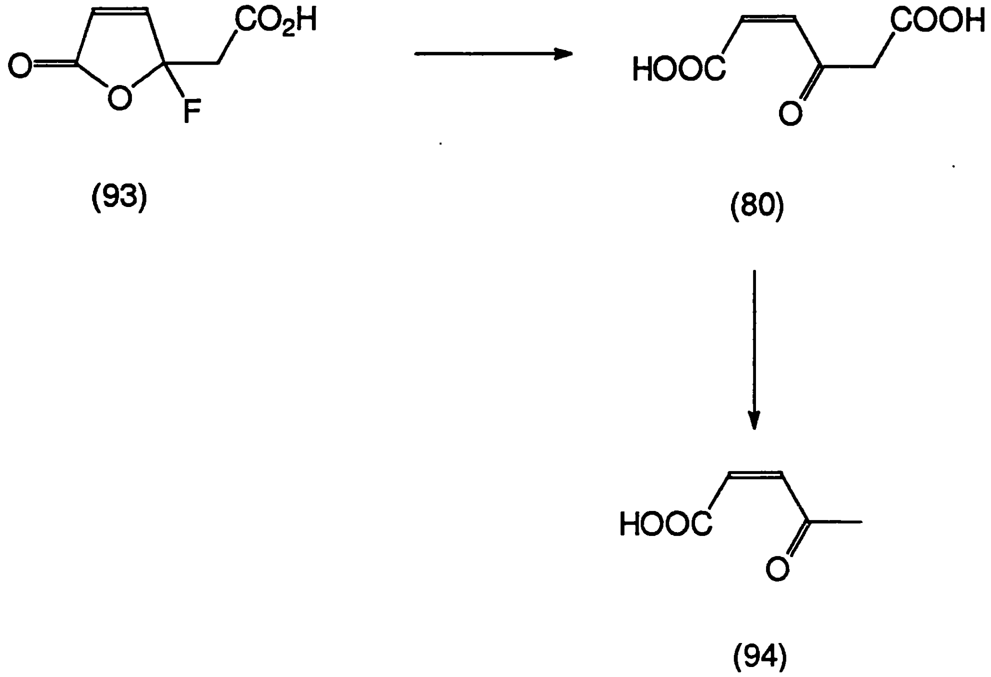
**Scheme 44**



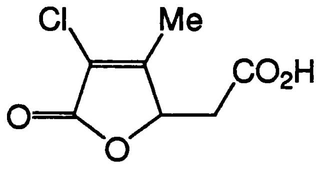
(91) (92) (93)

4-Fluoromuconolactone (93) decomposed non-enzymically at pH 7, mainly to maleylacetic acid (80), which then decarboxylated to give *cis*-acetylacrylic acid (94) (Scheme 45). Significant amounts of an unidentified compound were also formed, but hydrogen fluoride elimination to yield the two, isomeric diene lactones was negligible.[105] However, the non-enzymic reactions proceeded slowly and an enzymic conversion of 4-fluoromuconolactone *in vivo* was assumed. Participation of diene lactone hydrolases in this reaction was indicated by their induction during growth of various strains on 4-fluorobenzoic acid. However, experiments with cell extracts of *Pseudomonas putida* A3.12 suggested that at least one other hydrolytic enzyme was able to contribute to the 4-fluoromuconolactone conversion.[105]

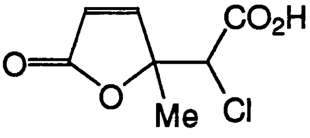
**Scheme 45**



Other halogenated muconolactones have been less well studied. It was found that 2-chloro-3-methylmuconolactone (95) was dechlorinated within 8 days in sludge from a lignin bleachery, whereas 4-methyl-5-chloromuconolactone monomethyl ester (96) did not undergo biological dechlorination, perhaps because it structurally-similar to 4-methylmuconolactone (50), a dead-end intermediate of the bacterial *ortho*-cleavage pathway.[106]



(95)

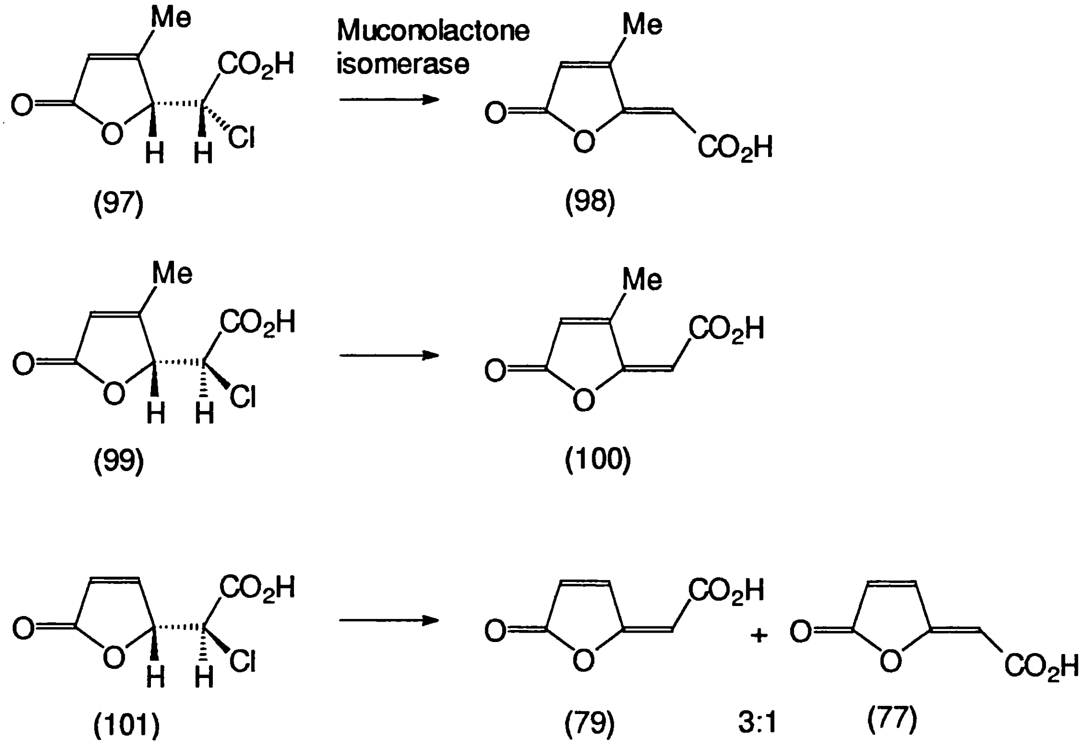


(96)

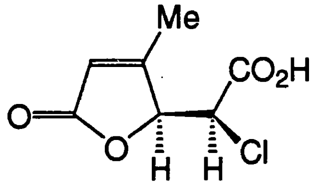
### 1.6.3 Stereochemistry of Halomuconolactones

Muconolactone isomerase of *Alcaligenes eutrophus* JMP 134, which normally catalyses isomerisation to the enol lactone (21) (see Section 1.3.3), catalyzed dechlorination of (4*R*,5*R*)- (97) and (4*R*,5*S*)-5-chloro-3-methyl-muconolactone (99) and (4*R*,5*S*)-5-chloromuconolactone (101) to give principally the 3-methyl-*trans*- (98) and 3-methyl-*cis*-diene lactone (100) and a 3:1 mixture of *cis*- (79) and *trans*-diene lactones (77), respectively.[50] The major products all arose by *syn*-elimination of HCl (Scheme 46).[107] The stereochemistry of the four stereoisomers of 5-chloro-3-methylmuconolactone was deduced from NMR data, stability data, and from comparison with authentic (4*R*,5*S*)-5-chloromuconolactone.[107]

Scheme 46



The enzyme was proposed to have a structure similar to that of muconolactone isomerase of *Pseudomonas putida* and a high degree of sequence similarity with other muconolactone isomerases was observed. It had a high activity with (4*S*)-muconolactone, although (4*R*,5*S*)-5-chloromuconolactone (101) was the preferred substrate. The (4*S*,5*S*)-lactone (102) was an inhibitor of (4*R*,5*R*)-5-chloro-3-methylmuconolactone (97) dehydrochlorination. Methyl substitution of the substrate resulted in a higher affinity for the enzyme, but a drastically lower velocity, resulting in a lower specificity constant.[50]



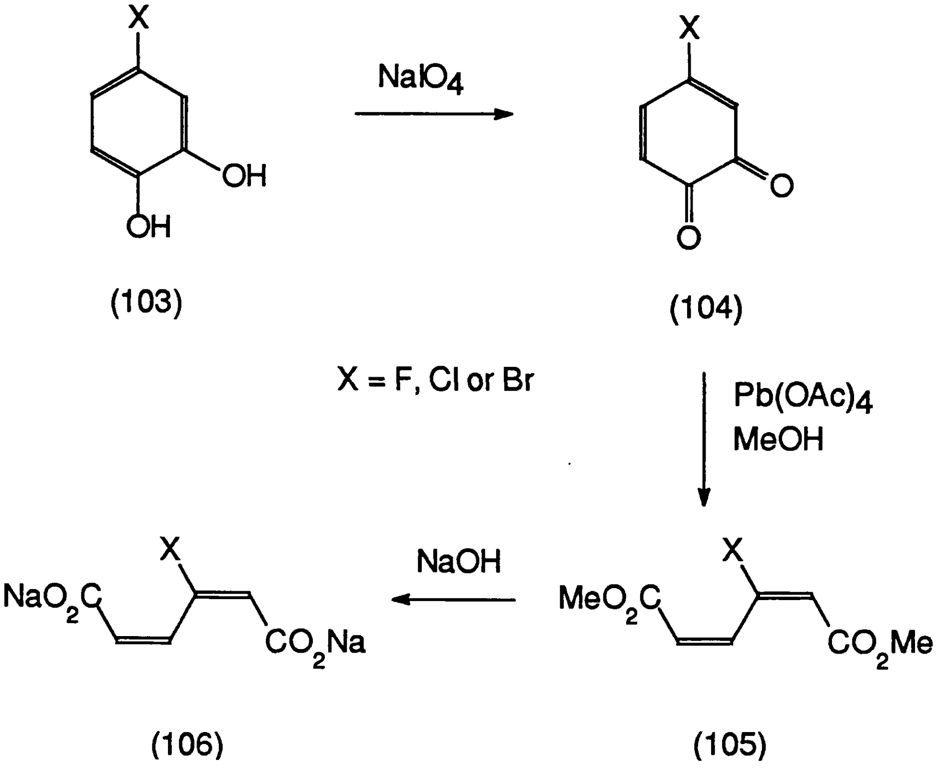
(102)

The absolute stereochemical courses of the *cis,cis*-muconic acid lactonizing enzyme from *Trichosporon cutaneum* and the chloromuconic acid cycloisomerase from *Pseudomonas* species B13 have also been determined.[45] The regiochemistry of cyclization of 3-halo-*cis,cis*-muconic acids by the enzyme from *Trichosporon cutaneum* and by the enzyme from *Pseudomonas* species B13 have been shown to differ in a halogen substituent-dependent manner, suggesting a different active site architecture of the two enzymes. The results parallelled those observed by Pieken and Kozarich[108] for the nonenzymic lactonization of the 3-halomuconates at pH 1-6 and in concentrated HCl, respectively (Section 1.6.4).

### 1.6.4 Non-Enzymic Formation of Halomuconic Acids and Halomuconolactones

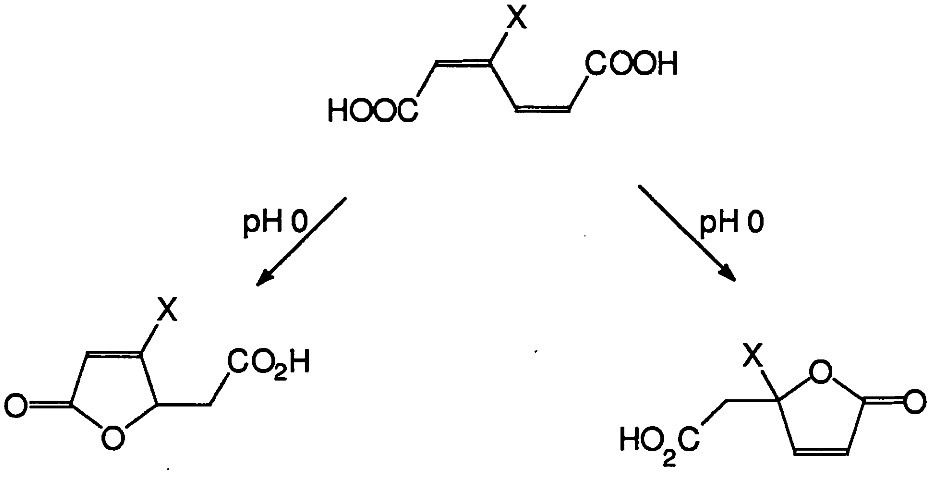
Dimethyl 3-halo-*cis,cis*-muconates (105) were prepared by Pieken and Kozarich[109] from 4-halopyrocatechols (103) by oxidation to the intermediate *o*-quinones (104) with sodium periodate, and subsequent oxidative cleavage with lead tetraacetate in the presence of methanol (Scheme 47). Alkaline hydrolysis of the dimethyl 3-halo-*cis,cis*-muconates (105) yielded acid-sensitive disodium salts of 3-halo-*cis,cis*-muconates (106). The authors examined the lactonization of *cis,cis*-3-fluoromuconate and its chloro and bromo analogs under acidic conditions.[108]

Scheme 47



At pH 0 *cis,cis*-3-fluoromuconic acid gave 4-fluoromuconolactone (107), while *cis,cis*-3-chloromuconic acid and *cis,cis*-3-bromomuconic acid gave 3-halomuconolactones (108) (Scheme 48). At pH 1-6, 4-carboxymethylenebut-2-en-4-olide (77) formed in all cases. At pH 3.2 and below, *cis,cis*-3-chloromuconic acid and *cis,cis*-3-bromomuconic acid also isomerised to the 3-halo-2-*cis*-4-*trans*-muconic acids (109) as additional products (Scheme 49).

**Scheme 48**



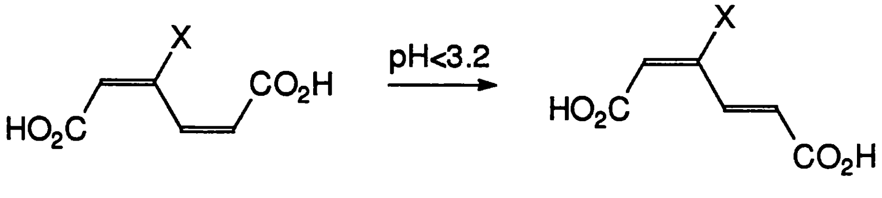
(108)

X = Cl, Br

(107)

X = F

**Scheme 49**



X = Cl, Br

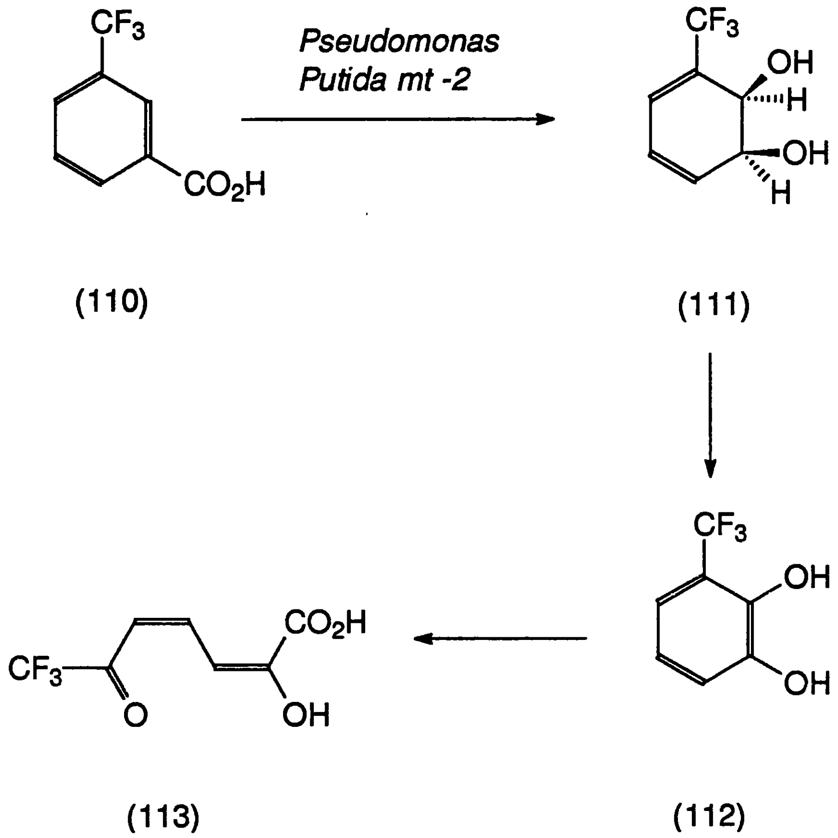
(109)

### 1.6.5 Degradation of Trifluoromethylpyrocatechols

The assimilation of trifluoromethyl-substituted aromatic compounds is especially interesting. The trifluoromethyl group has found widespread application in the biochemical and pharmaceutical industry in increasing the persistance and resistance to biological breakdown of the compounds into which it is incorporated. These properties are usually attributed to the fact that the trifluoromethyl group is not much more sterically-demanding than a methyl group, whereas it is much more electron-withdrawing. It was planned in the present work to synthesise and feed trifluoromethylated compounds to the organisms under investigation, for comparison with the alkyl analogues.

Both 3- and 4-trifluoromethylbenzoic acid have been subjected to degradation by bacteria, resulting in a common dead-end product, 2-hydroxy-6-oxo-7,7,7-trifluorohepta-2,4-dienoic acid (113).[110] 3-Trifluoromethylbenzoic acid (110) was metabolised in *Pseudomonas putida* mt-2 by *cis*-dioxygenation, followed by dehydrogenation and decarboxylation to 3-trifluoromethylpyrocatechol (112).[111] This was then cleaved to the dead-end product (113) by *meta*-fission (Scheme 50).[112] The cleavage by pyrocatechol-2,3-dioxygenase in *Pseudomonas putida* was strongly inhibited by the electronegative substituent, and it was not observed at all in *Rhodococcus rubropertincus*.[111]

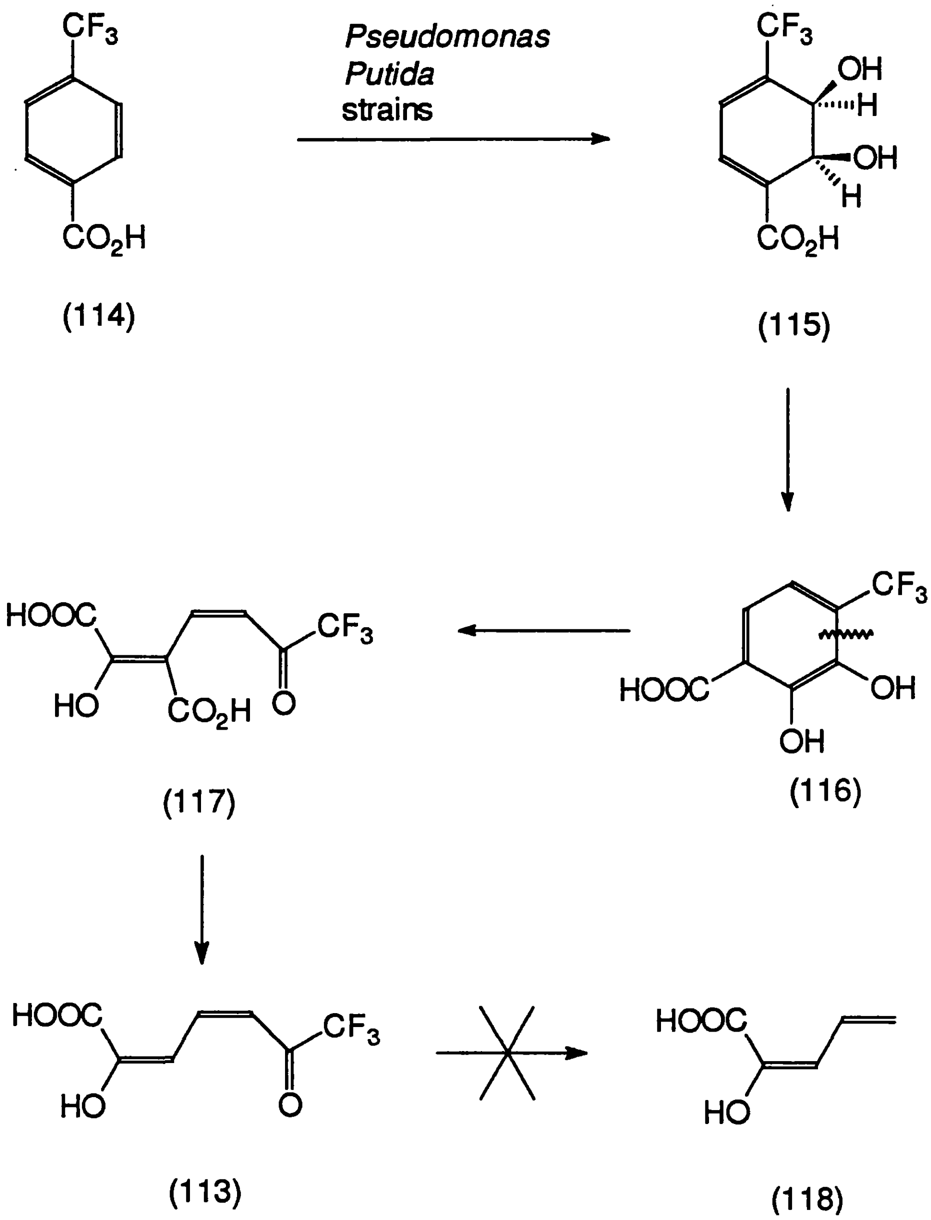
**Scheme 50**



(110) (111) (112) (113)

4-Trifluoromethylbenzoic acid (114) was dioxygenated to the *cis*-2,3-dihydroxy compound (115) in *Pseudomonas putida*, which was then transformed into 2,3-dihydroxy-4-trifluoromethylbenzoic acid (116).[113] *Meta* cleavage produced 3-carboxy-2-hydroxy-6-oxo-7,7,7-trifluorohepta-2,4-dienoic acid (117) (Scheme 51), which was decarboxylated to 2-hydroxy-6-oxo-7,7,7-trifluorohepta-2,4-dienoic acid (113). The *meta* cleavage was found to be retarded by electron withdrawal.[113] The decarboxylation was not affected by the trifluoromethyl group, but the hydrolysis of the product to (118) was affected.[113] This was accounted for in that the acid (113) showed strong binding to the hydrolase enzyme, but negligible turnover.[112] However, the acid (113) has been found to be further degraded by light.[110]

Scheme 51

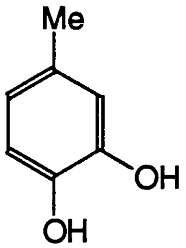


# CHAPTER 2 RESULTS AND DISCUSSION

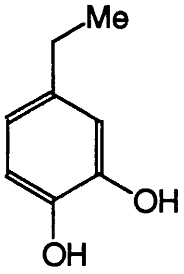
A major aim of the research described in this thesis was to explore the ability of micro-organisms to convert alkylpyrocatechols, other than 4-methylpyrocatechol, into the corresponding, chiral alkylmuconolactones. In this way, information about the substrate specificity, and hence active site geometry, of the pyrocatechase, (dioxygenase) and cycloisomerase enzymes would be obtained and it might also prove possible to develop useful microbial syntheses of the chiral lactones on a preparative scale. To this end, literature routes to pyrocatechols were surveyed and, where necessary, modified for the present purposes.

## 2.1 Synthesis of Pyrocatechols

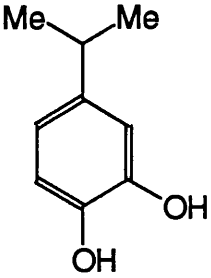
Appropriate methods were required to produce a variety of 4-substituted pyrocatechols, for testing as substrates for dioxygenase enzymes in bacteria and fungi. Of the pyrocatechol targets, only 4-methyl- (44) and 4-ethyl-pyrocatechol (119) were commercially available. The methyl derivative was used as a control for each experiment with other alkylpyrocatechols. 4-Isopropyl- (120) and 4-trifluoromethyl-pyrocatechol (121) were not commercially available, but it was believed that these compounds could be obtained from the corresponding phenols, which were available from commercial sources.



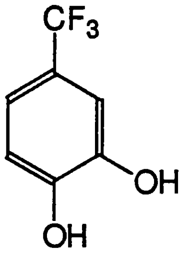
(44)



(119)



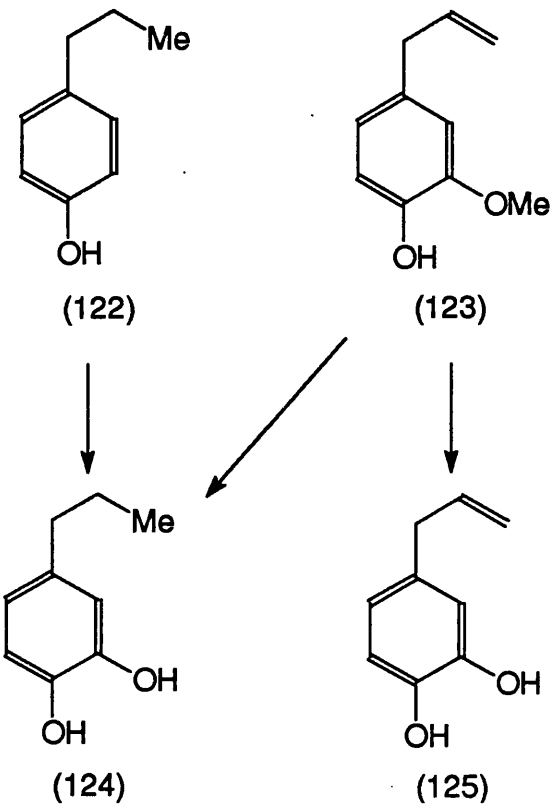
(120)



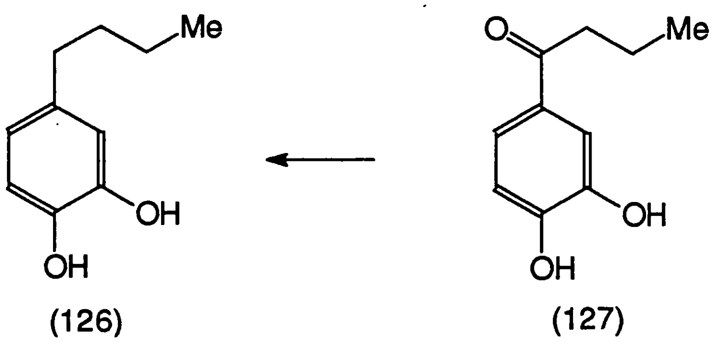
(121)

Both 4-propylphenol (122) and eugenol (123) could be utilised as starting materials to prepare 4-propylpyrocatechol (124), although eugenol had the advantage of having the correct substitution pattern already in place, and also served as a precursor for 4-allylpyrocatechol (125) (Scheme 52). For the synthesis of 4-*n*-butylpyrocatechol (126), the corresponding phenol was not available, but again a compound with the correct substitution pattern, 4-butyrylpyrocatechol (127), was accessible, supplied by Prof. R. B. Cain (Scheme 53).

**Scheme 52**

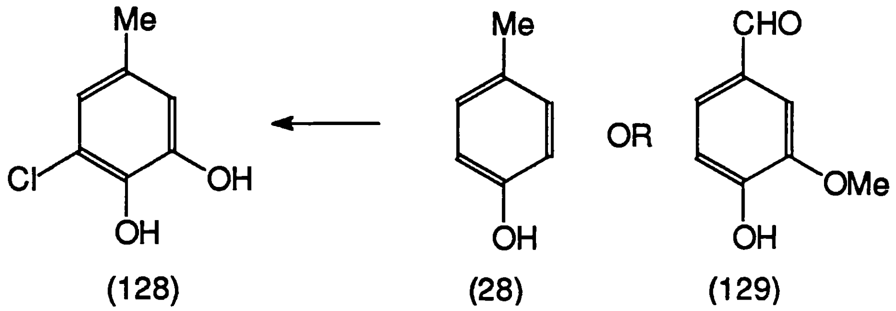


**Scheme 53**



Similarly, it was thought that 3-chloro-5-methylpyrocatechol (128) could be synthesised either from 4-methylphenol (28) or from vanillin (129), which had the oxygen substituents already in place (Scheme 54).

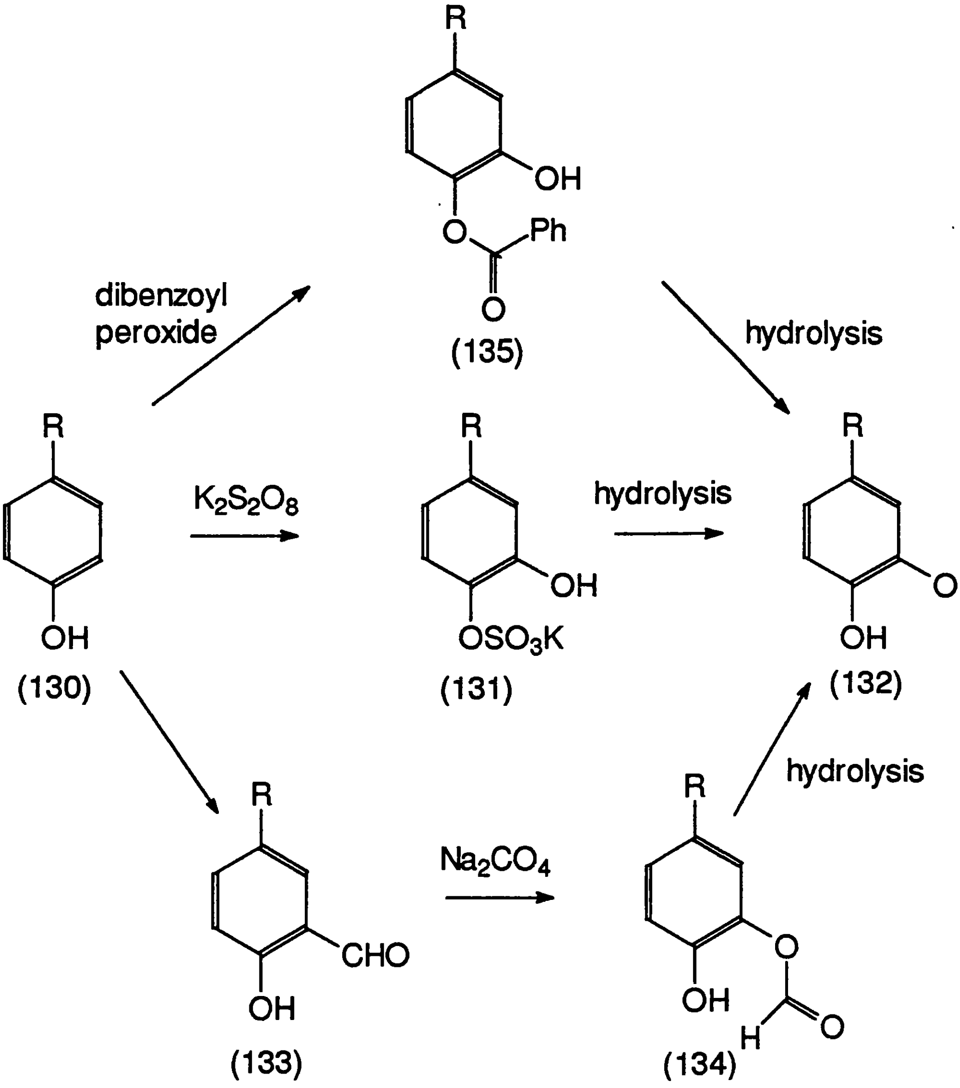
**Scheme 54**



When 4-substituted phenols were available, several potential methods were known for the synthesis of the corresponding pyrocatechols.[114,115]

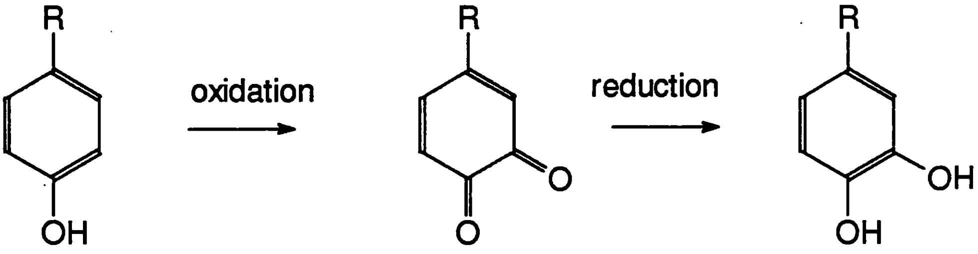
Elbs persulfate oxidation[116] involves the introduction of a sulfate group *ortho* to a phenolic hydroxy group, the product of which (131) can then be hydrolysed to a pyrocatechol (132) (Scheme 55).[117,118] Formylation *ortho* to the hydroxy group of a phenol allows oxidation of the resultant salicylaldehyde (133) by a Dakin reaction,[119] which can be performed with sodium percarbonate.[120] The resulting phenyl formate (134) can again be hydrolysed to a pyrocatechol (132). Another method of furnishing an acyloxy-substituted phenol which can be easily converted into a pyrocatechol utilises the reaction of benzoyl peroxide with 4-substituted phenols.[121,122] This gives *o*-hydroxyphenyl benzoate esters (135), which are hydrolysed to pyrocatechols (Scheme 55).

**Scheme 55**



Pyrocatechols can also be obtained indirectly by reduction of quinones (136) (Scheme 56). Quinones can be formed in a variety of ways[123] from 4-substituted phenols (130), including the use of Fremy's salt [$(KO_3S)_2NO$] (Teuber reaction),[124,125,126] chromic acid,[127] thallium trifluoroacetate[128] and diphenyldiselenic anhydride.[129] Reduction of the resultant quinones (136) can be effected with a variety of reagents,[130] such as sodium dithionite, diethyl hydroxylamine, sodium borohydride, trimethyl phosphite, zinc and acetic acid or potassium iodide in acetic acid.

Scheme 56



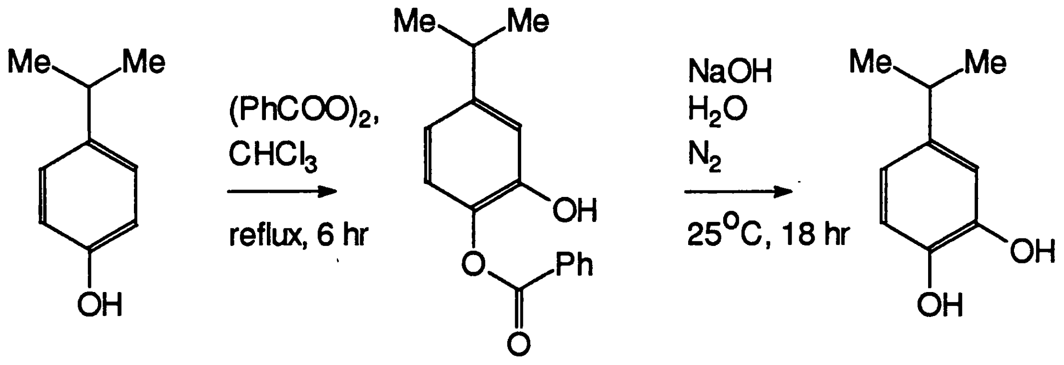
(130) (136) (132)

Some of the methods outlined above have been employed in the syntheses detailed in this work.

## 2.1.1 Synthesis of 4-Isopropylpyrocatechol (120)

Treatment of a phenol with a dibenzoyl peroxide is believed to give an intermediate phenyl benzoyl peroxide, which rearranges to give an (*o*-hydroxy)phenyl ester, and this can be hydrolysed to a pyrocatechol.[115] Following the recommended general method,[131] 4-isopropylphenol (137) was treated with dibenzoyl peroxide in chloroform at reflux for 6 h (Scheme 57), resulting in the formation of 2-benzoyloxy-5-isopropylphenol (138), which was isolated in 20 % yield following chromatographic separation. Cleavage of this ester group with aqueous sodium hydroxide under an inert atmosphere at ambient temperature for 18 hours gave 4-isopropylpyrocatechol (120), which was purified by distillation, giving an isolated yield of 33 %.

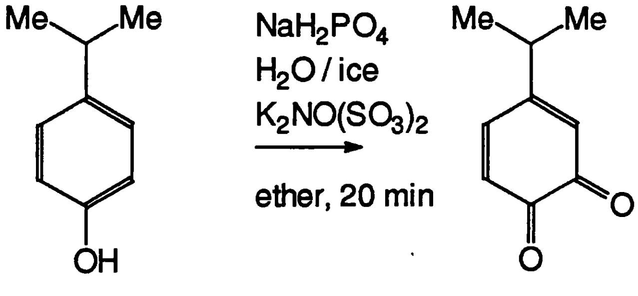
**Scheme 57**



(137) (138) (120)

The low yields associated with this first route to 4-isopropylpyrocatechol prompted investigation into another synthetic route. The formation of the desired product by reduction of the corresponding *ortho*-quinone (139) appeared attractive. Fremy's salt, potassium nitrosodisulfonate[132] was the oxidant of choice for the formation of this quinone from 4-isopropylphenol.[124,133] Thus, addition of an ethereal solution of 4-isopropylphenol (137) to a pH-buffered, cooled aqueous solution of potassium nitrosodisulfonate produced a highly coloured solution (Scheme 58). Following work-up, orange crystals were isolated, but analysis by $^1H$ NMR showed this material to be a complex mixture.

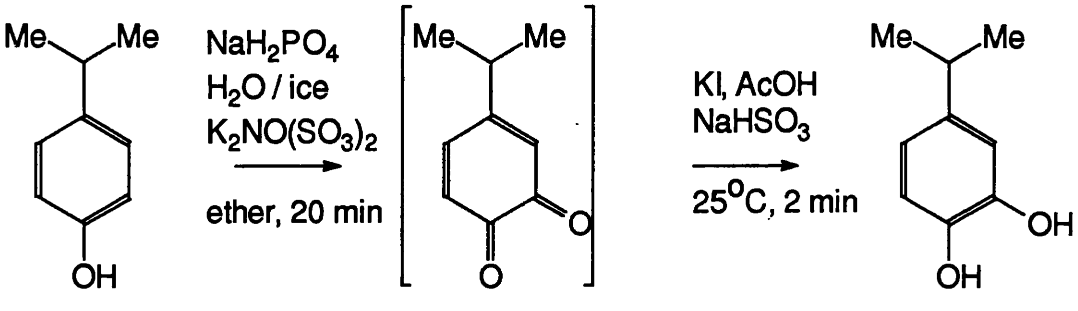
**Scheme 58**



(137) (139)

However, it was felt that this route would benefit if isolation of the *o*-quinone intermediate (139) was not attempted, but that it be generated and reacted *in situ*. *o*-Benzoquinones are known to undergo Diels-Alder dimerisation. Therefore, a chloroform solution of 4-isopropyl-*o*-benzoquinone (139) was formed as indicated above (Scheme 58). Treatment of this solution with potassium iodide in glacial acetic acid[134] effected a colour change from deep red to purple, indicating that the quinone had been reduced and that the iodide had been oxidised to iodine. Washing with sodium bisulfite reduced the iodine to iodide again prior to isolation of the resultant 4-isopropylcatechol (120) in 36 % yield after column chromatography (Scheme 59). The product had a melting point of 75-76 °C, which agreed with the literature value.[135]

Scheme 59

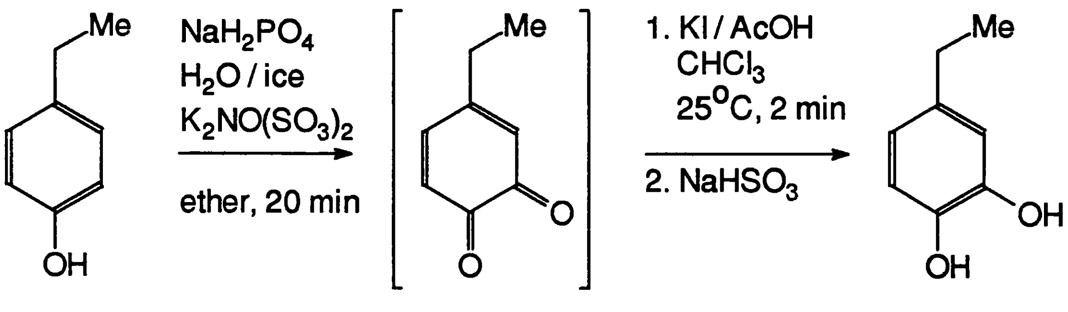


(137) (139) (120)

## 2.1.2 Synthesis of 4-ethylpyrocatechol (119)

4-Ethylpyrocatechol (119) was initially produced from 4-ethylphenol (140). Later, supplies became available commercially (Lancaster Synthesis). Again, oxidation of the phenol with Fremy's salt (potassium nitrosodisulfonate) gave the corresponding 4-ethyl-*o*-quinone (141), which was not isolated but reduced *in situ* with potassium iodide (Scheme 60). Distillation and chromatography were required to separate the product from the starting phenol. 4-Ethylpyrocatechol (119) was isolated as white crystals in 33 % yield. The melting point of the product was 38-39°C, and agreed with that reported in the literature.[136]

Scheme 60

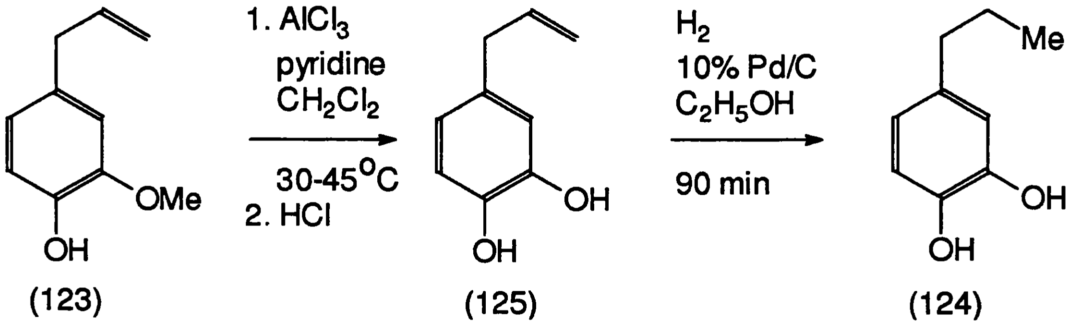


(140) (141) (119)

## 2.1.3 Synthesis of 4-allylpyrocatechol (125) and 4-*n*-propylpyrocatechol (124)

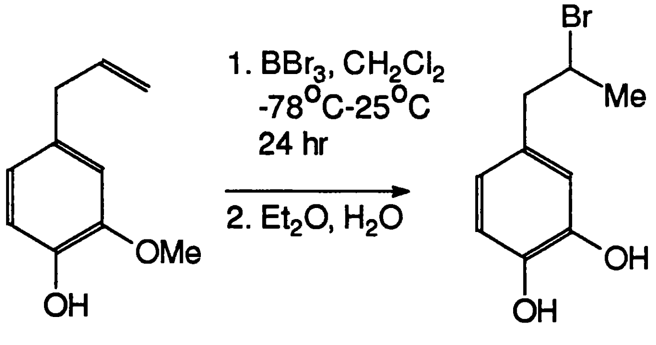
The commercial availability of eugenol (123) made this compound a desirable starting material for the synthesis of both 4-allylpyrocatechol (125) and 4-*n*-propylpyrocatechol (124). Both routes required the cleavage of the phenolic ether. Several reagents are known for this transformation,[130,137,138,139] including hydrogen bromide,[140] iodotrimethylsilane[141] and Lewis acids.[142] Cleavage of the methyl ether in eugenol was attempted with aluminium trichloride with pyridine in dichloromethane at reflux,[143] followed by acid hydrolysis of the aluminium complex formed (Scheme 61). This sequence yielded 4-allylpyrocatechol (125) in 26 % after chromatography, while 26 % of the starting eugenol was also recovered. The mass spectrum of 4-allylpyrocatechol (125) showed the molecular ion at $m/z$ = 150.0673 ($C_9H_{10}O_2$ requires $M$, 150.0681). Catalytic hydrogenation of the double bond of the 4-allyl group was then effected with 10 % palladium on carbon as catalyst, in ethanol for 90 minutes. Distillation of the crude product furnished the desired 4-*n*-propylpyrocatechol (124) in 93 % yield, with a melting point of 59-60°C, which agreed with the literature value.[144]

**Scheme 61**



The low yield of methyl ether cleavage with aluminium trichloride prompted investigation of an alternative reagent. Boron tribromide is often recommended for the cleavage of aryl methyl ethers.[145] A dichloromethane solution of eugenol (123) at -78 °C was treated with boron tribromide (Scheme 62).[137] The mixture was allowed to come to room temperature and stirred for 24 hours, then quenched with diethyl ether and hydrolysed with water. The major product, isolated in 40 % yield, was a brown gum, identified as 4-(2-bromoprop-1-yl)pyrocatechol (142). The $^1H$ NMR spectrum of this product showed a multiplet for a single proton at δ 4.11-4.28 ppm, assigned to the bromomethine group. It is suspected that this compound results from the hydrobromination in the acidic work-up of the allyl double bond in the initially-formed 4-allylcatechol.[146]

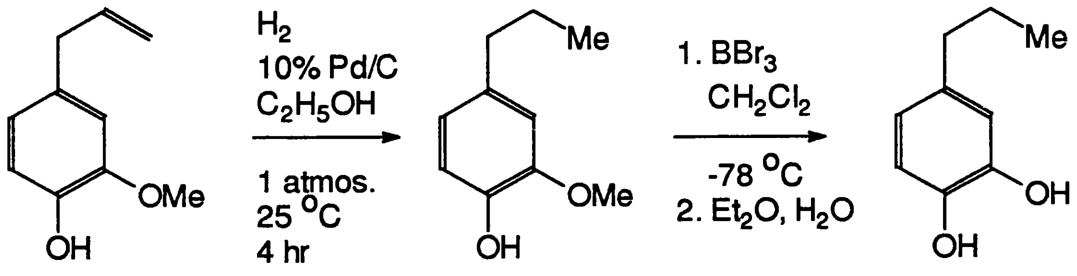
**Scheme 62**



(123) (142)

In order to avoid the bromination side reactions during the Lewis acid-mediated ether cleavage, reversal of the order of this and the hydrogenation step was attempted. Thus, hydrogenation of eugenol (123) at atmospheric pressure was accomplished over carbon-supported palladium in ethanol at ambient temperature (Scheme 63). 2-Methoxy-4-*n*-propylphenol (143) was formed completely after 4 hours, distilling to give a clear oil in 91 % yield. Cleavage of the methyl ether was then effected with boron tribromide to give 4-*n*-propylpyrocatechol (124) in 90 % yield after distillation.

**Scheme 63**

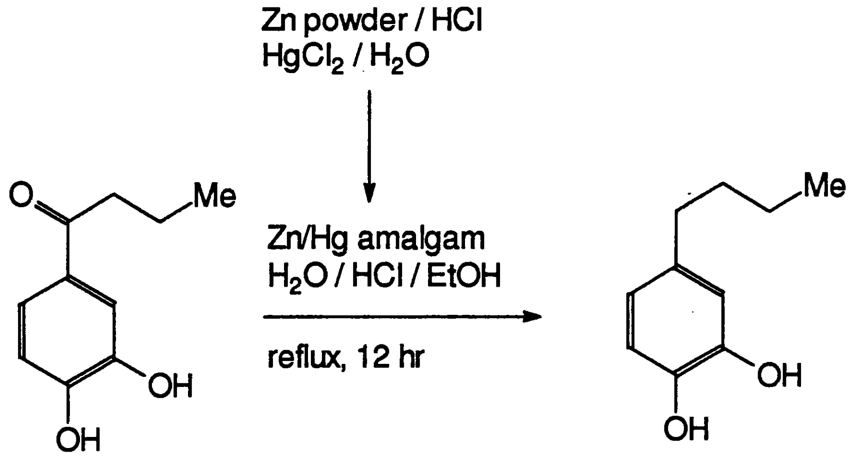


(123) (143) (124)

### 2.1.4 Synthesis of 4-*n*-butylpyrocatechol (126)

Clemmensen reduction[147,148] of the readily available 4-butyrylbenzene-1,2-diol (127) provided ready access to 4-*n*-butylpyrocatechol (126). A zinc-mercury amalgam[149] was prepared by mixing acid-washed zinc powder with mercuric chloride in water (Scheme 64). The resulting amalgam was slurried with aqueous acid and an ethanol solution solution of the ketone (127) for 12 hours at reflux.[150] Distillation of the resultant product gave 4-*n*-butylpyrocatechol (126) in 51 % yield as a colourless oil which crystallised on standing. The compound had a melting point of 39-41°C, which agreed with the literature value.[151]

Scheme 64



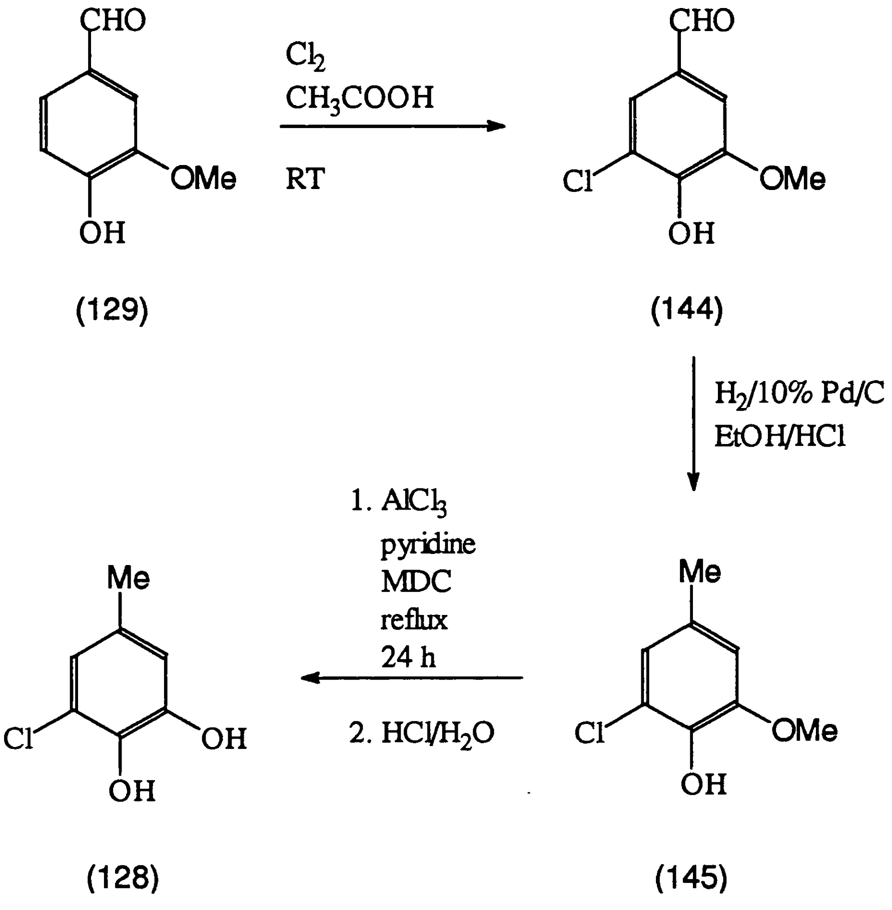
(127)

(126)

### 2.1.5 Synthesis of 3-chloro-5-methylcatechol (128)

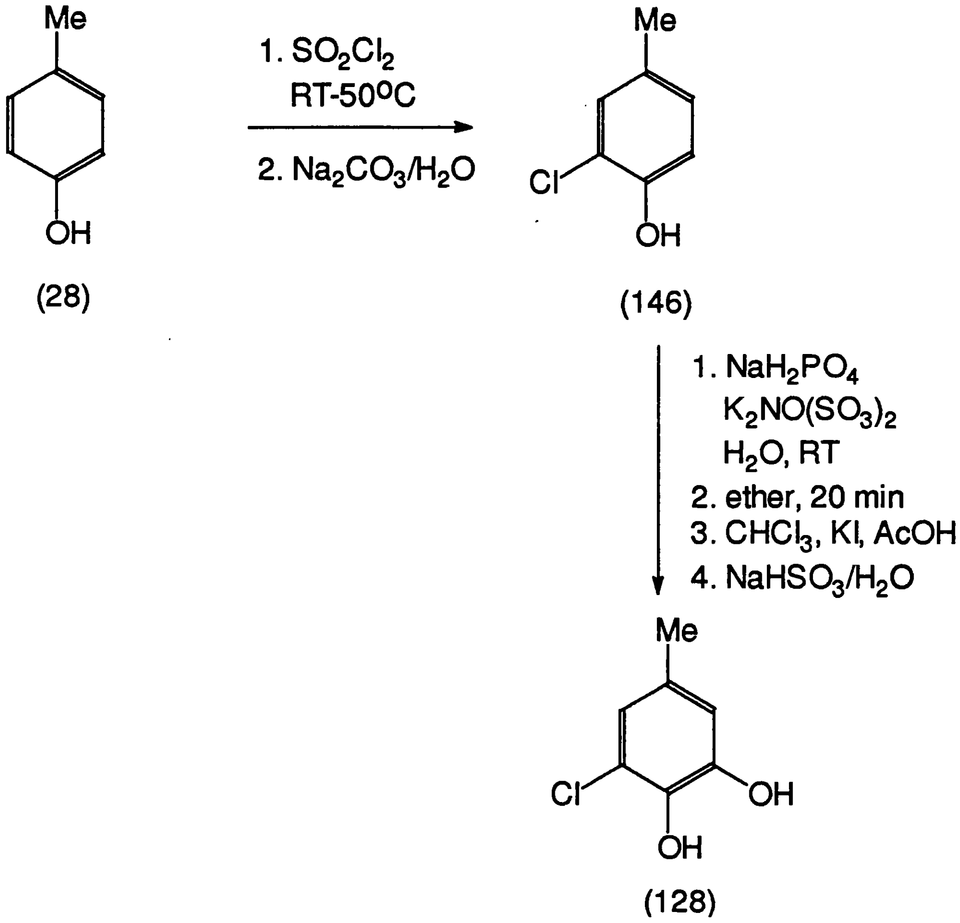
It was of interest in this project to observe the effect of chlorination of the aromatic substrates on their cleavage by bacterial dioxygenases. Also, interesting stereochemical deductions could be made from the cyclisation of the expected muconic acids (Section 1.6). 3-Chloro-5-methylpyrocatechol (128) was desired for comparison with 4-methylpyrocatechol, and a route to this compound was developed. Vanillin (129) was chlorinated with chlorine gas in glacial acetic acid at ambient temperature (Scheme 65).[152] The product crystallised from the solution, and was recrystallised from acetic acid to give 3-chlorovanillin (144) as a white solid in 87 % isolated yield. This material was then hydrogenated in acidified ethanol in the presence of 10 % palladium on carbon as catalyst. A 51 % yield of 2-chloro-6-methoxy-4-methylphenol (145) was obtained as an oil. Cleavage of the methyl ether was effected with anhydrous aluminium trichloride and pyridine in dichloromethane at reflux for 24 hours.[143] Acidification and work-up gave equal amounts of 3-chloro-5-methylpyrocatechol (128) and the starting methyl ether (145), which were separated by chromatography to yield the desired pyrocatechol in 22 %. The mass spectrum of the product showed molecular ions at *m/z* = 160 and 158 in a 1:3 ratio, while the $^1$H NMR spectrum showed the presence of the methyl group at δ 2.21 ppm.

Scheme 65



Again, the low yield in the final step of this route was undesirable. A possible alternative route to 3-chloro-5-methylpyrocatechol involved *o*-chlorination of fused *p*-cresol (28). Treatment of this phenol with neat sulfuryl chloride and warming the mixture to 50 °C gave 2-chloro-4-methylphenol (146) as an oil, after alkaline work-up.[153] Purification by distillation furnished the desired product in 62 % yield (Scheme 66). Oxidation of 2-chloro-4-methylphenol (146) with potassium nitrosodisulfonate in a buffered solution, followed by potassium iodide-mediated reduction of the quinone intermediate in chloroform yielded a mixture of products. After chromatography and distillation, a 34 % yield of 3-chloro-5-methylpyrocatechol (128) was obtained as an off-white solid.

Scheme 66

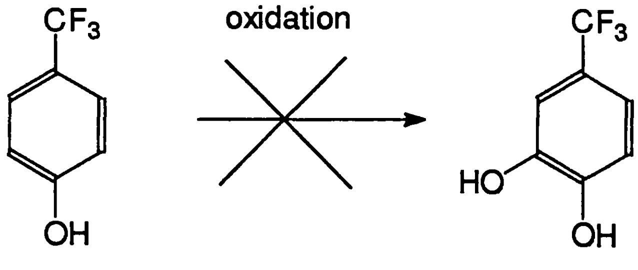


### 2.1.6 Synthesis of 4-trifluoromethylpyrocatechol (121)

The influence of the trifluoromethyl group on the cleavage and subsequent enzymic reactions of some pyrocatechols has already been outlined (Section 1.6.5). Trifluoromethyl-substituted aromatic compounds are becoming increasingly widespread pollutants. Their resistance to biodegradation in mammals is useful in the pharmaceutical industry, but causes problems in the environment. It was therefore of interest to investigate the hitherto unexplored degradation of 4-trifluoromethylpyrocatechol by bacteria, in comparison to the sterically-similar 4-methyl analogue.

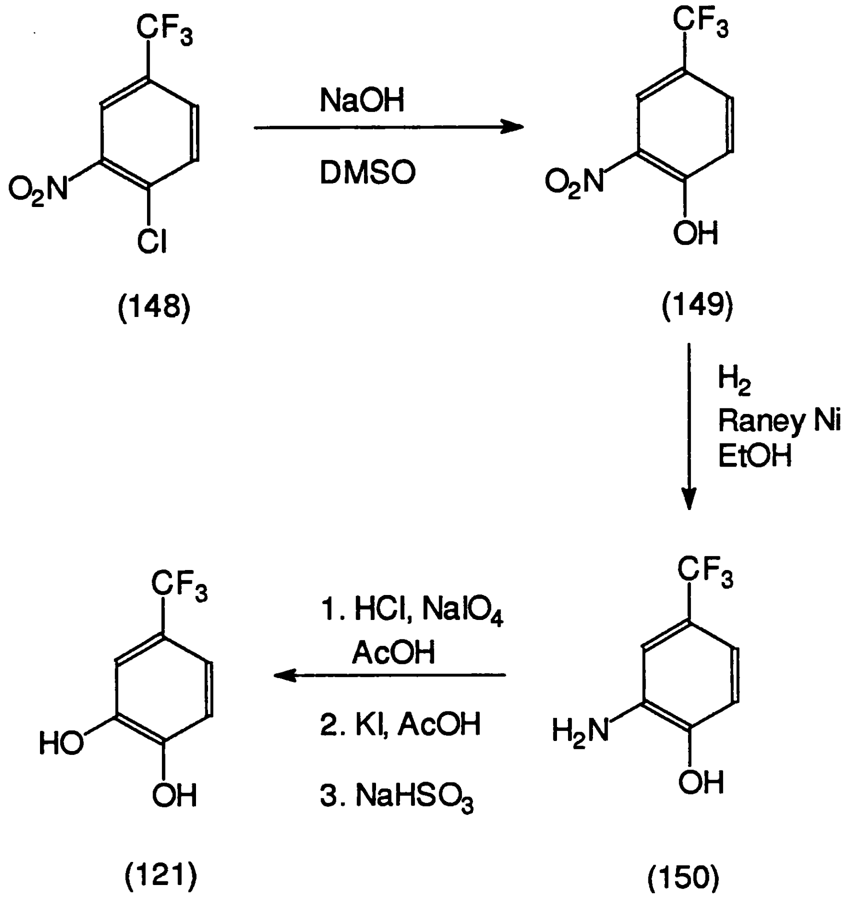
Attempts to synthesise 4-trifluoromethylpyrocatechol (121) proved very difficult. Oxidation of 4-trifluoromethylphenol (147) to the corresponding benzoquinone with Fremy's salt and reduction with potassium iodide failed to give the desired pyrocatechol, with starting material being recovered quantitatively. Attempted oxidation of this phenol (147) with benzoyl peroxide also failed to change the starting material (Scheme 67). Presumably, the powerful electron-withdrawing effect of the trifluoromethyl group prevented electrophilic attack by either reagent.

Scheme 67



Instead, activation of the ring towards nucleophilic attack was expected in a route to 4-trifluoromethylpyrocatechol (121) from the commercially available 4-chloro-3-nitro-1-trifluoromethylbenzene (148). This compound was treated with powdered sodium hydroxide in dimethyl sulfoxide for 24 hours at ambient temperature.[154] Acidification to pH 1 and extraction with ether gave 2-nitro-4-trifluoromethylphenol (149) in 87 % yield as a red oil (Scheme 68). Hydrogenation of the nitro group was effected with hydrogen gas over Raney nickel in ethanol at ambient temperature.[155] The resultant 2-amino-4-trifluoromethylphenol (150) was crystallised from toluene as an off-white solid in 76 % yield. The same compound was also obtained from the nitrophenol (149), by hydrogen transfer from formic acid, catalysed by 10 % palladium on carbon,[156] at 100 °C and acidic work-up. 2-Amino-4-trifluoromethylphenol (150) was converted into 4-trifluoromethylpyrocatechol (121) by treatment with acidified sodium periodate at ambient temperature in acetic acid.[134] This oxidised the amino phenol to the corresponding *o*-quinone mono-imine, and then hydrolysed the imine group. The resultant *o*-quinone was reduced with potassium iodide in acetic acid to the desired 4-trifluoromethylpyrocatechol (121), isolated in 17 % yield as a colourless oil after distillation. Sodium bisulfite was used to reduce the iodine formed to iodide ion. This new pyrocatechol was characterised by NMR spectroscopy. The $^{13}$C NMR spectrum showed a quartet at δ 124.0 assigned to the trifluoromethyl group, while the $^{1}$H NMR spectrum showed a broad singlet for two OH protons at δ 6.52 ppm which were exchanged for deuterium on shaking with $D_2O$. The high resolution mass spectrum for (121) gave a value for the molecular ion of 178.0241 ($C_7H_5O_2F_3$ requires *M*, 178.0242)

**Scheme 68**



## 2.2 Synthesis of Metabolic Intermediates

In order to investigate the muconolactone methyl isomerase enzyme, which overcomes the metabolic dead-end in the bacterial assimilation of 4-methylpyrocatechol (Section 1.5), several targets were identified as possible racemic substrates or inhibitors for the enzyme of

*Rhodococcus rhodocrous*. These included (±)-3-alkylmuconolactones, *e.g.* (126), and (±)-1-alkylmuconodilactones, *e.g.* (154).

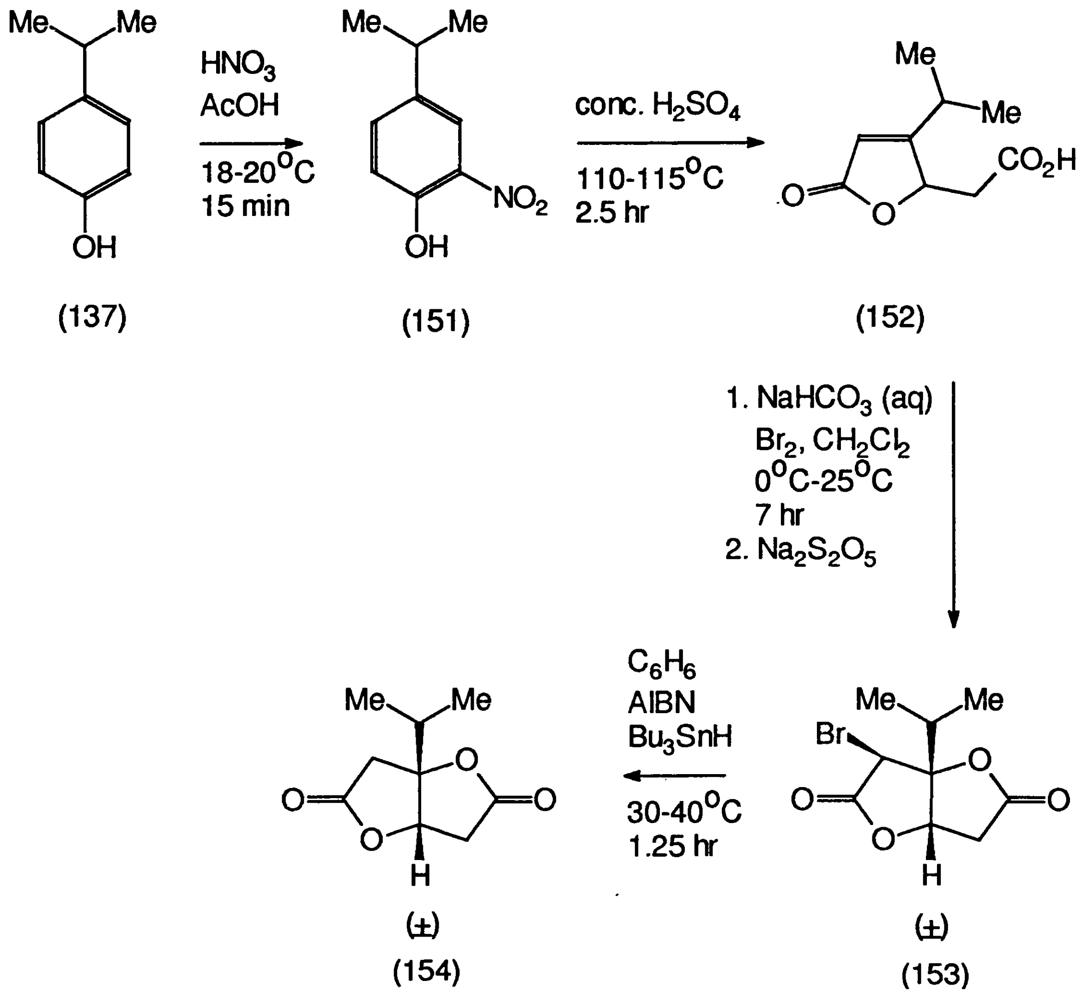
3-Alkyl-*cis-cis* muconic acids were thought to be useful precursors to these lactones and they could also be used to examine the substrate specificity of the cycloisomerase enzyme in *Pseudomonas putida,* which converts these muconic acids to 4-substituted muconolactones.

Thus, investigations were carried out into potential synthetic routes to 3-alkyl-*cis,cis*-muconic acids or their derivatives. A possible route involved the oxidative cleavage of *o*-quinones, which can be formed from pyrocatechols or as mentioned previously (Section 2.1). Oxidation of pyrocatechols to *o*-quinones has been executed with a variety of reagents,[157] including chromium trioxide, iron (III) salts, silver oxide,[158] silver carbonate on Celite[159] and ceric ammonium nitrate.[160] The *o*-quinones could then be cleaved to *cis,cis*-muconic acid derivatives with peracids[161] or an oxidised cobaltocene adduct.[162] A direct preparation of alkylmuconolactones from *o*-nitrophenols in hot sulfuric acid is also known.[163]

### 2.2.1 Synthesis of (±)-3-Isopropylmuconolactone (152) and (±)-1-isopropylmuconoldilactone (154)

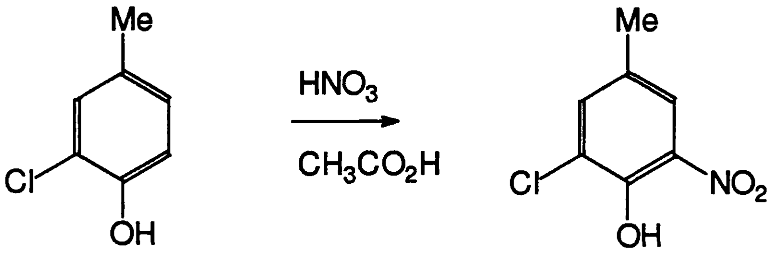
4-Isopropylphenol (137) was nitrated at the 2-position when kept in a solution of concentrated nitric acid in acetic acid at 20 °C for 15 minutes (Scheme 69).[163] 4-Isopropyl-2-nitrophenol (151) was isolated in 59 % yield after chromatography and distillation, with the remaining 41 % consisting mainly of the starting phenol. Treatment of 4-isopropyl-2-nitrophenol with concentrated sulphuric acid at 110-115 °C for 2.5 hours cleaved the carbon-carbon bond between the hydroxy group and the nitro group, and hydrolysed the carbon-nitrogen bond. Recyclisation then occurred to give the racemic 3-isopropylmuconolactone (152) in 56 % isolated yield.[164] This new lactone showed a band in the IR spectrum at 1746 $cm^{-1}$, which is characteristic of the carbonyl group in an $\alpha\beta$-unsaturated 5-membered ring lactone. The high resolution mass spectrum for (152) gave a value for the molecular ion of 184.0735 ($C_9H_{12}O_4$ requires *M*, 184.0736). Bromolactonisation was performed on this muconolactone with bromine in the presence of base, followed by bisulfite washes to remove excess bromine.[165] (±)-8-Bromo-1-isopropylmuconodilactone (153) was isolated as a brown gum in 87 % yield. The $^1H$ NMR spectrum of the product showed a single proton at $\delta$ 4.55 ppm, indicating that it was bonded to the carbon bearing the bromine atom. The high resolution mass spectrum for (153) gave values for the molecular ions of 263.9816 and 261.9839 ($C_9H_{11}O_4{}^{81}Br$ requires *M*, 263.9821, and $C_9H_{12}O_4{}^{79}Br$ requires *M*, 261.9841). Reduction of the bromodilactone was accomplished with tributylstannane in benzene at 30-40 °C in the presence of azoisobutylnitrile as radical initiator,[165] furnishing (±)-1-isopropylmuconodilactone (154) in 22 % isolated yield. The high resolution mass spectrum for the new dilactone (154) gave a value for the molecular ion of 184.0738 ($C_9H_{12}O_4$ requires *M*, 184.0736), while two carbonyl carbons were observed at $\delta$ 172.8 and 173.0 in the $^{13}C$ NMR spectrum.

Scheme 69



Thus, this route was successfully used to give the racemic 3-isopropylmuconolactone and the racemic 1-isopropylmucondilactone. It was hoped that it could also be used to give other racemic 3-alkylmuconolactones and dilactones. Thus, 2-chloro-4-methylphenol (146) was converted into 2-chloro-4-methyl-6-nitrophenol with nitric acid in acetic acid at 0 °C without agitation (Scheme 70). The desired product was isolated in 61 % yield after addition of water gave a yellow precipitate. However, treatment of this nitrophenol with hot, concentrated sulfuric acid gave only intractable mixtures.

**Scheme 70**



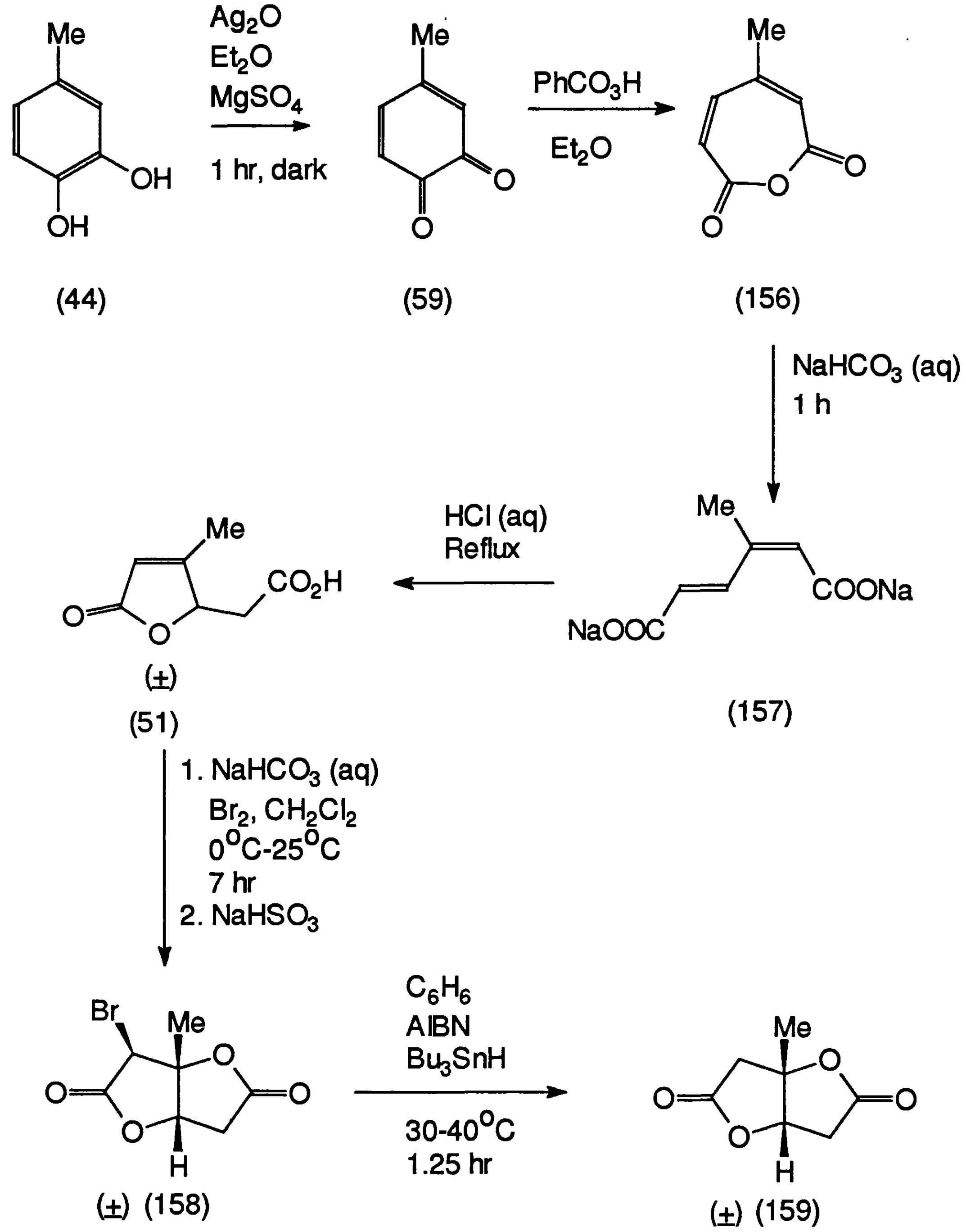
(146) (155)

Cleavage of 2-nitro-4-trifluoromethylphenol (149) in this manner gave mainly starting material and 3-trifluoromethylmuconolactone was not isolated from the reaction.

### 2.2.2 Synthesis of alkylmuconolcatones by oxidation of corresponding *o*-quinones with monoperphthalic acid

As a model study for this route, racemic 3-methylmuconolactone (51) and 1-methylmuconodilactone (158) were synthesised from 4-methylpyrocatechol (44) (Scheme 71) following the published methods.[80] 4-Methylpyrocatechol was oxidised with a suspension of silver oxide in ether.[166] Careful removal of the solvent by low temperature distillation gave 4-methyl-*o*-benzoquinone (59), in 71 % yield as red-brown crystals. The quinone was then treated with monoperphthalic acid, prepared from phthalic anhydride and aqueous alkaline hydrogen peroxide,[167] in ether for 24 hours.[168] 3-Methylmuconic anhydride (156) was isolated and was then cleaved in aqueous base to form disodium 3-methyl-2-*cis*-4-*trans*-muconate (157).[168]

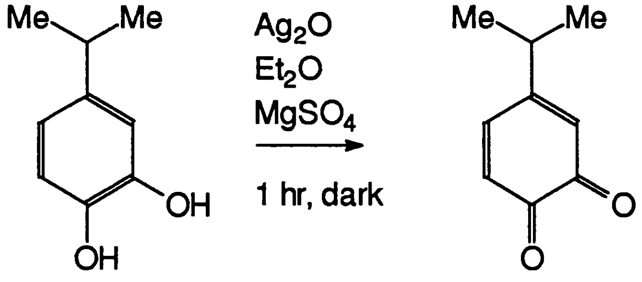
Scheme 71



3-Methylmuconolactone (51) was obtained by heating the salt (157) under reflux in hydrochloric acid.[169] The racemic 3-methylmuconolactone was isolated and converted with bromine and aqueous base in dichloromethane[165] into the racemic 1-methyl-8-bromomuconodilactone (158), obtained as a white solid in 87 % yield. Treatment of this compound with tributylstannane in benzene, warmed in the presence of azoisobutylnitrile,[170] replaced the bromine atom with hydrogen, to give racemic 1-methylmuconodilactone (159) as a white crystalline solid in 22 % yield.

Silver oxide,[171] prepared from the action of sodium hydroxide on silver nitrate solution, oxidised 4-isopropylcatechol (120) in dry ether in the presence of anhydrous magnesium sulfate and in the absence of light. 4-Isopropyl-*o*-benzoquinone (139) was produced in 71 % yield as a deep red oil (Scheme 72), but it decomposed on storage. The $^1$H NMR spectrum of (139) showed three protons in the alkene region at δ 6.07, 6.27 and 6.94 ppm. The $^{13}$C NMR spectrum showed the presence of two quinone carbonyls at δ 179.6 and 180.1.

Scheme 72

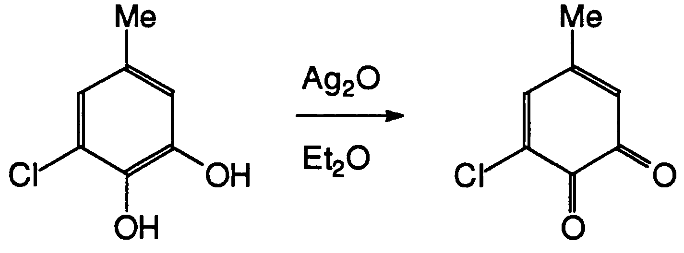


(120) (139)

However, attempted Baeyer-Villiger oxidation of this quinone to the corresponding 3-isopropyl-*cis,cis*-muconic acid anhydride, with monoperphthalic acid, resulted in intractable tars, and the route was not pursued further.

It was intended that 3-chloro-5-methylpyrocatechol be used as a precursor to 3-methyl-5-chloromuconolactone, for $^1$H NMR studies, with deuterium labelling, into the stereochemistry of enzymic cyclisation. To this end, 3-chloro-5-methylpyrocatechol was oxidised with silver oxide in ether to give 3-chloro-5-methyl-*o*-quinone (160), isolated as a red oil in 40 % yield (Scheme 73). The $^{13}$C NMR spectrum of (160) showed the presence of two carbonyl groups at δ 173.3 and 179.9 ppm. However, attempted Baeyer-Villiger oxidation of this quinone to the corresponding 2-chloro-4-methyl-*cis,cis*-muconic acid anhydride, using monoperphthalic acid, again resulted in intractable tars.

**Scheme 73**



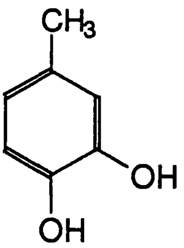
(128) (160)

## 2.3 METABOLISM OF 4-ALKYLPYROCATECHOLS

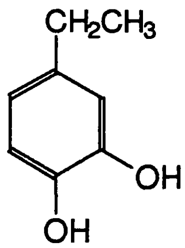
### 2.3.1 In *Pseudomonas putida*

Once the 4-alkylpyrocatechols shown in Figure 1, had been successfully synthesised, they were fed along with commercially available 4-t-butylpyrocatechol (161) to cultures of *Pseudomonas putida.* The feeding of 4-methylpyrocatechol (44) was used as a control each time.

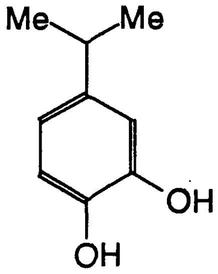
**Figure 1**



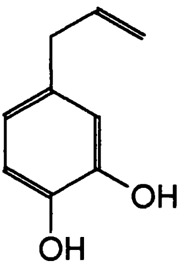
(44)



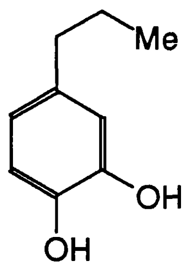
(119)



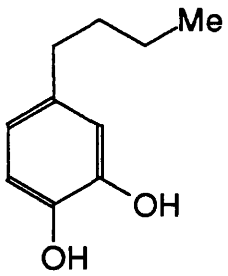
(120)



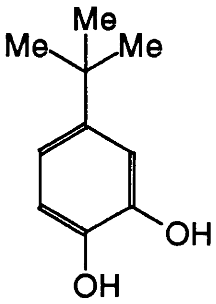
(125)



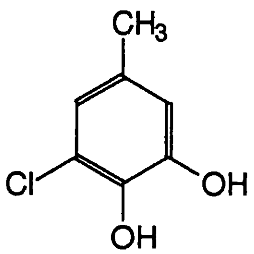
(124)



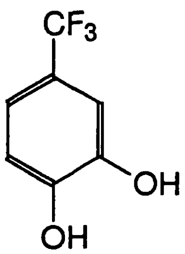
(126)



(161)



(128)

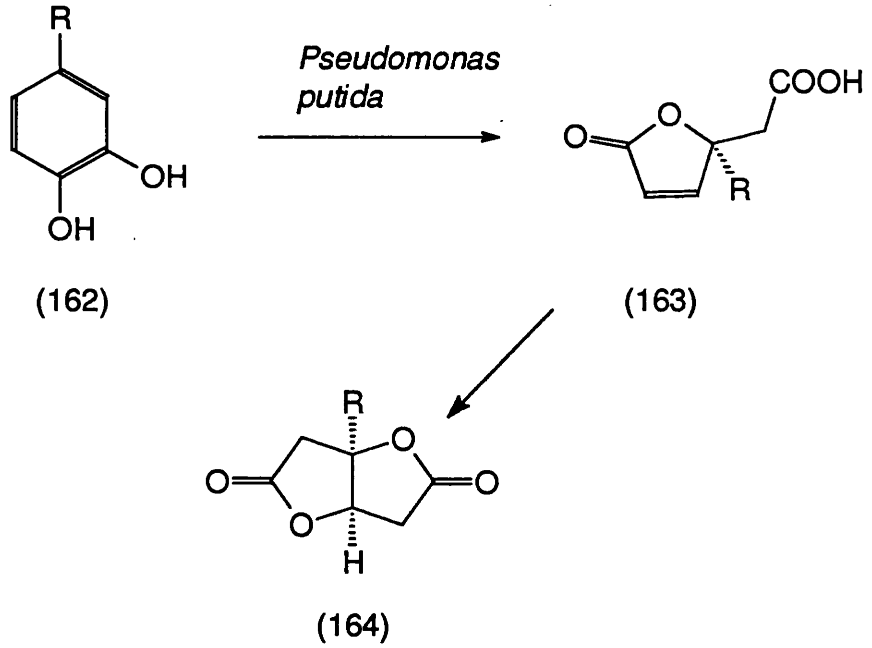


(121)

These compounds were fed for the following reasons (Scheme 74):

1. To test the capability of the dioxygenase and cycloisomerase enzymes in *Pseudomonas putida* cultures to use these compounds, and the derived muconic acids, as substrates, for the biosynthesis of the corresponding muconolactones. This would give insight into the shape and size of the enzymes active sites by showing which groups are easily accommodated.
2. If the corresponding 4-alkylmuconolactone was formed, this would be converted into the corresponding dilactone, and both compounds would be tested as substrates for methylmuconolactone methyl isomerase of *Rhodococcus rhodocrous* N75.

**Scheme 74**



(162) (163) (164)

R = Alkyl

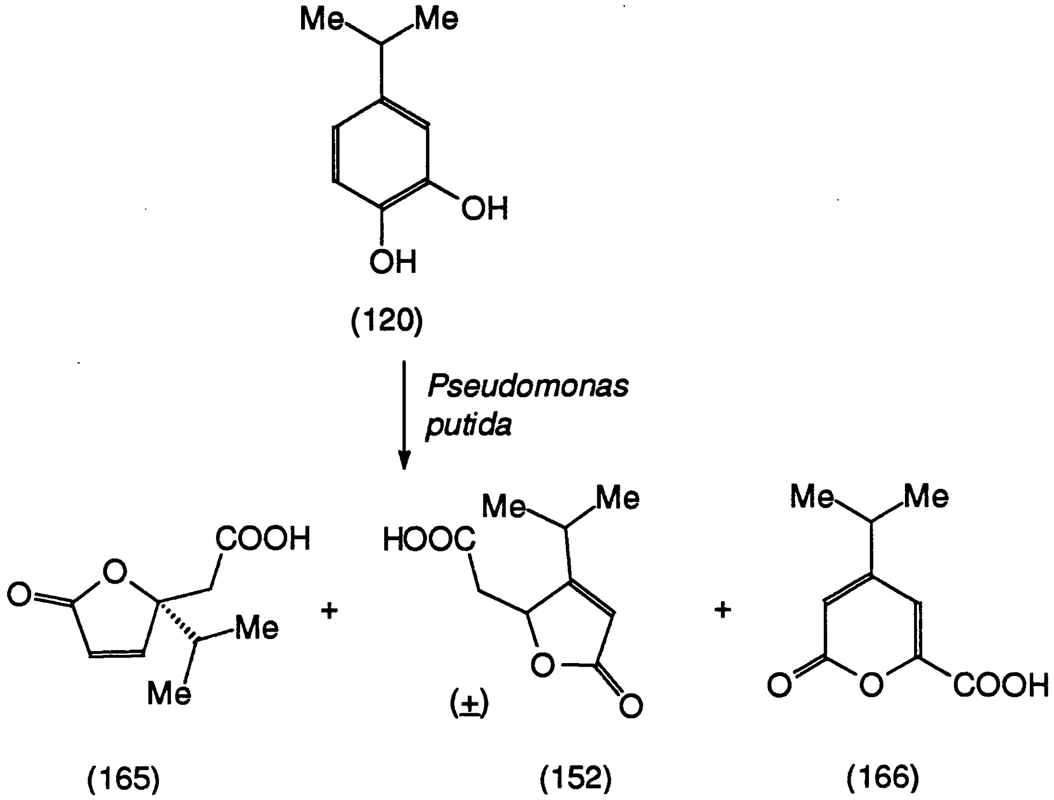
All the pyrocatechol derivates were fed to cultures of *Pseudomonas putida* under the conditions employed successfully for 4-methylpyrocatechol.[172] All substrates were fed in 3 batches over 24 hours, to 3 day old cultures of *Pseudomonas putida.* The entire culture medium was combined together 24 hours after the last feeding and adjusted to pH 7.5 and extracted with ether, to extract any non-acidic material present in the broth which included any pyrocatechol that had not been cleaved by the dioxygenase enzyme. The ether extracts were analysed by $^1$H NMR spectroscopy. The culture medium was then adjusted to pH 2.5 and extracted with ether. These ether extracts contained any muconolactones that had been produced by the organism. An area of concern was the quantification of substrate specificity of the enzymes for each pyrocatechol. Proof was required that the enzymes were being produced and working effectively each time. To overcome this problem, 4-methylpyrocatechol was fed in parallel each time to demonstrate that the bacterial and fungal enzymes were operating consistently throughout the feeding experiments.

### 2.3.1.1 Metabolism of 4-Isopropylpyrocatechol (120)

4-Isopropylpyrocatechol (120) was the first in the series of 4-alkylpyrocatechols that was fed to *Pseudomonas putida.* The culture medium was combined and adjusted to pH 7.5 with sodium hydroxide solution. The entire culture medium was extracted with ether, dried and concentrated to give a dark brown oil which was analysed by $^1$H NMR spectroscopy. This showed that small quantities of unreacted 4-isopropylpyrocatechol (120) had been recovered. Acidification, extraction into ether and evaporation of the ether gave a red-brown gum. The total extract was analysed by $^1$H NMR spectroscopy in $(CD_3)_2CO$ to ensure that all the extract had fully dissolved. The $^1$H NMR spectrum showed doublets (*J* 5.8 Hz) at δ 6.12 and 7.76, corresponding to the vinylic protons (2- and 3-H), confirming that 4-isopropylmuconolactone (165) was the major product. The minor metabolites 3-isopropylmuconolactone (152) and 3-isopropyl-2-pyrone-5-carboxylic acid (166) were also present. 3-Isopropylmuconolactone (152) was identified by comparison of its spectrum with that of the synthetic racemate. In particular, signals were observed at 5.40 (ddd, *J* 8.6, 3.6 and 1.7 Hz) and δ 5.86 (s with fine splitting) for 4-H and 2-H respectively. 3-Isopropyl-2-pyrone-5-carboxylic acid (166) was tentatively identified by singlets with fine splitting at δ 7.14 and 6.30 corresponding to the vinylic protons (Scheme 75). The corresponding *tert*-butyl derivative, which had been isolated from the bacterial degradation of 4-*tert*-butylpyrocatechol, gave similar signals. (See Section 2.3.1.6, page 110).

The total extract was separated on 20 x 20 cm silica plates using diisopropyl ether : formic acid : water (200:7:3) as eluent. Examination of the TLC plate by UV light and development with iodine vapour showed approximately 20 separate bands. Each band was removed, stirred with acetone for several hours and filtered. The filtrate was evaporated and the residue was dissolved in ether and washed with water to remove any formic acid present, dried over sodium sulphate and then evaporated under reduced pressure. The extract from each band was examined by $^1$H NMR spectroscopy. 4-Isopropylmuconolactone (165), $R_F$ 0.4, and 3-isopropylmuconolactone (152), $R_F$ 0.2, were obtained as off - white oils, which failed to crystallise. Examination of the other bands showed few $^1$H NMR signals and none which corresponded to any possible metabolites.

Scheme 75



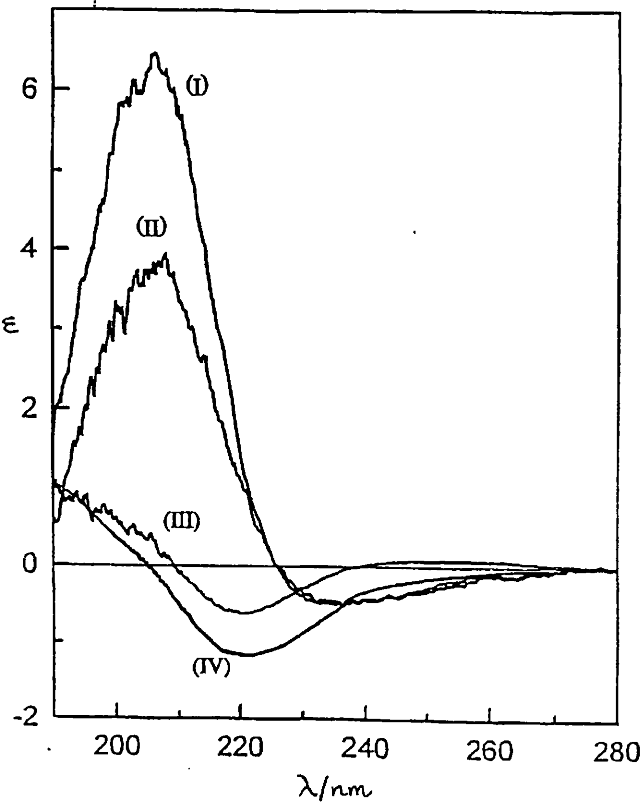
4-Isopropylmuconolactone (165) was isolated as an oil, $[\alpha]_D$ + 21 ° (*c*, 0.7 in methanol). The IR spectrum showed the carbonyl absorption at 1745 $cm^{-1}$ and the high resolution mass spectrum gave a molecular weight of 184.0751 in good agreement with a theoretical value of 184.0736 for $C_9H_{12}O_4$. The $^1H$ NMR spectrum showed the characteristic doublet of doublets for the vinyl protons at δ 6.12 and δ 7.76 with *J* 5.8 Hz, while the AB quartet corresponding to 5-methylene protons was observed at δ 2.79 and 2.97 with *J* 15.9 Hz.

The absolute configurations of (*S*)-muconolactone (20), (*S*)-3-methylmuconolactone (48), (*S*)-4-methylmuconolactone (50) and (*S*)-3-carboxymuconolactone (38) have all been determined unambiguously. Although it was likely that the cycloisomerase of *Pseudomonas putida* would produce the new 4-alkylmuconolactone with the same absolute stereochemistry, this point was checked by comparing the CD spectra (measured by Professor N.C. Price, Stirling University) of the known and new derivatives.

It was found [173] that the muconolactones having hydrogen at C-4 all showed positive Cotton effects (CEs) near 200 nm, corresponding to the strong, UV, $\pi$ - $\pi^*$ absorptions, and negative CEs at longer wavelength, corresponding to weak n - $\pi^*$ absorptions not normally detectable in the UV spectra. A similar conclusion for other butenolides has recently be reported [174]. As expected, the positive CE, near 200 nm, for 4-methylmuconolactone was much less well defined, since the two groups at the chiral centre now have similar sizes and polarisabilities. Nevertheless, the negative CE (at 220 nm) was still apparent. It was of interest therefore to observe the effect of the larger, isopropyl group at C-4.

The amorphous 4-isopropylmuconolactone (165) was accurately weighed and the CD spectrum run in acetonitrile. The same solution was used directly to measure the UV spectrum. No CE peak was observable near 200 nm, although the curve was still positive in this region (Figure 2). Unexpectedly, a strong negative CE was observed at 217 nm, presumably arising from an n - $\pi^*$ transition. It is not clear why this CE is stronger than the negative CEs of the other muconolactones. Whether fortuitously or not, the CE curve shows the same change in sign, positive to negative, with increasing wavelength characteristic of all the other muconolactones with the absolute configuration. The UV spectrum showed the strong absorption expected for a butenolide, $\lambda_{max}$ 204 nm ($\Sigma$ 7330)

**Figure 2 CD Spectrum of 4-Alkylmuconolactones**

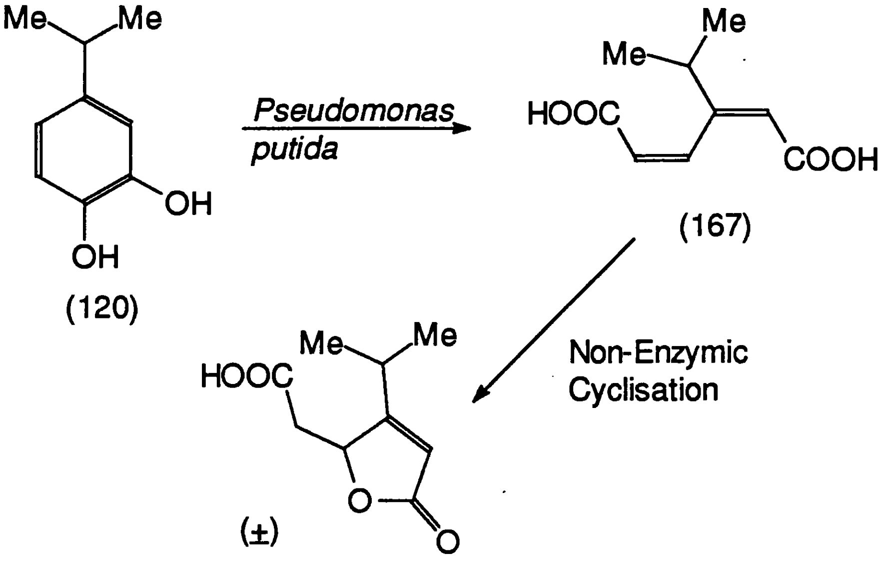


(I) 2-Methylmuconolactone, (II) 3-Methylmuconolactone (48), (III) 4-Methylmuconolactone (50) and (IV) 4-Isopropylmuconolactone (165)

3-Isopropylmuconolactone (152) was also isolated as an oil and identified by comparison of its spectrum with that of the synthetic racemate. In particular, signals were obtained as a multiplet at δ 5.35 - 5.45 and δ 5.86 (s, with fine splitting) for 4-H and 2-H, respectively. An IR absorption band was observed at 1720 $cm^{-1}$ and a molecular weight of 184.0722, ($C_9H_{12}O_4$ requires *M*, 184.0736). The 3-isopropylmuconolactone did not give any significant optical rotation and is thought to be formed from the non-enzymic cyclisation of the 3-isopropylmuconic acid (167) formed *in vivo* from 4-

isopropylpyrocatechol (Scheme 76). The absence of any signals for 3-isopropyl-*cis,trans*-muconic acid in the total extract showed that the cycloisomerase enzyme had efficiently cyclised the 3-isopropyl-*cis,cis*-muconic acid. It is known that 3-methyl-*cis,cis*-muconic acid isomerises readily, non-enzymically to the *cis,trans* isomer at pH 7.[80]

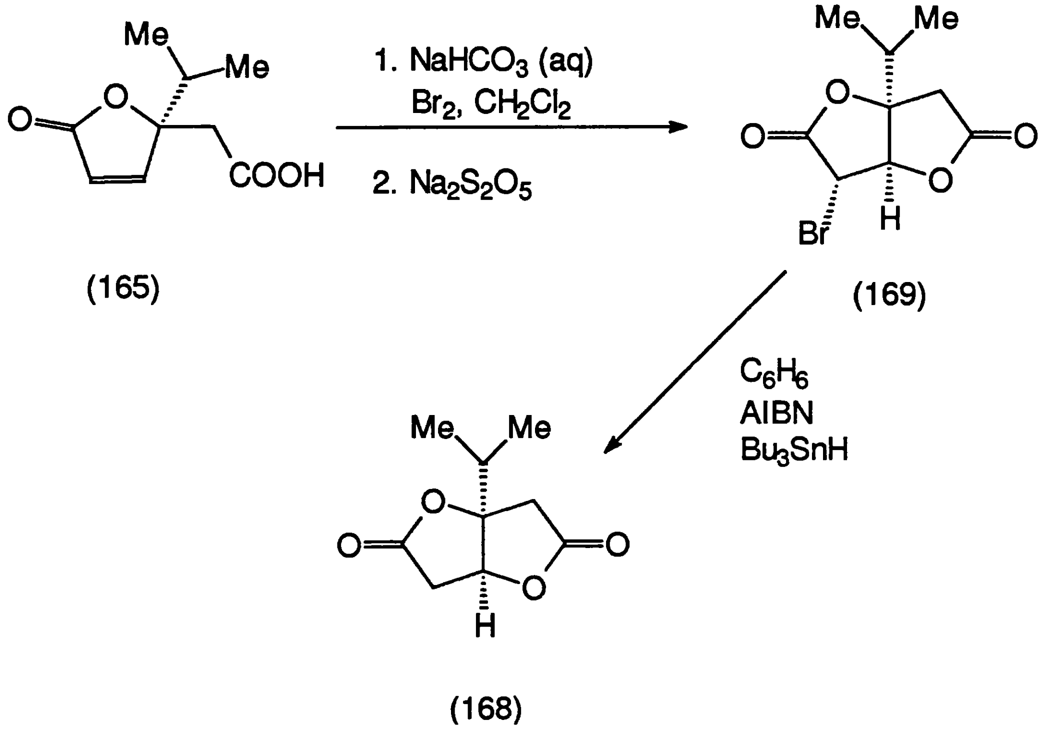
Scheme 76



(152)

(+)-4-isopropylmuconolactone (165) was then converted into the corresponding 1-isopropylmuconodilactone (168) *via* 1-isopropyl-4-bromomuconodilactone (169) which was required as a potential substrate for the cycloisomerase enzyme of *Rhodococcus rhodocrous* N75 (Scheme 77).

Scheme 77

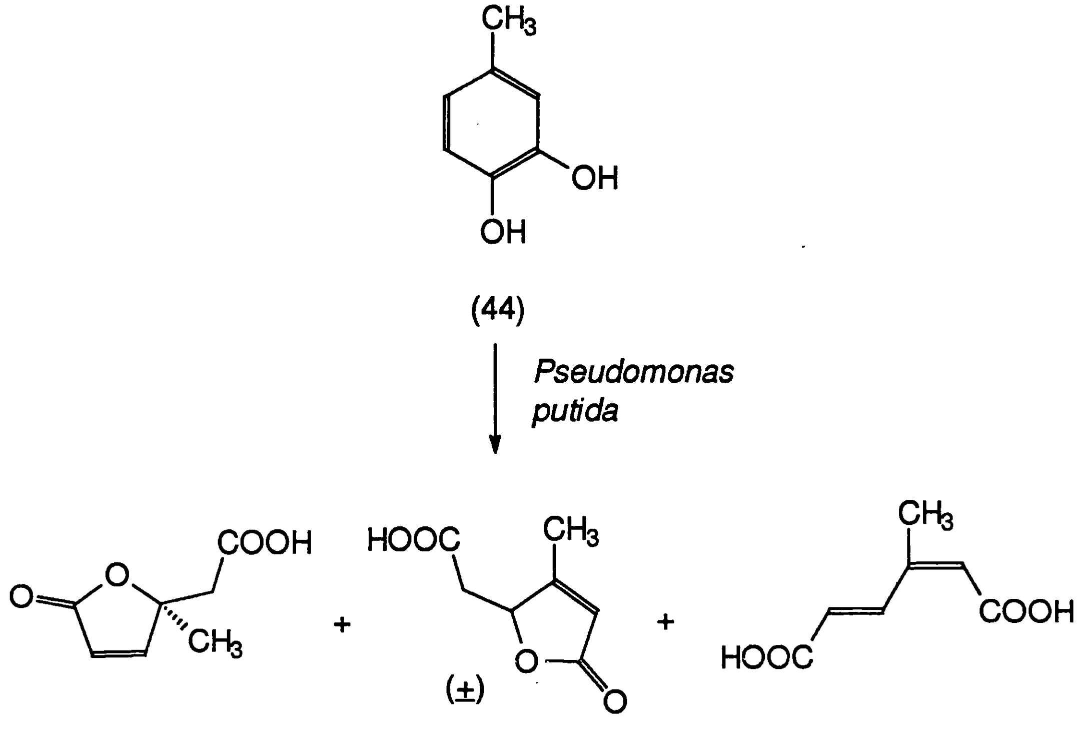


The lactone (165) was treated with bromine and sodium hydrogen carbonate in a two phase, water-dichloromethane system to give the 1-isopropyl-4-bromomuconodilactone (169) in high yield (91%) with an $[\alpha]_D$ - 49 ° (*c*, 0.6 in methanol). The $^1$H NMR spectrum of this product showed singlets at $\delta$ 4.49 and 5.08 expected for the *trans* protons 4-H and 5-H respectively, which have a torsion angle of 90 °. This bromodilactone (169) was treated at room temperature with tributyltin hydride, the reaction being initiated by warming with azoisobutrylnitrile, to give 1-isopropylmuconodilactone (168) as an oil with $[\alpha]_D$ - 117 ° (*c*, 0.3 in methanol). This dilactone (168) was characterised by the IR carbonyl stretching band at 1788 cm$^{-1}$. The high resolution mass spectrum gave a value for the molecular ion of 184.0746, ($C_9H_{12}O_4$ requires *M*, 184.0736).

As mentioned previously, 4-methylpyrocatechol (44) was fed during each feeding experiment as a control to ensure that the organism was functioning effectively. The 4-methylpyrocatechol (44) was fed in the same manner as described for 4-isopropylpyrocatechol (120). At pH 7.5 the ether extracts contained only trace quantities of the 4-methylpyrocatechol (44), whereas large quantities were recovered when the organism was not functioning effectively. At pH 2.5, the ether extracts were evaporated to give an off-white, oily solid. This oily solid would not dissolve fully in $(CD_3)_2CO$ or $CDCl_3$ to enable the total extract to be examined by $^1$H NMR. Therefore a portion of the total extract was dissolved in $(CD_3)_2SO$. In this way, the major component was identified as 4-methylmuconolactone (50). Also observed were quantities of 3-methylmuconolactone (51) and 3-methyl-2-*cis*-4-*trans*-muconic acid (157) (Scheme 78). Surprisingly, none of the corresponding *cis-trans*-isopropylmuconic acid was detected in the preceding experiment.

3-Methyl-2-*cis*-4-*trans*-muconic acid (157) was separated from the 4- and 3-methylmuconolactones (50) and (51) by centrifrugiation with acetone. This muconic acid (157) was obtained as a white crystalline solid, m.p. 176-177 °C. The $^1$H NMR spectrum showed the characteristic doublets at $\delta$ 6.15 and 8.45 with $J$ 16 Hz corresponding to the *trans* vinylic protons, while the *cis* proton gave a singlet at $\delta$ 5.96. Once this acid had been removed, the rest of the extract was purified by preparative TLC using the same method and conditions as for the products from 4-isopropylpyrocatechol (120). On examination of the TLC plates by UV light and after development with iodine vapour, approximately 10 bands were observed. Each of the bands were extracted as before and the extracts examined by $^1$H NMR spectroscopy.

**Scheme 78**



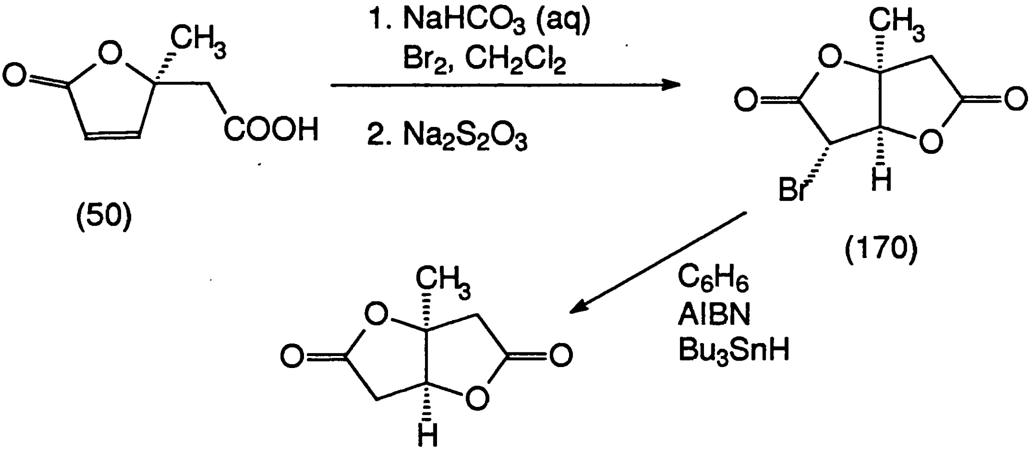
(44)

(50) (51) (157)

4-Methylmuconolactone (50), $R_F$0.4, and 3-methylmuconolactone (51), $R_F$ 0.2, were isolated successfully as white gums. Examination of the other bands showed few $^1$H NMR signals and none which corresponded to any possible metabolites. (*S*)-4-Methylmuconolactone (50) had $[\alpha]_D$ + 31 ° (*c*, 0.9 in methanol). This agrees with a literature value of $[\alpha]_D$ + 32 ° (*c*, 10.0 in $H_2O$).[74] In some literature references the (S)-4-methylmuconolactone (50) was successfully isolated as a white crystalline solid, m.p 49-51 °C.[175] However the optical rotation has the same value irrespective of the physical form. Once again, 3-methylmuconolactone (51) did not have an optical rotation and was thought to be formed from the non-enzymic cyclisation of 3-methyl-2-*cis*-4-*trans*-muconic acid (157) formed *in vivo* from 4-methylpyrocatechol (44).

As a trial for the conversion of the 4-isopropylmuconolactone (165) into the corresponding dilactone (168), cyclisation of 4-methylmuconolactone (50) was attempted first (Scheme 79). (*S*)-4-Methylmuconolactone (50) was treated with bromine as before to give the 1-methyl-4-bromomuconodilactone (170) as a white crystalline solid with m.p 123-124 °C with $[\alpha]_D$ - 68 ° (*c*, 0.9 in methanol). This bromodilactone was converted into the dilactone by debromination as before to produce 1-methylmuconodilactone (54) as a white crystalline compound with m.p 108-109 °C and $[\alpha]_D$ - 132 ° (*c*, 0.2 in methanol), which is in agreement with literature values.[175]

**Scheme 79**



(54)

The feeding of 4-isopropylpyrocatechol (120) to *Pseudomonas putida* was carried out on several occasions to determine the reproducibility of the experiment and to accumulate enough material to use as a substrate for *Rhodococcus rhodocrous* N75. Each time, 4-methylpyrocatechol (44) was fed as a control, which was important as on several occasions during this period it was found that the organism was not working effectively. Lower levels of enzymically oxidised products were produced and consequently larger quantities of unreacted pyrocatechols were recovered. Thus, 4-methylpyrocatechol served as an effective indicator of the efficiency of the organism.

### 2.3.1.1.1 Quantification of Metabolites by NMR Spectroscopy

A concern in the execution of the feeding experiments was the accurate quantification of the different metabolites produced. This was previously measured directly by the amount of metabolite recovered from preparative TLC. To assess the accuracy of quantification by preparative TLC, quantitative NMR measurements were carried out on the total metabolites extracted at pH 2.5. Thus an aliquot of the total extract was evaporated, completely dried and then weighed. This aliquot was dissolved in an appropriate deuteriated solvent and the $^1$H NMR spectrum was recorded. An known quantity of iodoform ($CHI_3$) was added to act as an internal standard for the proton integrals. The weight of $CHI_3$ was approximately equal to that of the 4-substituted muconolactone present. The $^1$H NMR spectrum of this mixture, when integrated allowed absolute quantities of the metabolites to be calculated. Iodoform proved to be an excellent compound for use as an internal standard as it has a high molecular weight per proton and is soluble in all the deuteriated solvents tested. By this quantatitive method, 4-isopropylmuconolactone (165) was found to be formed from the corresponding pyrocatechol in 31 % yield. Similarly, the yields of 3-isopropylmuconolactone (152) and 3-isopropyl-2-pyrone-5-carboxylic acid (166) were 6 and 5 %, respectively. These figures are consistent with those obtained by direct weighing of the metabolites after separation by preparative TLC, carried out on the same extract.

Quantitative NMR measurements were also carried out on a portion of the total material extracted at pH 2.5 from the feeding of 4-methylpyrocatechol (44) to *Pseudomonas putida*. Thus, 4-methylmuconolactone (50) was obtained in 46 % yield. This agrees with previously reported results.[162] 3-Methylmuconolactone (51) was produced in 6 % yield, and 3-methyl-2-*cis*-4-*trans*-muconic acid (157) in 15 % yield. Previous experiments with 4-methylpyrocatechol in *Pseudomonas putida* had not detected 3-methyl-2-*cis*-4-*trans*-muconic acid (157).[162] This is probably because the muconic acid is completely insoluble in the normal $CDCl_3$ and $(CD_3)_2CO$, the solvents previously used for examining these extracts.

In conclusion quantitative NMR measurements agree well with weighing after separation, but are more direct and not subject to losses during preparative TLC. Preparatitive TLC is an adequate method for quantification of some metabolites e.g. 4 and 3- substituted muconolactones. However some metabolites e.g. 3- substituted - *cis,trans-* muconic acids are lost during TLC and consequently cannot be quantified. The results of the feeding experiments with 4-isopropylpyrocatechol (120) are shown in Table 1 on page 99.

Table 1: Feedings of 4-isopropylpyrocatechol (120) to cultures of *Pseudomonas putida*

| Experiment | 1 | Methyl Control[b] | 2[c] | Methyl Control[a,c] | 3 | Methyl Control[c] | 4 | Methyl Control[c] |
|---|---|---|---|---|---|---|---|---|
| Total number of flasks used[a] | 15 | 8 | 16 | 8 | 14 | 6 | 14 | 6 |
| Amount (mg) of pyrocatechol fed | 375 | 200 | 400 | 200 | 350 | 150 | 350 | 150 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 91<br>(23%) | - | 90<br>(23%) | 5<br>(3%) | 72<br>(20%) | 2<br>(1%) | 104<br>(30%) | 6<br>(4%) |
| Total metabolites (mg) extracted at pH 2.5 | 245 | 181 | 185 | 185 | 204 | 125 | 153 | 110 |
| Yield (mg) of 4-alkyl-muconolactone | 160<br>(33%) | 122<br>(48.3%) | 149<br>(31%) | 115<br>(46%) | 132<br>(31%) | 71<br>(38%) | 111<br>(26%) | 64<br>(34%) |
| Yield (mg) of 3-alkyl-muconolactone | 34<br>(5%) | 18<br>(7%) | 26<br>(5%) | 15<br>(6%) | 16<br>(4%) | 18<br>(10%) | 8<br>(2%) | 14<br>(8%) |
| Yield (mg) of 3-alkyl-*cis-trans* muconic acid | - | 16<br>(6%) | - | 38<br>(15%) | - | 12<br>(7%) | - | 18<br>(10%) |
| Yield (mg) of 3-alkyl-pyrone acid | - | - | 22<br>(5%) | - | - | - | - | - |

[a] Each incubation flask contained 100 $cm^3$ medium.

[b] 4-Methylpyrocatechol was fed in parallel

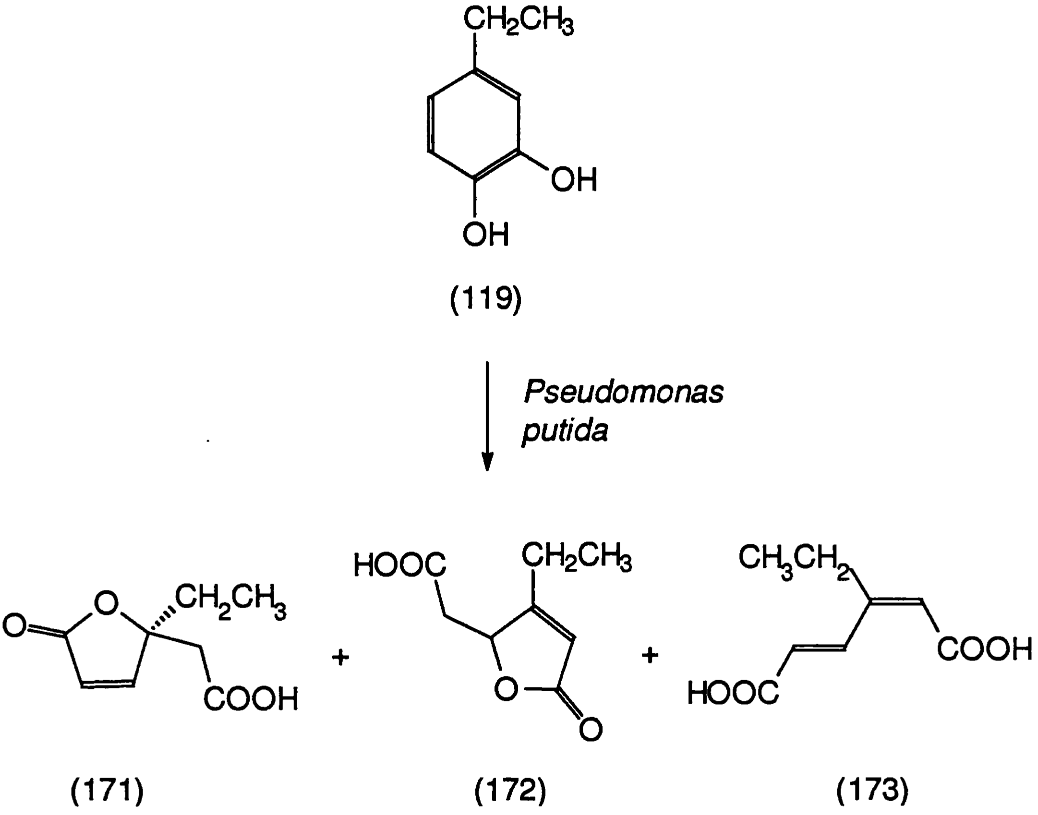
[c] The figures for metabolite yield have been measured from quantitative $^1H$ NMR spectroscopy.

### 2.3.1.2 Metabolism of 4-Ethylpyrocatechol (119)

4-Ethylpyrocatechol (119) has been fed on several previous occasions to *Pseudomonas putida* with different quantities of metabolites produced each time.[165, 172] It was therefore necessary to accurately quantify the amounts of metabolites produced using quantitative $^1$H NMR measurements and using 4-methylpyrocatechol (44) as a control to ensure the organism was functioning effectively.

Unmetabolised 4-ethylpyrocatechol (119) was extracted at pH 7.5. The ether extracts of the medium at pH 2.5 were evaporated *in vacuo* to give a mid-brown, oily solid. The $^1$H NMR in $(CD_3)_2CO$ showed the presence of three metabolites. 4-Ethylmuconolactone (171) was the major metabolite, showing a characteristic doublet of doublets at $\delta$ 6.05 and 7.56 ($J$ 5.7 Hz) for 2- and 3-H. 3-Ethylmuconolactone (172) was identified by the characteristic signals at $\delta$ 5.19 - 5.25 (multiplet, 4-H) and $\delta$ 5.80 (s, 2-H). This product had been detected by $^1$H NMR spectroscopy before but had not been isolated.[165] Also observed was 3-ethyl-2-*cis*-4-*trans*-muconic acid (171) with characteristic doublets at $\delta$ 6.27 and 8.66 ($J$ 15.7 Hz) corresponding to the protons on the *trans* double bond, and a singlet at $\delta$ 6.02 for the proton of the *cis* double bond (Scheme 80). As 4-ethylmuconolactone (171) had been previously isolated and characterised it was not necessary to purify the extract by TLC.[172] Quantification was instead executed by $^1$H NMR spectroscopy using iodoform as an internal standard.

**Scheme 80**



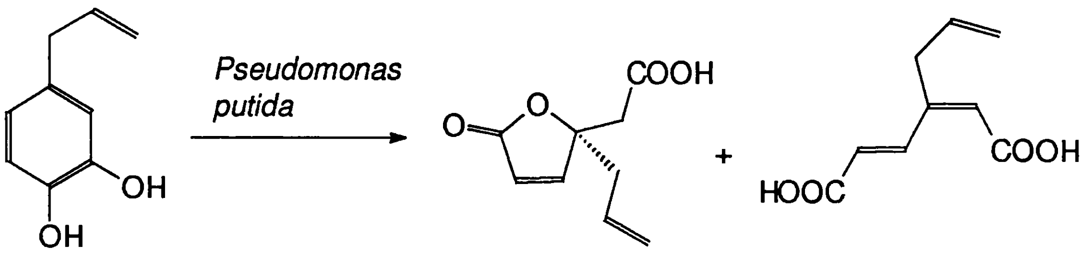
Quantitative NMR measurements on the total metabolites extracted at pH 2.5 showed conversion of 4-ethylpyrocatechol (119) into 4-ethylmuconolactone (171) in 46 % yield. 3-Ethylmuconolactone (172) and 3-ethyl-2-*cis*-4-*trans*-muconic acid (173) were formed in 7 and 6 % yield respectively. These results are in broad agreement with work previously carried out for 4-ethylmuconolactone, although no figures were available for the conversion into 3-ethylmuconolactone (172) and there was no indication that 3-ethyl-2-*cis*-4-*trans*-muconic acid (173) was present.[165, 172] The results of the feeding data for this experiment with 4-ethylpyrocatechol (119) are shown in Table 4 on page 109.

### 2.3.1.3 Metabolism of 4-Allylpyrocatechol (125)

Since 4-isopropylpyrocatechol (120) was efficiently metabolised by *Pseudomonas putida* it was of interest to find if the organism would accommodate a longer chain length in the 4- position of pyrocatechol. It was decided to feed 4-allylpyrocatechol (125) to the organism as outlined previously. Unreacted 4-allylpyrocatechol (125) was recovered during the ether extracts of the culture medium at pH 7.5. At pH 2.5, the ether extracts were evaporated *in vacuo* to give a dark brown gum. The total extract was examined by $^1$H NMR spectroscopy, giving a complex spectrum partly due to the vinyl protons on the allyl group having shifts comparable to the vinyl protons in the muconolactone. 4-Allylmuconolactone (174) was observed as one of the major metabolites with the characteristic doublet of doublets at $\delta$ 6.11 and 7.74 ($J$ 5.7 Hz). Also observed were the characteristic signals for the olefinic protons due to 3-allyl-2-*cis*-4-*trans*-muconic acid (175), namely a doublet of doublets at $\delta$ 6.29 and 8.64 ($J$ 16.2 Hz), and a singlet at $\delta$ 6.02. 3-Allylmuconolactone could not be observed in the $^1$H NMR of the total extract at pH 2.5 (Scheme 81).

The total extract was then purified by preparative TLC using the same procedure as 4-isopropylpyrocatechol (120). 4-Allylmuconolactone (174) was isolated as a viscous gum with an $[\alpha]_D$ + 10 ° ($c$, 0.5 in methanol). 3-Allyl-2-*cis*-4-*trans*-muconic acid (175) and 3-allylmuconolactone were not isolated although (175) was detected in the $^1$H NMR spectrum of the total extract at pH 2.5.

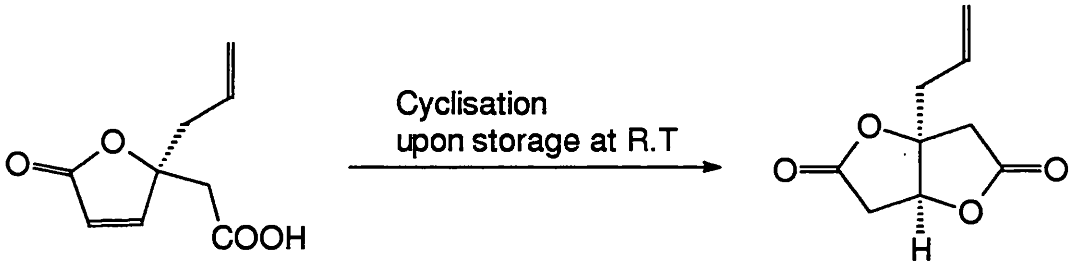
**Scheme 81**



(125) (174) (175)

It was found that the solid gum 4-allylmuconolactone (174) spontaneously cyclised upon storage at ambient temperature to give (-)-1-allylmuconodilactone (176), a white crystalline compound with m.p 114 -115 °C and $[\alpha]_D$ - 82 ° (*c*, 0.3 in methanol) (Scheme 82). As the allylmuconodilactone had been obtained this way there was no requirement to attempt its synthesis *via* the bromodilactone, with the concomitant problem of bromination of the allylic double bond.

**Scheme 82**



(174) (176)

The results of the feeding experiments with 4-allylpyrocatechol (125) are shown in Table 3 on page 104.

Table 2: Feedings of 4-allylpyrocatechol (125) to cultures of *Pseudomonas putida*

| Experiment | 1 | Methyl Control[b] | 2 | Methyl Control[b] | 3 | Methyl Control[b] | 4 | Methyl Control[b] |
|---|---|---|---|---|---|---|---|---|
| Total number of flasks used[a] | 15 | 8 | 15 | 8 | 17 | 6 | 15 | 8 |
| Amount (mg) of pyrocatechol fed | 375 | 200 | 375 | 200 | 425 | 150 | 375 | 200 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 40<br>(11%) | - | 20<br>(5%) | 4<br>(2%) | 31<br>(7%) | - | 57<br>(15%) | 3<br>(1%) |
| Total metabolites (mg) extracted at pH 2.5 | 150 | 181 | 196 | 175 | 280 | 143 | 130 | 147 |
| Yield (mg) of 4-alkyl-muconolactone | 48<br>(11%) | 122<br>(48.3%) | 43<br>(9%) | 108<br>(43%) | 61<br>(12%) | 115<br>(61%) | 36<br>(8%) | 80<br>(32%) |
| Yield (mg) of 3-alkyl-muconolactone | - | 18<br>(7%) | - | 11<br>(4%) | - | 16<br>(9%) | - | 19<br>(7%) |
| Yield (mg) of 3-alkyl-*cis-trans* muconic acid | 40[c]<br>(9%) | 16<br>(6%) | - | 15<br>(6%) | 45[c]<br>(9%) | 10<br>(5%) | - | 22<br>(9%) |

[a] Each incubation flask contained 100 $cm^3$ medium.

[b] 4-Methylpyrocatechol was fed in parallel

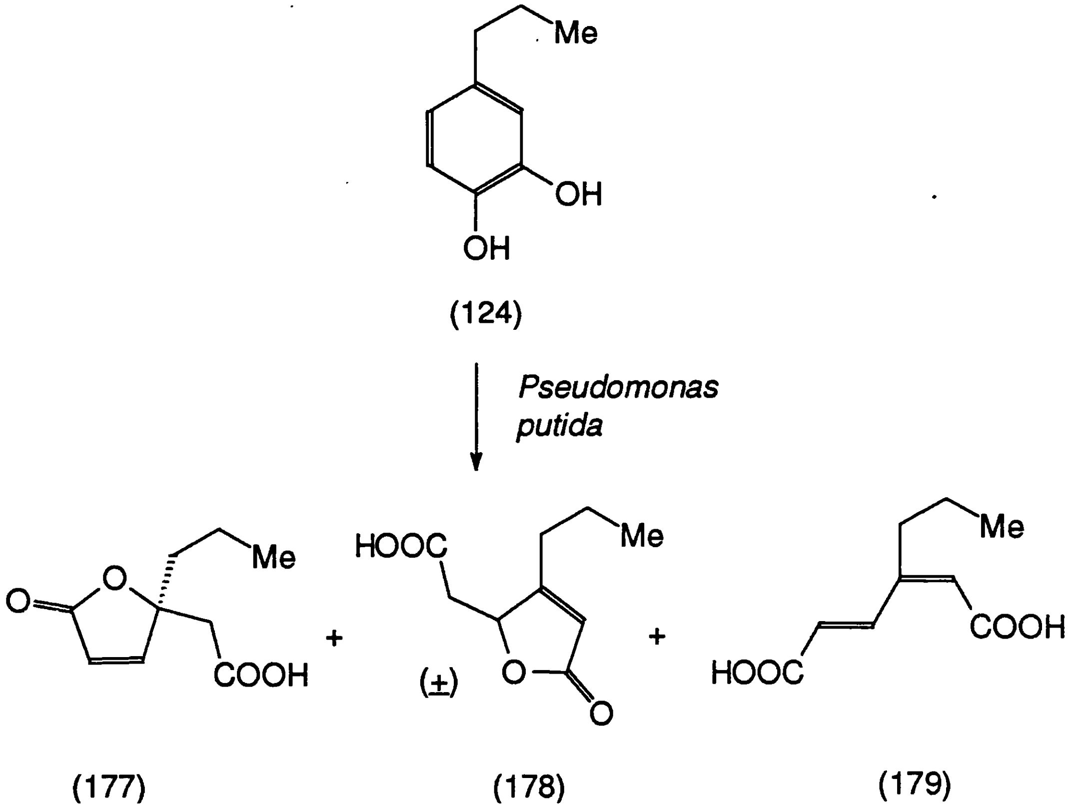
[c] The figures for metabolite yield have been measured from $^1H$ NMR spectroscopy.

### 2.3.1.4 Metabolism of 4-Propylpyrocatechol (124)

To further investigate the effect of the size of the hydrocarbon chain on the 4-position in the degradation of pyrocatechol by *Pseudomonas putida* it was decided to feed 4-propylpyrocatechol (124). It was expected that cleavage would occur in the same manner as for 4-allylpyrocatechol (125). The unmetabolised 4-propylpyrocatechol (124) was recovered at pH 7.5 as usual. The ether extracts of the medium at pH 2.5 were evaporated *in vacuo* to give a dark-brown, tar-like gum. The $^1$H NMR spectrum in $(CD_3)_2CO$ showed 3 metabolites present in equal amounts (Scheme 83). 4-Propylmuconolactone (177) was recognised by the characteristic doublet of doublets at δ 6.08 and 7.73 (*J* 5.7 Hz), corresponding to 2- and 3-H respectively. 3-Propylmuconolactone (178) was also observed by signals at 5.30 (ddd, *J* 8.5, 3.7 and 1.6 Hz) and δ 5.86 (s with fine splitting) for 4-H and 2-H respectively. 3-Propyl-2-*cis*-4-*trans*-muconic acid (179) gave doublets at δ 6.25 and 8.63 (*J* 16.2 Hz) from the *trans* olefinic protons, and the *cis* olefinic proton gave a singlet at δ 5.99.

As the total extract was isolated as a tar-like gum, it was not possible to remove the 3-propyl-2-*cis*-4-*trans*-muconic acid (179) by suspension in acetone and centrifugation, as was the case with 3-methyl-2-*cis*-4-*trans*-muconic acid (179). The total extract was instead purified by preparative TLC as before.

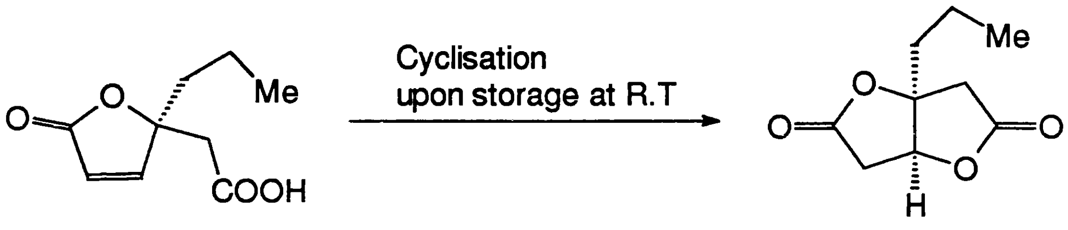
**Scheme 83**



4-Propylmuconolactone (177) was obtained as an off-white gum, $[\alpha]_D$ + 11 ° (*c*, 0.7 in methanol). The IR spectrum showed the carbonyl absorption at 1750 $cm^{-1}$, and a high resolution mass spectrum gave a molecular weight of 184.0745 ($C_9H_{12}O_4$, requires *M*, 184.0735).

3-Propylmuconolactone (178) was obtained as a yellow gum, and had no optical rotation. Again it was believed to arise from the non-enzymic cyclisation of the intermediate 3-propyl-*cis-cis*-muconic acid. As with the (+)-4-allylmuconolactone it was found that the (+)-4-propylmuconolactone (177) cyclised spontaneously upon storage at ambient temperature to give the 1-propyldilactone (180) as a gum with $[\alpha]_D$ - 98 ° (*c*, 0.5 in methanol) (Scheme 84). This dilactone (180) was characterised by the IR carbonyl stretching frequency at 1789 cm$^{-1}$. The high resolution mass spectrum gave a value for the molecular ion of 184.0728 ($C_9H_{12}O_4$, requires *M*, 184.073. Unfortunately no 3-propyl-2-*cis*-4-*trans*-muconic acid (179) was recovered. Having acquired the (-)-1-propylmuconodilactone, it was not neccessary to synthesise this dilactone *via* the bromodilactone.

**Scheme 84**



(177) (180)

The results of these feeding experiments are contained in the Table 3 on page 108.

Table 3: Feedings of 4-n-propylpyrocatechol (124) to cultures of *Pseudomonas putida*

| Experiment | 1 | Methyl Control[b] | 2 | Methyl Control[b] | 3 | Methyl Control[b] | 4 | Methyl Control[b] |
|---|---|---|---|---|---|---|---|---|
| Total number of flasks used[a] | 15 | 8 | 17 | 6 | 14 | 6 | 14 | 6 |
| Amount (mg) of pyrocatechol fed | 375 | 200 | 425 | 150 | 350 | 150 | 350 | 150 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 72<br>(20%) | 3<br>(1%) | 84<br>(20%) | - | 86<br>(25%) | - | 105<br>(30%) | 5<br>(4%) |
| Total metabolites (mg) extracted at pH 2.5 | 274 | 147 | 280 | 143 | 261 | 103 | 247 | 89 |
| Yield (mg) of 4-alkyl-muconolactone | 72<br>(16%) | 80<br>(32%) | 69<br>(13%) | 115<br>(61%) | 74<br>(16%) | 94<br>(50%) | 52<br>(12%) | 69<br>(37%) |
| Yield (mg) of 3-alkyl-muconolactone | 66<br>(15%) | 19<br>(7%) | 62<br>(12%) | 16<br>(9%) | 62<br>(15%) | 16<br>(9%) | 48<br>(11%) | 21<br>(11%) |
| Yield (mg) of 3-alkyl-*cis-trans* muconic acid | 72<br>(16%)[c] | 22<br>(9%) | - | 10<br>(5%) | - | 8<br>(4%) | - | 13.6<br>(7%) |

[a] Each incubation flask contained 100 $cm^3$ medium.

[b] 4-Methylpyrocatechol (44) was fed in parallel

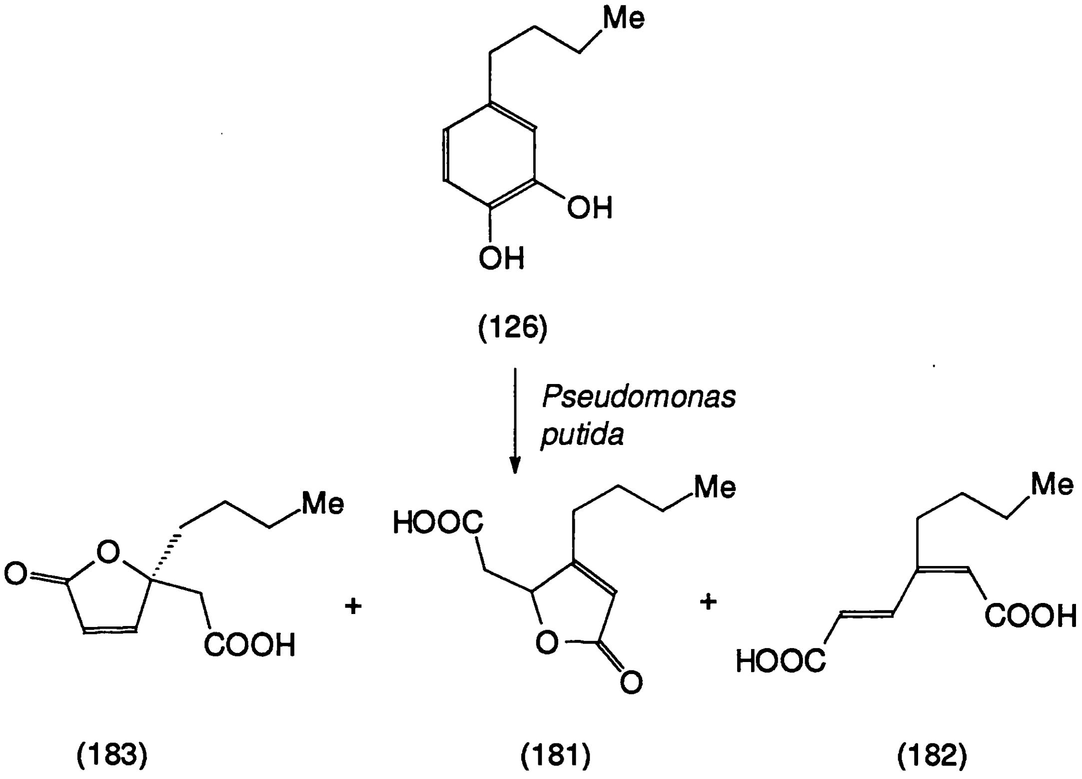
[c] The figures for metabolite yield have been measured from $^1H$ NMR spectroscopy.

### 2.3.1.5 Metabolism of 4-n-Butylpyrocatechol (126)

Since 4-propylpyrocatechol was less efficiently metabolised than the derivatives with shorter alkyl groups, 4-n-butylpyrocatechol (126) was tested as a substrate.

Unmetabolised 4-butylpyrocatechol (126) was recovered at pH 7.5. The total metabolites extracted at pH 2.5 the total were examined by $^1$H NMR spectroscopy. This spectrum indicated that the major components were 3-butylmuconolactone (181) and 3-butyl-2-*cis*-4-*trans*-muconic acid (182). Also present were much smaller amounts of 4-butylmuconolactone (183). However, the combined amount of metabolites was distinctly less than with the foregoing pyrocatechols and a larger amount of 4-butylpyrocatechol was recovered (Scheme 85).

**Scheme 85**



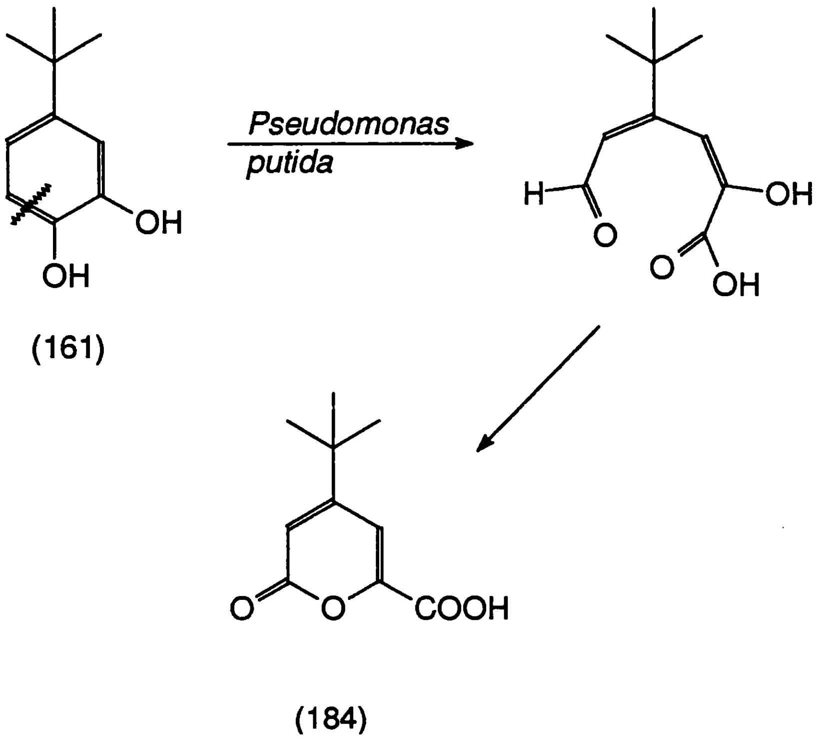
The $^1$H NMR spectrum indicated that the metabolites (183), (181) and (182) were present in a ratio of 1:3:3. As the 4-n-butylmuconolactone (183) was present in very small quantities in the total extract it was decided not to pursue the separation of these components by preparative thin layer chromatography.

The results of this feeding experiment is contained in Table 4 on page 114.

### 2.3.1.6 Metabolism of 4-t-butylpyrocatechol (161)

Having fed 4-butylpyrocatechol (126) and discovered that the chain length of the 4 carbons is not easily accomodated by the dioxygenase enzyme, we decided to feed 4-t-butylpyrocatechol (161) to see if the bulkier but shorter (two carbon) group would be more acceptable to the organism. 4-t-Butylpyrocatechol (161) was recovered from the medium at pH 7.5. The $^1$H NMR spectrum of the metabolites extracted at pH 2.5 showed that few products were present. Neither the 4- or 3- substituted t-butylmuconolactones were present, nor was the 3-t-butyl-2-*cis*-4-*trans*-muconic acid. Several signals were present at $\delta$ 7.1 and 6.2. It was decided to chromatograph this extract in order to identify the metabolites present. Preparative TLC using the standard conditions gave 3-t-butyl-2-pyrone-5-carboxylic acid (184) as a yellow gum with $R_F$ 0.5. The $^1$H NMR spectrum showed two singlets with fine splitting at $\delta$ 7.24 and 6.33 corresponding to the vinyl protons. The structure (184) was established by IR and $^1$H and $^{13}$C NMR spectroscopy and accurate mass measurement. This product is thought to have been formed *via meta* cleavage rather than *ortho* cleavage, suggesting that the tertiary butyl group was not accommodated by the 1,2-dioxygenase. Therefore, 2,3-dioxygenase activity was induced instead (Scheme 86).

Scheme 86



(161)

(184)

The results of this feeding experiment are contained in Table 4 on page 114.

### 2.3.1.7 Metabolism of 4-Trifluoromethylpyrocatechol (121)

4-Trifluoromethylpyrocatechol (121) was fed to *Pseudomonas putida* to investigate the effect on dioxygenase cleavage of a trifluoromethyl group as opposed to a simple methyl group. The trifluoromethyl group and methyl group are very similar in size but have very different electronic properties. Thus the Hammett constants, $\sigma_m$, are + 0.46 and - 0.06 for $CF_3$ and $CH_3$ respectively.

Unmetabolised 4-trifluoromethylpyrocatechol (121) was recovered at pH 7.5. The metabolites extracted at pH 2.5 gave a dark-brown tar. The total extract was examined by $^1$H NMR spectroscopy, which showed few distingishable proton signals. This extract was then chromatographed to identify any metabolites present.

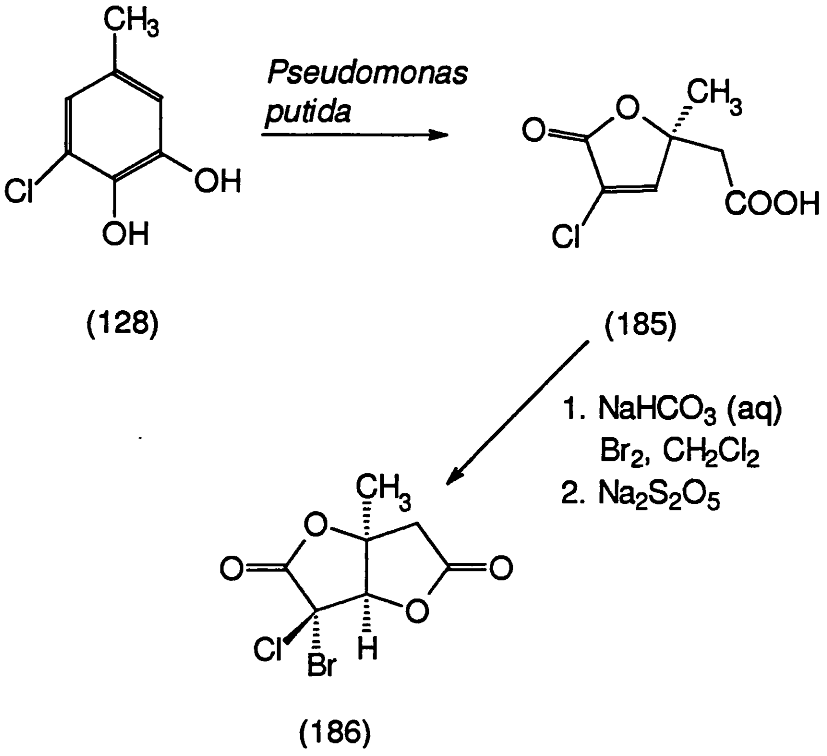
However, preparative TLC using the standard conditions did not yield any metabolites. Several bands were extracted but the NMR spectra of the extracts showed few proton signals. It was therefore concluded that the electron-withdrawing character of the trifluoromethyl group was unacceptable to the dioxygenase enzyme.

The results of this feeding experiment are contained in Table 5 on page 115.

### 2.3.1.8 Metabolism of 3-Chloro-5-methylpyrocatechol (128)

3-Chloro-5-methylpyrocatechol (128) was fed to *Pseudomonas putida* to examine the effect on ring cleavage of a chlorine in the 3 position of 4-methylpyrocatechol. It was also hoped that this would lead to the formation of 2-chloro-4-methylmuconolactone (185) which could then be converted into the corresponding bromodilactone (186) to determine the configuration of lactones acquired enzymically from the feeding of 2-chloro-4-methylphenoxyacetate to *Pseudomonas sp.* B13 by D.H.Pieper [50, 96, 102, 107] (Scheme 87).

Scheme 87



However, 3-chloro-5-methylpyrocatechol (128) was recovered in large quantities, thus suggesting that the dioxygenase enzyme did not readily accept this pyrocatechol as a substrate. The ether extracts of the medium at pH 2.5 gave a dark-brown oil. The total extract was examined by $^1$H NMR spectroscopy. Few proton signals present corresponded to any of the metabolites expected to have arisen. Preparative TLC did not give any metabolites. It was therefore concluded that the dioxygenase enzyme of *Pseudomonas putida* would not accept the chloro derivative (128) as a substrate.

The results of this feeding experiment are contained in Table 5 on page 115.

**Table 4: Feedings of other 4-Alkylpyrocatechol to cultures of *Pseudomonas putida***

| Experiment | 4-Ethyl pyrocatechol (119)[c] | Methyl Control[b] | 4-t-Butyl-pyrocatechol (161) | Methyl Control[b] | 4-n-Butyl-pyrocatechol (126) | Methyl Control[b] |
|---|---|---|---|---|---|---|
| Total number of flasks used[a] | 16 | 8 | 15 | 8 | 15 | 7 |
| Amount (mg) of pyrocatechol fed | 400 | 200 | 375 | 200 | 375 | 175 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 2<br>(1%) | 5<br>(3%) | 238<br>(68 %) | 10.2<br>(5%) | 242<br>(64 %) | 6<br>(2%) |
| Total metabolites (mg) extracted at pH 2.5 | 382 | 185 | 86 | 155 | 108 | 117 |
| Yield (mg) of 4-alkyl-muconolactone | 224<br>(46%) | 115<br>(46%) | - | 107<br>(43%) | 1[d] | 91<br>(41%) |
| Yield (mg) of 3-alkyl-muconolactone | 36<br>(7%) | 15<br>(6%) | - | 10<br>(4%) | 3[d] | 3<br>(2%) |
| Yield (mg) of 3-alkyl-*cis-trans* muconic acid | 28<br>(6%) | 38<br>(15%) | - | 16<br>(6%) | 3[d] | 10<br>(4%) |
| Yield (mg) of 3-alkyl pyrone acid | - | 38<br>(15%) | 11<br>(3%) | - | - | - |

[a] Each incubation flask contained 100 $cm^3$ medium.

[b] 4-Methylpyrocatechol was fed in parallel

[c] The figures for metabolite yield have been measured from quantitative $^1H$ NMR spectroscopy.

[d] Ratio of metabolities calculated from $^1H$ NMR spectroscopy (1:3:3).

Table 5: Feedings of other 4-Alkylpyrocatechol to cultures of *Pseudomonas putida*

| Experiment | 2-Chloro-5-methyl pyrocatechol (128) | Methyl Control[b] | 4-Triflurometbyl-pyrocatechol (121) | Methyl Control[b] |
|---|---|---|---|---|
| Total number of flasks used[a] | 15 | 8 | 15 | 7 |
| Amount (mg) of pyrocatechol fed | 375 | 200 | 180[c] | 175 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 50<br>(13%) | 10<br>(5%) | 31<br>(17%) | 6<br>(2%) |
| Total metabolites (mg) extracted at pH 2.5 | 185 | 155 | 26 | 117 |
| Yield (mg) of 4-alkyl-muconolactone | - | 107<br>(43%) | - | 91<br>(41%) |
| Yield (mg) of 3-alkyl-muconolactone | - | 10<br>(4%) | - | 3<br>(2%) |
| Yield (mg) of 3-alkyl-*cis-trans* muconic acid | - | 16<br>(6%) | - | 10<br>(4%) |

[a] Each incubation flask contained 100 $cm^3$ medium.

[b] 4-Methylpyrocatechol was fed in parallel.

[c] Quantity of 4-Triflurometbylpyrocatechol (121) that was available.

### 2.3.2 In *Aspergillus niger*

Several pyrocatechols were fed to an *Aspergillus niger* mutant (that accumulates 3-methylmuconolactone) for the following reasons:

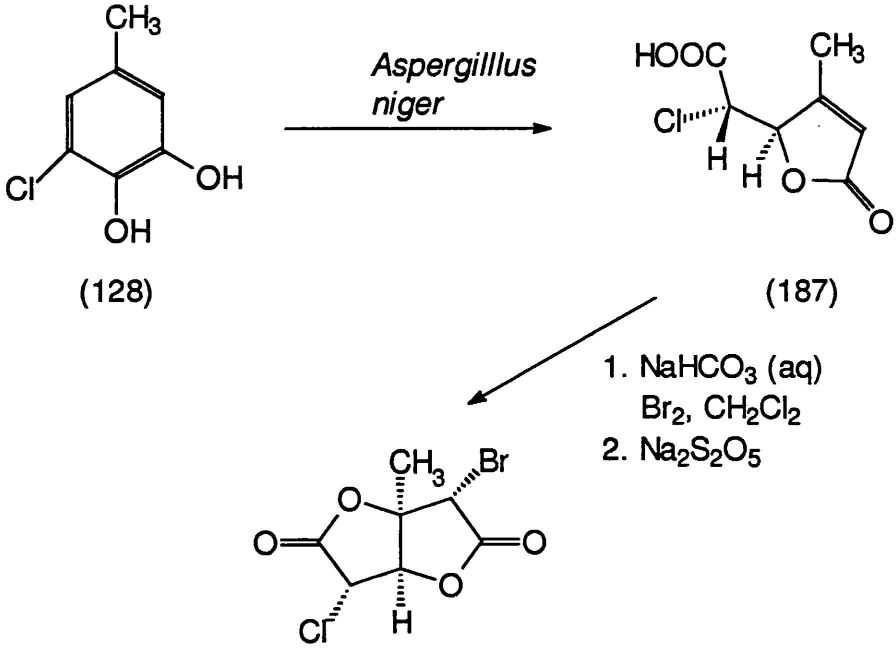
1. 3-Chloro-5-methylpyrocatechol (128) was fed to elucidate the configuration of the 5-chloro-4-methylmuconolactones produced by Pieper.[50, 96, 102, 107]
2. 4-Isopropylpyrocatechol (120) and 4-propylpyrocatechol (124), which were efficiently metabolised by *Pseudomonas putida*, were fed as possible substrates for the dioxygenase and cycloisomerase enzymes in *Aspergillus niger*.

All the pyrocatechols were fed to cultures of *Aspergillus niger*. The utilization of glucose in the original glucose-salts medium was monitored by UV spectrometry and when all the glucose had been consumed the substrates were fed in two batches over a period of 24 hours. The pH of the broth was maintained at pH 5.5 throughout the feeding experiment. The contents of the culture flasks were combined 24 hours after the second feeding and filtered to remove the mycelium. The pH was adjusted to 7.2 and extraction with ether was performed to remove any non-acidic material. The broth was then adjusted to pH 2.0 with phosphoric acid and extracted with ether. After this manual extraction the broth was then continuously extracted with ether for 48 hours. As with *Pseudomonas putida*, 4-methylpyrocatechol was fed as a control each time.

### 2.3.2.1 Metabolism of 3-Chloro-5-methylpyrocatechol (128) and 4-Methylpyrocatechol (44)

3-Chloro-5-methylpyrocatechol (128) was fed to *Aspergillus niger* in an attempt to produce the corresponding 'fungal' lactone 5-chloro-3-methylmuconolactone (187), in order to assist with the determination of the configuration of the lactones isolated in work carried out by Pieper (Scheme 88).[50,96,102,107] In structure (187), *syn* cyclisation onto the usual face of the double bond has been assumed.

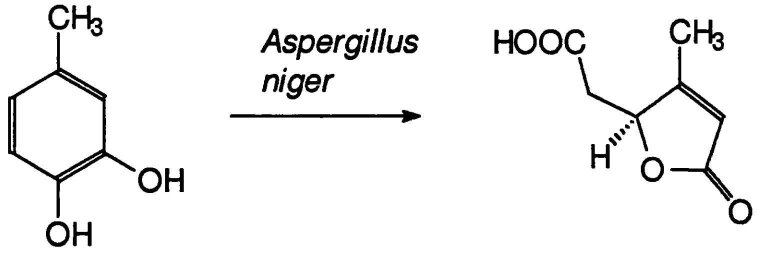
**Scheme 88**



Extraction at pH 7.2 gave 3-chloro-5-methylpyrocatechol (128) in large amounts. The ether extracts of the medium at pH 2.0 were evaporated to give a mid-brown oily solid. After manual extraction of the medium at pH 2.0 the remaining aqueous broth was extracted continuously with ether for 48 hours. The extracts were dried and evaporated to give a further oily solid. Both extracts were examined separately by $^1$H NMR spectroscopy. However, neither spectrum showed proton signals that corresponded to any likely metabolic products. Preparative TLC on both the manual and continuous extract using the standard solvent system, of diisopropyl ether: formic acid: water (200:7:3), failed to give any metabolites, although some highly coloured bands were removed which showed few proton signals when examined by $^1$H NMR spectroscopy. 3-Chloro-5-methylpyrocatechol (128) was fed several times to *Aspergillus niger*, but each time the fungus failed to convert any of the pyrocatechol into recognisable metabolites.

4-Methylpyrocatechol (44) was fed as a control each time to *Aspergillus niger* to ensure that the organism was functioning as normal. This was particularly important with *Aspergillus niger*, as on several occasions it failed to metabolise 4-methylpyrocatechol (45) and a new batch of the organism had to be used. Unmetabolised 4-methylpyrocatechol (44) was recovered from the medium at pH 7.2. At pH 2.0, manual extraction gave an oily solid. The remaining broth was then continuously extracted with ether. $^1$H NMR spectra of both extracts showed 3-methylmuconolactone (48) to be present as expected.

**Scheme 89**

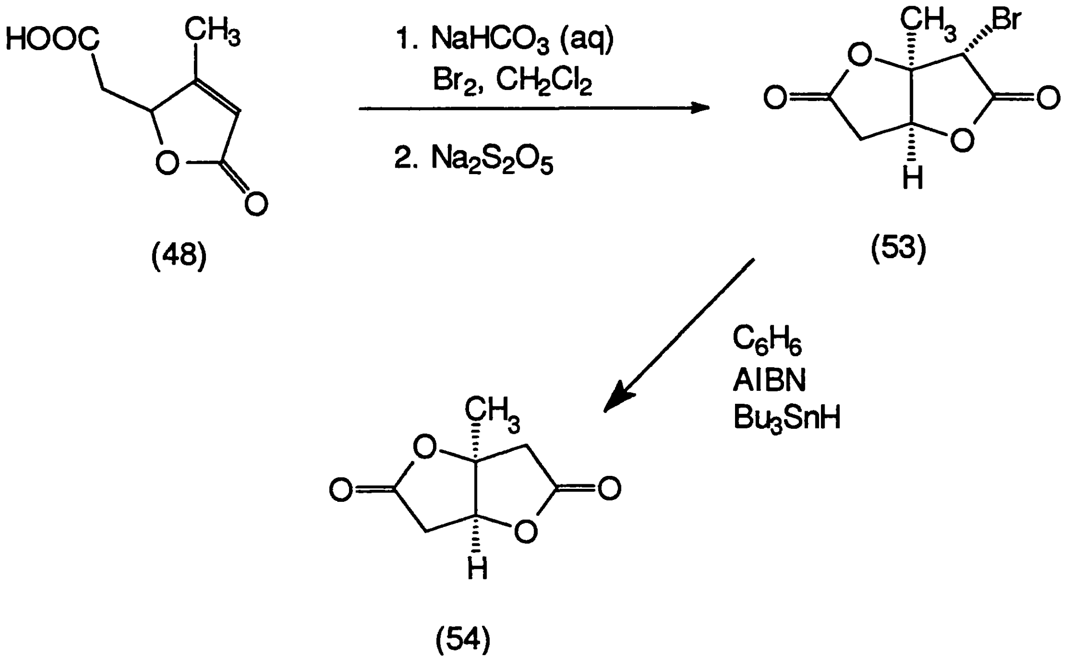


(44) (48)

Preparative TLC under the standard conditions recovered 3-methylmuconolactone (48) as an off-white gum with $[\alpha]_D$ - 26 ° (*c*, 0.3 in methanol). However, this rotation is low in comparison to the literature value, $[\alpha]_D$ - 36 ° (*c*, 10.9 in water).[177] The IR spectrum showed the characteristic carbonyl stretching frequency at 1732 $cm^{-1}$, while high resolution mass spectroscopy gave a molecular weight of 156.0419, ($C_7H_8O_4$, requires *M*, 156.0422).

It was of concern that, after dioxygenase cleavage of the pyrocatechol in *Aspergillus niger*, the resulting *cis,cis*-muconic acid would cyclise non-enzymically to form the racemic 3-methylmuconolactone (51). Therefore further confirmation of the functional activity of the enzyme in the organism was achieved by conversion of (-)-3-methylmuconolactone (48) to the corresponding bromodilactone (53) using the standard method.[152] Debromination using tributyltin hydride gave the (-)-1-methylmuconodilactone (54) with $[\alpha]_D$ - 130 ° (*c*,1.2 in methanol), which is in accordance with literature values.[175]

Scheme 90



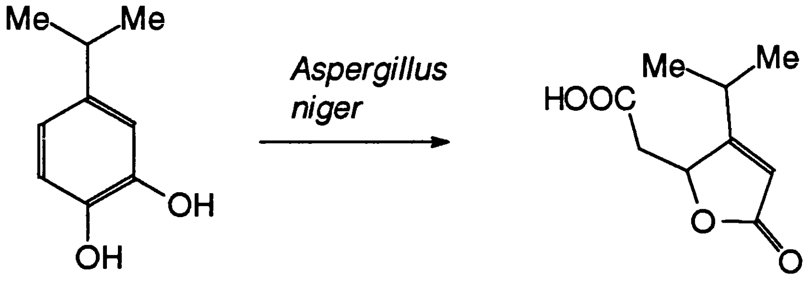
The results of these feeding experiments are contained in Table 6 on page 123.

### 2.3.2.2 Metabolism of 4-Isopropylpyrocatechol (120)

4-Ethylpyrocatechol (119) had been shown previously into be converted into the corresponding ‘fungal’ lactone 3-ethylmuconolactone in a modest yield of 7 %.[172] It was decided that 4-isopropylpyrocatechol (120) should be fed to see if the organism could cope with a larger alkyl group, of the same chain length (C2) in the 4-position.

Extraction at pH 7.2 gave unmetabolised 4-isopropylpyrocatechol (120). The ether extracts of the medium at pH 2.0 were evaporated to give a dark brown oily solid. Continuous ether extraction of the aqueous broth after this manual extraction produced further quantities of a brown oily solid. $^1$H NMR spectra of both of these extracts showed very small quantities of 3-isopropylmuconolactone (189) with characteristic $^1$H NMR signals at δ 5.36 (ddd, *J* 8.5, 3.9 and 1.6 Hz) and 5.85 (s with fine splitting) for 4-H and 2-H respectively. Preparative TLC of both of these extracts gave small quantities of 3-isopropylmuconolactone (189) as an off-white gum.

**Scheme 91**



(120) (189)

3-Isopropylmuconolactone (189) was characterised by the IR carbonyl stretching frequency at 1757 cm$^{-1}$. The high resolution mass spectrum gave a molecular weight of 184.0727 ($C_9H_{12}O_4$ requires *M*, 184.0736). Unfortunately, such small quantities of (189) were obtained that an accurate $[\alpha]_D$ reading could not be obtained. It was therefore not determined whether the 3-isopropylmuconolactone (189) was formed by the methylmuconolactone cycloisomerase or by non-enzymic cyclisation. It can, however, be concluded that the dioxygenase enzyme present in *Aspergillus niger* was capable to some extent of cleaving 4-isopropylpyrocatechol (120).

The results of this feeding experiment are shown in Table 6 on page 123.

### 2.3.2.3 Metabolism of 4-n-Propylpyrocatechol (124)

Although 4-isopropylpyrocatechol (120) was only metabolised to a very small extent, 4-n-propylcatechol (124) was fed to complete the experiments with the fungus.

4-Propylpyrocatechol (124) was recovered upon manual extraction with ether of the medium at pH 7.2. Acidification to pH 2.0 and manual ether extraction followed by continuous extraction for 48 hours produced black, tar-like solids in small quantities. Both extracts were examined by $^1H$ NMR spectroscopy, but showed few signals that corresponded to any expected metabolites. Attempts were not made to purify these extracts by TLC as the $^1H$ NMR spectra suggested that no enzymic metabolites were present.

The results of this feeding experiment are shown in Table 6 on page 123.

**Table 6: Feedings of 4-alkylpyrocatechols to cultures of *Aspergillus niger*.**

| Experiment | 2-chloro-5-methyl pyrocatechol (128) | Methyl Control[b] | 4-isopropyl-pyrocatechol (120) | Methyl control[b] | 4-n-propyl-pyrocatechol (124) | Methyl Control[b] |
|---|---|---|---|---|---|---|
| Total number of flasks used[a] | 19 | 9 | 24 | 12 | 18 | 18 |
| Amount (mg) of pyrocatechol fed | 475 | 225 | 300 | 300 | 200 | 450 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 228<br>(48%) | 32<br>(14%) | 217<br>(72%) | 49<br>(16%) | 121<br>(61%) | 24<br>(5%) |
| Total metabolites (mg) extracted at pH 2.5 | 63 | 56 | 65 | 63 | 69 | 42 |
| Yield (mg) of 4-alkyl-muconolactone | - | 32<br>(11%) | 16<br>(4%) | 39<br>(10%) | - | 39<br>(7%) |

[a] Each incubation flask contained 100 $cm^3$ medium.

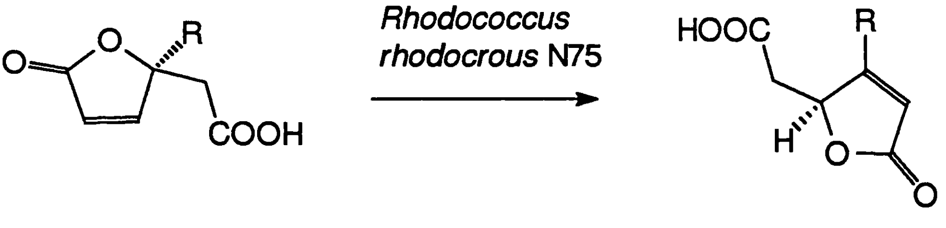
[b] 4-Methylpyrocatechol was fed in parallel.

### 2.3.3 Studies with *Rhodococcus rhodocrous* N75 (in collaboration with the University of Newcastle)

The pyrocatechols, 4-alkylmuconolactones (163) and 1-alkylmuconodilactones prepared in this study were made available to Professor R.B. Cain's group (University of Newcastle) to test as substrates for the enzymes of *Rhodococcus rhodocrous* N75, as part of a continuing, collaborative project on the muconic acid pathways. The conversion of the enzymic 4-alkylmuconolactone (163) to 3-alkylmuconolactone (190) is shown in Scheme 92.

The bacterium *R. rhodocrous* N75 has the special ability to produce a methylmuconolactone methyl-isomerase, which catalysises the isomerisation of 4-methylmuconolactone (50) into 3-methylmuconolactone (48) (See Scheme 24, Section 1.5.4). So far, the following results have been obtained at Newcastle with the present author's substrates (see Table 7). [178] Not all the enzymic assays have yet been completed.

**Scheme 92**



(163) (190)

Table 7. Relative activities of the methylmuconolactone methyl-isomerase from *Rhodococcus rhodocrous* N75.

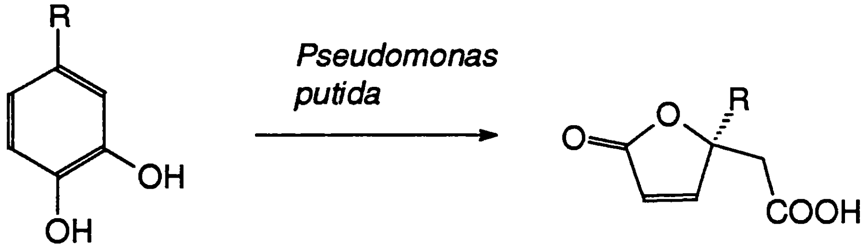
| R | Relative rates of enzyme activity |
|---|---|
| Methyl | 100 |
| Ethyl | 34 |
| Isopropyl | 22 |
| n-Propyl | 12 |
| Allyl | 1 |

### 2.3.4 CONCLUSIONS

*Pseudomonas putida*

Six 4-alkylpyrocatechols (162) were fed, many several times, to intact cells of the bacterium in parallel with 4-methylpyrocatechol (44) as a control. The aim was to probe the effect of the size and shape of the alkyl group on the activity of the two relevant enzymes, the dioxygenase (pyrocatechase) and the cycloisomerase (lactonising), acting in succession. The yields of the 4-alkylmuconolactones (163) in Table 8 are average values from Tables 1-5 for those pyrocatechols fed on more than one occasion. Gratifyingly, the average, methyl control conversion (46%) was the same as that measured precisely by $^1$H NMR integration with the iodoform standard for the feeding experiments with the 4-ethyl and 4-isopropyl - pyrocatechols.

Scheme 93



(162) (163)

Table 8. 4-Alkylmuconolactones (162) from 4-alkylpyrocatechols (163) in *Pseudomonas putida*

| R | Average % Yield of Lactone |
|---|---|
| Methyl | 46 |
| Ethyl | 46 |
| Isopropyl | 31 |
| n-Propyl | 14 |
| Allyl | 10 |
| n-Butyl | trace |
| t-Butyl | - |

Since two enzymes are responsible for the production of the 4-alkyl lactones, it is not possible to make independent quantitative assessments of their individual activities. However, several broad conclusions clearly emerge. Thus, the methyl and ethyl derivatives behaved the same within experimental error. The yield from 4-isopropylpyrocatechol was significantly less, and substantially more pyrocatechol was recovered than in the control feedings. A much larger drop in yield was observed with the propyl and allyl derivatives, and again substantial amounts of the pyrocatechols were recovered. 4-Butylpyrocatechol was a very poor substrate (64% recovered) and only traces of metabolites were detected. Finally, 4-*tert*-butylpyrocatechol gave no detectable product of *ortho* cleavage and 68% of this substrate was recovered.

Two of the pyrocatechols fed to *Pseudomonas putida,* 4-trifluoromethylpyrocatechol (121) and 3-chloro-5-methylpyrocatechol (128) failed to act as substrates. Clearly 4-trifluoromethylpyrocatechol (121) was not a substrate for the dioxygenase enzymes, presumably due to the electron-deficient nature of the aromatic ring, but we can draw no conclusions about the cycloisomerase specificity. That 3-chloro-5-methylpyrocatechol (128) also failed to act as substrate for the dioxygenase enzyme, perhaps is due to electronic or steric reasons.

The findings can be most simply interpreted in terms of the size and shape of the region in the active site of the dioxygenase accomodating the 4-alkyl group. Ethyl and isopropyl groups, having chains of two carbons, clearly fit well even though the latter is much wider. However, the tert-butyl group is too bulky to fit the appropriate pocket in the active site. Also, the three-carbon chains of the propyl and allyl groups are less well accommodated than the shorter chains of the methyl, ethyl and isopropyl groups.

It is not possible to draw detailed conclusions about the cycloisomerase. However, the yields of 3-alkylmuconolactones, products of non-enzymic cyclisation of the *cis, cis*-muconic acids, and of the *cis, trans*-muconic acids, metabolically inert products of non-enzymic *cis-trans* isomerism, give some indication of reduced cyclioisomerase activity. Broadly, the cyclisomerase tolerates ethyl and isopropyl groups well but appears to show less activity, for propyl and allyl groups. Interestingly, competitive *meta* cleavage of the pyrocatechols, leading to the pyrone carboxylic acids has been observed for the first time, even with 4-methylpyrocatechol.

From the purely preparative viewpoint, these experiments show that optically active 4-alkylmuconolactones having Me, Et and $Pr^i$ groups can be synthesised in acceptable yields from pyrocatechols. Extraction of the crude products with chloroform selectively dissolves the 4-alkylpyrocatechols, the other metabolites being less soluble. At present, there is no high-yielding, chemical synthesis of even racemic 4-alkylmuconolactones, so the microbial synthesis of even the propyl and allyl derivatives is the method of choice.

Quantitative assessment of the substrate specificity of the cycloisomerase would require a different approach. It would be necessary first to synthesise the labile 3-alkyl-*cis,cis*-muconic acids as their disodium salts. So far, only the methyl derivative has been synthesised. Then, measurements of cyclase activity would require supplies of at least partially purified enzyme since competitive, non-enzymic, *cis-trans* isomerism of the muconic acids occurs even at pH 7. Also it is possible that efficient uptake of the *cis-cis*-muconic acids into intact cells would not take place; these intermediates are normally formed intracellularly. Experiments of this kind are planned for the future.

### *Aspergillus niger*

In general, the fungus was not as efficient as the bacteria in metabolising 4-alkylpyrocatechols. As with *Pseudomonas putida*, an increase in the length of the 4-alkyl chain of the pyrocatechol reduced the yield of the corresponding 3-alkylmuconolactone (Scheme 94) (Table 9).

**Scheme 94**

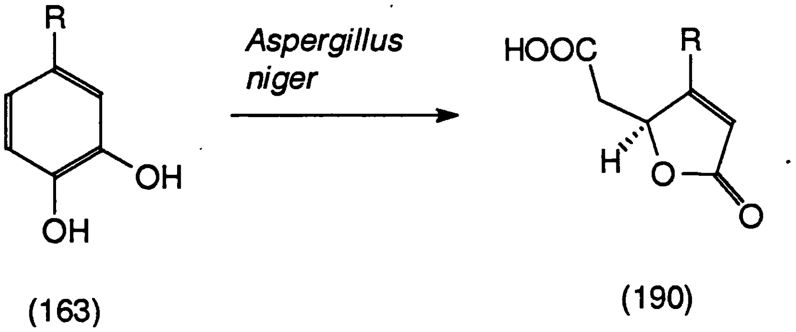


Table 9. Conversions of 4-alkylpyrocatechols (163) into 3-alkylmuconolactones (190) in *Aspergilllus Niger.*

| R | % Yield of Lactone |
|---|---|
| Methyl | 11 |
| Ethyl | 7 |
| Isopropyl | 4 |
| n-Propyl | - |

## Studies with *Rhodococcus rhodocrous* N75 (in collaboration with the University of Newcastle)

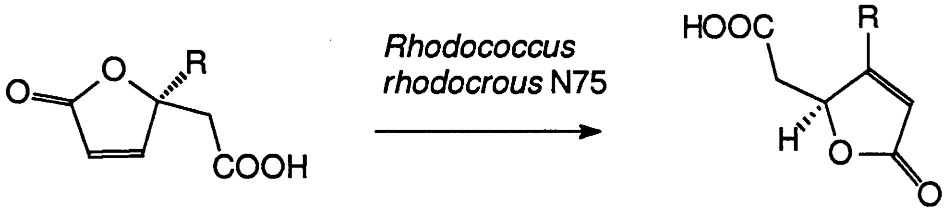
The pyrocatechols, 4-alkylmuconolactones and 1-alkylmuconodilactones prepared in this study were made available to Professor R.B. Cain's group (University of Newcastle) to test as substrates for the enzymes of *Rhodococcus rhodocrous* N75, as part of a continuing, collaborative project on the muconic acid pathways. Not all the enzymic assays have yet been completed.

The bacterium *R. rhodocrous* has the special ability to produce a methylmuconolactone methyl-isomerase, which catalysis the isomerisation of 4-methylmuconolactone (50) into 3-methylmuconolactone (48) (See Scheme 24, Section 1.5.4 ). So far, the following results have been obtained at Newcastle with the present author's substrates.

**Dioxygenase / pyrocatechase.** The cleavage of the pyrocatechols (162) by cell-free extracts of *p*-toluate-grown *R. rhodocrous* N75 was studied by measuring the rates of oxygen uptake (Table 7). The dependence of rate upon the length and size of the 4-alkyl group corresponded remarkably well with that observed with *P. putida*, with the unexpected exception that 4-*tert*-butylpyrocatechol was cleaved at about the same rate as the derivatives with propyl and allyl groups. It appears that the 'alkyl pocket' on the enzyme from *Rhodococcus* is wider than that on the *Pseudomonas* enzyme, but has approximately the same length.

**Methyl-isomerase.** The rates of isomerisation of the 4-alkylmuconolactones, to give the 3-alkyl isomers, were studied with the methyl-isomerase from *Rhodococcus* (Table 7). These relative rates correspond broadly with those tentatively assigned to the cycloisomerase from *P.putida*, with the notable exceptions that the ethyl lactone was isomerised significantly more slowly than the methyl standard and the allyl derivative was a much poorer substrate than its dihydro form. Nevertheless, somewhat similar restrictions apply to the size of the alkyl group accommodated by this enzyme with a distinctly different function.

Scheme 92



(163) (190)

Table 7. Relative activities of the methylmuconolactone methyl-isomerase from *Rhodococcus rhodocrous* N75.

| R | Relative rates of enzyme activity |
|---|---|
| Methyl | 100 |
| Ethyl | 34 |
| Isopropyl | 22 |
| n-Propyl | 12 |
| Allyl | 1 |

# CHAPTER 3 EXPERIMENTAL

## 3.1 GENERAL

Melting points were measured on a Kofler hot-stage apparatus and are uncorrected. Boiling points refer to the oven temperature of a Büchi GKR-50 Kugelrohr. Infrared (IR) spectra were obtained on a Perkin Elmer 580 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Perkin Elmer R32 spectrometer operating at 90 MHz, or a Bruker WP200-SY spectrometer operating at 200 MHz ($^{1}$H) or 50.3 MHz ($^{13}$C). The multiplicities of the $^{13}$C NMR spectra were determined using DEPT spectra with pulse angles of $\theta = 90°$ and $\theta = 135°$. Mass spectra were obtained using A.E.I. MS 12 or 902 spectrometers. Elemental analyses were obtained with a Carlo-Erba 1106 elemental analyser. Optical rotations were determined on a AA/100 Polarimeter (Optical Activity Ltd) at 20°C.

Flash column chromatography was carried out on Merck Kieselgel silica $HF_{254}$ under reduced pressure. Analytical thin layer chromatography (TLC) was carried out on precoated Merck Kieselgel silica $GF_{254}$. Spots were viewed under a UV lamp (254 nm) and developed by iodine vapour. Preparative TLC was carried out on 20 x 20 cm Merck Kieselgel silica $GF_{254}$ glass plates of 0.25 mm thickness in diisopropyl ether - formic acid - water (100 : 7 : 1).

All solvents were purified by standard techniques. Ether refers to diethyl ether.

## 3.2 CHEMICAL SYNTHESIS

### 3.2.1 4-Isopropylpyrocatechol (120) and Metabolites

**4-Benzoyloxy-3-hydroxyisopropylbenzene (138)**

A mixture of 4-isopropylphenol (137) (6.8 g, 0.05 mol), benzoyl peroxide (12.11 g, 0.05 mmol) and dried chloroform (50 $cm^3$) was heated under reflux for 6 h. The mixture was cooled to ambient temperature and the resultant brown solution was washed with saturated sodium bicarbonate (3 x 50 $cm^3$) until pH of the washes remained alkaline. The chloroform layer was dried over sodium sulfate, filtered and evaporated to dryness under reduced pressure. The resulting dark brown oil was found to be a mixture of the desired ester and unreacted starting material. The mixture was purified by column chromatography on basic alumina and elution with ethyl acetate : light petrol (b.p. 40-60 °C) (7:3). Kugelrohr distillation gave the required ester (138) as an off-white solid gum (2.6 g, yield 20 %); $\nu_{max}$ ($CHCl_3$) 3027, 2964, 1740, 1268, 1229,1220, 1176, 1113 and 1024 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.24 (6H, d, *J* 7 Hz, $Me_2$), 2.78 (1H, septet, *J* 7 Hz, $\underline{\underline{CH}}Me_2$), 6.79 - 7.1, 7.47 - 7.64 and 8.18- 8.23 (8H, m, Ar-H); *m/z* (Found: $M^+$ 256.1097. $C_{16}H_{16}O_3$ requires 256.1099).

**4-Isopropylpyrocatechol (120)**

The ester (138) (2.56 g, 10 mmol) was added slowly portionwise with stirring to sodium hydroxide (0.8 g, 20 mmol) in water (100 $cm^3$) under nitrogen. The solution became dark in colour and was stirred for 16 h. The resulting solution was acidified with concentrated hydrochloric acid, saturated with sodium chloride and extracted with ether (3 x 100 $cm^3$). The extract was dried and evaporated under reduced pressure. Kugelrohr distillation gave a pale yellow liquid which crystallised to form the desired 4-isopropylpyrocatechol (120) as pale yellow crystals (0.5 g, yield 33 %), m.p 76-77 °C (lit.,[135] 78 °C); $\nu_{max}$ (KBr disc) 3431, 3316, 2957, 1522 and 1286 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.14 (6H, d, *J* 10 Hz, $Me_2$), 2.72 (1H, septet, *J* 10 Hz, C$\underline{H}$$Me_2$), 5.9 (1H, br.s, OH exch. with $D_2O$) and 6.60-6.79 (3H, m, Ar-H) ; $\delta_C$ (50 MHz) ($CDCl_3$) 24.1 ($Me_2$), 33.4 ($\underline{C}$H$Me_2$), 113.9, 115.6, 119.1 (Ar-H), 141.0 (C-4), 142.7 and 143.2 (C-1 and C-2); *m/z* 152 ($M^+$) (Found: $M^+$ 152.0821. $C_9H_{12}O_2$ requires 152.0837) (Found : C, 71.2; H, 7.65 %. $C_9H_{12}O_2$ requires C, 71.05; H, 7.9 %).

### Potassium Nitrosodisulfonate - Fremy's Salt [133]

The procedure given by Singh[133] was followed with some modifications. In a 5L three neck - round bottomed flask was placed distilled water (1000 $cm^3$) and crushed ice (500 g), sodium bicarbonate (79.2 g, 0.94 mol) and sodium nitrite (66 g, 0.96 mol). The mixture was thoroughly mixed with a mechanical stirrer and was externally cooled to - 5 °C by a mixture of salt and ice. Sulfur dioxide was passed into the mixture rapidly, and the temperature was maintained at - 5 °C to - 2 °C. When the colour changed to orange - brown (50 min), the passage of sulfur dioxide was immediately stopped. The mixture was stirred for an additional 10 min, during which time it became colourless. Nitrogen was bubbled through the solution for 5 min and the solution was adjusted to pH 10.1 with 18 % ammonium hydroxide (50 $cm^3$). The solution was stirred at -2

°C for a further 30 min. Potassium permanganate (120 g) in water (360 cm$^3$) was cooled to - 3 °C and then added portionwise at a rate which kept the reaction 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 water (140 cm$^3$). The solution was cooled and yielded an orange crystalline precipitate which was collected by vacuum filtration. The solid was washed with saturated potassium chloride and ammoniacal methanol (containing 10 % concentrated ammonia) and dried under vacuum over calcium oxide, in the presence of ammonium carbonate in a separate dish to provide an ammonia atmosphere. The product was stored in a refrigerator (107 g, yield 42 %, based on sodium bicarbonate).

**CAUTION**: This salt must not be stored in a sealed vessel; decomposition may cause a pressure explosion.

### Attempted Preparation of 4-Isopropyl-1,2-benzoquinone (139)

A solution of sodium dihydrogen phosphate (1.5 g) in water (500 cm$^3$) was placed in a conical flask. To this solution was added potasssium nitrosodisulfonate (Fremy's salt) (9 g, 34 mmol) and the mixture was shaken to dissolve the inorganic radical. A solution of 4-isopropylphenol (137) (1.6 g, 11.8 mmol) in ether (35 cm$^3$) was added quickly to the purple solution and the mixture was stirred vigorously for 20 min. The colour of the solution changed to red - brown and the solution was extracted with chloroform (3 x 200 cm$^3$). The combined organic layers were dried over anhydrous sodium sulfate for 5 min, filtered, and evaporated under reduced pressure at 20 - 23 °C (higher temperatures may accelerate dimerization of the product) to give an oil. The oil was slurried twice with ether (2 x 15 $cm^3$) at 0 °C and the orange / yellow crystals produced collected by filtration (0.5 g). The $^1H$ NMR spectrum was complex and could not be interpreted.

### 4-Isopropylpyrocatechol (120)

A solution of 4-isopropyl-1,2-benzoquinone (139) in chloroform (600 $cm^3$) was prepared as before. Potassium iodide (3 g, 18.1 mmol) in acetic acid (200 $cm^3$) was added to the dark red solution and the mixture stirred for 2 min. The resultant mixture was washed with sodium bisulfite solution (100 $cm^3$ of 5 % w/v). The chloroform layer was washed once with water (500 $cm^3$), dried with sodium sulfate, filtered and evaporated under reduced pressure to give a dark red oil which was a mixture of the desired 4-isopropylpyrocatechol (120) and 4-isopropylphenol (137), as judged by TLC. The mixture was purified by column chromatography on basic alumina and elution with diethyl ether : light petrol (b.p. 40 - 60 °C) (1:5). The product (120) was obtained as a dark red oil which was Kugelrohr distilled to produce a pale yellow crystalline solid (0.6 g, yield 36 %), m.p 75-76 °C (lit.,[135] 78 °C); $\nu_{max}$ (KBr disc) 3425, 3305, 2958, 1519, 1376 and 1281 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.12 (6H, d, *J* 7 Hz, $Me_2$), 2.7 (1H, septet, *J* 7 Hz, $C\underline{H}Me_2$) and 6.57 - 6.80 (3H, m, Ar-H) ; $\delta_C$ (50 MHz) ($CDCl_3$) 23.9 ($Me_2$), 33.2 ($\underline{C}HMe_2$), 113.8, 115.6, 118.7 (Ar-H), 141.1 (C-4) and 142.2 and 143.2 (C-1 and C-2); *m/z* 152 ($M^+$) (Found: $M^+$ 152.0836. $C_9H_{12}O_2$ requires 152.0837)

**4-Isopropyl-2-nitrophenol (151)**

4-Isopropylphenol (137) (2.8 g, 20.6 mmol) was dissolved in glacial acetic acid (50 $cm^3$) and the mixture cooled to 18 - 20 °C. Nitric acid (2.5 $cm^3$, sp.gr 1.42) was dissolved in acetic acid (10 $cm^3$) and the resultant solution was added dropwise with stirring. After the addition the reaction mixture was allowed to stand for 15 min at 18 - 20 °C. Water (500 $cm^3$) was added and the solution neutralised with sodium hydroxide (20%). The aqueous solution was extracted with ether (3 x 250 $cm^3$). The organic layer was washed with water (2 x 400 $cm^3$), dried with sodium sulfate, filtered and evaporated under reduced pressure to give a dark yellow oil (2.9 g) which was a mixture of the desired 4-isopropyl-2-nitrophenol (151) and the by-product 4-isopropyl-2,6-dinitrophenol. The mixture was purified by column chromatography on basic alumina and elution with ethyl acetate - light petrol (b.p. 40 - 60 °C) (1:5). The *product* (151) was obtained as a dark yellow oil which was then Kugelrohr distilled to produce a pale yellow oil (2.2 g, yield 59 %); $\nu_{max}$ (thin film) 3252, 2964, 2932, 2872, 1630 and 1537 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.22 (6H, d, *J* 7 Hz, $Me_2$), 2.9 (1H, septet, *J* 7 Hz, $C\underline{H}Me_2$), 7.04 (1H, d, *J* 8.6 Hz, 6-H), 7.44 (1H, d with fine splitting, *J* 8.6 Hz, 5-H), 7.89 (1H, s with fine splitting, 3-H) and 10.4 (1H, s, OH,); $\delta_C$ (50 MHz) ($CDCl_3$) 23.6 ($Me_2$), 33.0 ($\underline{C}HMe_2$), 119.6 (C-6), 121.7 (C-5), 136.4 (C-3), 133.1 (C-4), 141.1 (C-1) and 153.2 (C-2); *m/z* 181 ($M^+$) (Found: $M^+$ 181.0735. $C_9H_{11}O_3N$ requires 181.0739). The by-product 2,6-dinitro-4-isopropylphenol (0.7 g, yield 15 %) was obtained as a dark red oil; $\delta_H$ (200 MHz) ($CDCl_3$) 1.29 (6H, d, *J* 7 Hz, $Me_2$), 3.00 (1H, septet, *J* 7 Hz, $C\underline{H}Me_2$) and 8.17 (2H, s, 3-H and 5-H); $\delta_C$ (50 MHz) ($CDCl_3$) 23.4 ($Me_2$), 33.1 ($\underline{C}HMe_2$), 129.4 (C-3 and C-5), 137.5 (C-4), 140.3 (C-1) and 147.5 (C-2 and C-6).

### (±)-3-Isopropylmucolactone (152)

4-Isopropyl-2-nitrophenol (151) (4.4 g, 24 mmol) was added in portions to concentrated sulfuric acid (15 $cm^3$) at 110 - 115 °C over 2 h with stirring. The black mixture was stirred for a further 30 min at 110 - 115 °C, then cooled to room temperature and carefully poured onto crushed ice (200 g). 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 over sodium sulfate, filtered and evaporated to give the *lactone (152)* as a dark red oil (2.5 g, yield 56 %); $\nu_{max}$ (thin film) 2972, 1746, 1634, 1364, 1273, 1233 and 1172 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.10 (3H, d, *J* 7 Hz, Me), 1.17 (3H, d, *J* 7 Hz, Me), 2.00 (1H, septet, $C\underline{H}Me_2$), 2.86 (1H, dd, *J* 16.4 and 3.7 Hz, 5-H), 2.47 (1H, dd, *J* 16.4 and 8.6 Hz, 5-H), 5.31 (1H, ddd, *J* 8.6, 3.7 and 1.5 Hz, 4-H), 5.76 (1H, s with fine splitting, 2-H), 10.4 (1H, s, OH); $\delta_C$ (50 MHz) ($CDCl_3$) 19.5 and 20.8 ($Me_2$), 26.7 ($\underline{C}HMe_2$), 36.3 (C-5), 78.5 (C-4), 113.7 (C-2), 172.3 (C=O) and 177.4 (C=O); *m/z* 184 ($M^+$) (Found: $M^+$ 184.0735. $C_9H_{12}O_4$ requires 184.0736).

### (±) -1-Isopropyl-8-bromomuconodilactone (153)

(±)-3-Isopropylmuconolactone (152) (1.84g, 10 mmol) was dissolved in aqueous sodium hydrogen carbonate (30 $cm^3$, 0.33 M). Bromine (1.6 g, 10 mmol) in dichloromethane (50 $cm^3$) was added to the solution at 0 °C with stirring. The mixture was warmed to ambient temperature and stirred for 7 h. Aqueous sodium thiosulfate was added to remove traces of bromine. The mixture was extracted with dichloromethane (3 x 100 $cm^3$) and the combined extracts were dried with magnesium sulfate. Evaporation of the solvents gave the *bromodilactone (153)* as a brown oily solid, which failed to crystallise from chloroform - hexane, (2.3 g, yield 87 %); $\nu_{max}$ ($CHCl_3$) 3026, 1799, 1226 and 1205 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 0.96 (3H, d, *J* 6.7 Hz, Me), 1.09 (3H, d, *J* 6.7 Hz, Me), 2.65 (1H, septet, *J* 7 Hz, C$\underline{H}$$Me_2$), 2.90 (1H, d, *J* 19.2 Hz, 4-H), 3.10 (1H, dd, *J* 19.2 and 5.0 Hz, 4-H), 4.55 (1H, s, 8-H) and 5.20 (1H, d, *J* 4.8 Hz, 5-H); $\delta_C$ (50 MHz) ($CDCl_3$) 15.9 and 16.0 ($Me_2$), 31.7 ($\underline{C}HMe_2$), 35.7 (C-4), 42.1 (C-8), 80.2 (C-5), 92.8 (C-1), 169.5 (C=O) and 172.0 (C=O); *m/z* 264 ($M^+$,$^{81}Br$ ), 262 ($M^+$, $^{79}Br$) (Found: $M^+$ 263.9816. $C_9H_{11}O_4{}^{81}Br$ requires 263.9821) (Found: $M^+$ 261.9839. $C_9H_{11}O_4{}^{79}Br$ requires 261.9841).

**(±)-1-Isopropylmuconodilactone (154)**

To a suspension of the bromodilactone (153) (263 mg, 1.0 mmol) in dry benzene (4 $cm^3$) was added azoisobutyronitrile (14 mg, 0.1 mmol) under nitrogen and the flask sealed with a subaseal. Tributyltin hydride (0.54 $cm^3$, ca. 1.2 mmol) was introduced with syringe, and the mixture was warmed to 30 - 40 °C to give a clear solution. The mixture was heated for another 15 min and then kept at room temperature for 1 h with stirring. Hexane (3 $cm^3$) was added and a white precipitate formed, which was filtered off to give the *dilactone (154)* as an off-white wax, which failed to recrystallise from chloroform-hexane (0.04 g, yield 22 %). $\nu_{max}$ (thin film) 2972, 1787, 1392, 1282, 1235, 1159, 1123 and 1026 $cm^{-1}$; $\delta_H$ (200

MHz) ($CDCl_3$) 0.98 (3H, d, *J* 6.8 Hz, Me), 1.05 (3H, d, *J* 6.8 Hz, Me), 2.02 (1H, septet *J* 6.8 Hz, C$\underline{H}$$Me_2$), 2.62 - 3.00 (4H, m, 4-$H_2$ and 8-$H_2$) and 4.96 (1H, d with fine splitting, *J* 4.9 Hz, 5-H); $\delta_C$ (50 MHz) ($CDCl_3$) 16.6 and 17.4 (Me), 34.1 ($\underline{C}HMe_2$), 35.9 and 37.4 (C-4 and C-8), 80.2 (C-5), 93.3 (C-1), 172.8 (C=O) and 173.0 (C=O); *m/z* 184 ($M^+$); (Found: $M^+$ 184.0738. $C_9H_{12}O_4$ requires 184.0736).

**Silver (I) Oxide**

Silver nitrate (10 g, 0.059 mmol) was dissolved in water (25 $cm^3$). Sodium hydroxide pellets (2.5 g, 0.063 mmol) were dissolved in water (20 $cm^3$) and the solution was added to the silver nitrate solution. A brown precipitate was produced immediately which was filtered off, washed with hot water (2 x 10 $cm^3$), acetone (10 $cm^3$) and dry ether (3 x 10 $cm^3$) and stored in a desiccator over phosphorus pentoxide for 1 h (7.7 g, yield 56 %).

**4-Isopropyl-1,2-benzoquinone (139)**

4-Isopropylpyrocatechol (102) (0.1 g, 0.66 mmol) was dissolved in dry ether (5 $cm^3$) and placed in a dry flask fitted with a silica gel guard tube. To this solution was added an ethereal suspension (10 $cm^3$) of silver (I) oxide (0.85 g, 3.66 mmol) followed by anhydrous magnesium sulfate (0.85 g, 7.08 mmol). This mixture was stirred for 1 h in the dark. The solution was filtered through Celite and dry sodium sulfate. The filtrate was concentrated to ~ 20 $cm^3$ and refrigerated in the dark for 16 h. The solution appeared dark red - green and no crystals were observed. The solution was evaporated to dryness *in vacuo* without heating. The *o*-

quinone (139) was produced as a dark red oil, which was unstable when stored at -5 °C (0.07 g, yield 71 %); $\delta_H$ (200 MHz) ($CDCl_3$) 1.09 (6H, d, *J* 7 Hz, $Me_2$), 2.51 (1H, septet, *J* 7 Hz, C$\underline{H}Me_2$), 6.07 (1H, s with fine splitting, 3-H), 6.27 (1H, d, *J* 10.2 Hz, 6-H ) and 6.94 (1H, d with fine splitting, *J* 10.2 Hz, 5-H ); $\delta_C$ (50 MHz) ($CDCl_3$) 20.0 ($Me_2$), 33.9 ($\underline{C}HMe_2$), 124.0 (C-3), 129.5 (C-6), 141.6 (C-5), 160.5 (C-1), 179.6 (C=O) and 180.1 (C=O).

### 3.2.2 4-Alkyl Substituted Pyrocatechols

**4-Allylpyrocatechol (125)**

Anhydrous aluminium chloride (9.7 g, 72.7 mmol) was suspended in a solution of eugenol (123) (10.8 g, 65.9 mmol) in dry dichloromethane (100 $cm^3$), in an apparatus protected from atmospheric moisture. Pyridine (22.9 g, 0.29 mol) was added slowly while the reaction was cooled to maintain the temperature at 30 - 45 °C. The mixture was then heated to reflux for 24 h. The solution was cooled to 25 °C and dilute hydrochloric acid added until the mixture was acidic to Congo Red indicator. The two layers were separated and the aqueous phase was washed with ether. The organic layers were dried over sodium sulfate, filtered and evaporated. The crude product was purified by column chromatography on a silica column using ether- light petrol (b.p. 40-60 °C) (1:5) to give equal amounts of eugenol (123) and the desired 4-allylpyrocatechol (125). The title compound was Kugelrhor distilled to give a clear oil which failed to crystallise (2.3 g, yield 26 %), (lit.,[179] m.p. 48 °C); $\nu_{max}$ (thin film) 3352, 3080, 1518, 1444, and 1282 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 3.21 (2H, d, *J*

7 Hz, $C\underline{H}_2CH=CH_2$), 4.96 - 5.06 (2H, m, $CH_2CH=C\underline{H}_2$), 5.77 - 5.97, (1H, m, $CH_2C\underline{H}=CH_2$) and 6.54-6.78 (3H, m, Ar-H) ; $\delta_C$ (50 MHz) ($CDCl_3$) 39.4 ($\underline{C}H_2CH=CH_2$), 115.6 ($CH_2C\underline{H}=CH_2$), 115.6 and 115.8 (C-3 and C-6), 121.1 (C-5), 134.0 (C-4), 137.6 ($CH_2CH=C\underline{H}_2$), 141.3 and 143.4 (C-1 and C-2); *m/z* 150 ($M^+$) (Found: $M^+$ 150.0673. $C_9H_{10}O_2$ requires 150.0681).

**4-n-Propylpyrocatechol (124)**

4-Allylpyrocatechol (125) (2.19 g, 14.6 mmol) was dissolved in ethanol (30 $cm^3$) and hydrogenated in the presence of palladium on carbon catalyst (0.7 g, 10 % Pd /C). Hydrogen uptake ceased after 90 min. The solution was filtered through Celite and evaporated to give a dark oil. Kugelrohr distillation gave the title compound as a white crystalline solid (2.07 g, yield 93 %), m.p 59-60 °C (lit.,[144] 60 °C); $\nu_{max}$ (KBr disc) 3389, 1604, 1525, 1522, 1518 and 1286 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 0.91 (3H, t, *J* 7 Hz, $CH_2CH_2C\underline{H}_3$), 1.57 (2H, sextet, *J* 7 Hz $CH_2C\underline{H}_2CH_3$), 2.45 (2H, t, *J* 7 Hz $C\underline{H}_2CH_2CH_3$), 5.86 (2H, br.s, OH exch. with $D_2O$), 6.57 (1H, d with fine splitting, *J* 8 Hz, 5-H), 6.68 (1H, s with fine splitting, 3-H) and 6.76 (1H, d, *J* 8 Hz, 6-H); $\delta_C$ (50 MHz) ($CDCl_3$) 13.7 ($CH_2CH_2C\underline{H}_3$), 24.6 ($CH_2C\underline{H}_2CH_3$), 37.2 ($C\underline{H}_2CH_2CH_3$), 115.4, 115.7, 121.0 (Ar-H), 136.2 (C-4), 141.0 and 143.1 (C-1 and C-2); *m/z* 152 ($M^+$) (Found: $M^+$ 152.0847. $C_9H_{12}O_2$ requires 152.0837). (Found : C, 71.2; H, 7.9 %. $C_9H_{12}O_2$ requires C, 71.05; H, 7.9 %).

**4-(2-bromoprop-1-yl)pyrocatechol (142)**

Eugenol (123) (1.0 g, 6.10 mmol) was dissolved in dichloromethane (40 $cm^3$) and cooled to - 78 °C in an acetone / dry ice bath. To this solution was added boron tribromide (neat) (1 $cm^3$, 9.22 mmol). The dry ice / acetone bath was removed and the mixture warmed to ambient temperature and stirred for 24 h. The solution was then cooled in an ice bath and ether (50 $cm^3$) added with care. Water (50 $cm^3$) was added to the reaction mixture. Two layers formed and the ether layer was removed and was washed with water (3 x 50 $cm^3$). The organic layer was separated, dried with sodium sulfate, filtered and the filtrate evaporated *in vacuo* to leave a dark brown oily solid (0.56 g, yield 40 %); $\nu_{max}$ (thin film) 3476, 3348, 1531, 1452, 1444, 1363, 1286, 1259, 1203 and 1172 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.64 (3H, d, *J* 7 Hz, $CH_2CHBrC\underline{H_3}$), 2.97 (2H, ddd, *J* 7 Hz $C\underline{H_2}CHBrCH_3$), 4.20 (1H, sextet, *J* 7 Hz $CH_2C\underline{H}BrCH_3$), 6.60 (1H, d with fine splitting, *J* 8 Hz, 5-H), 6.62 (1H, s, with fine splitting, 3-H) and 6.78 (1H, d, *J* 8 Hz, 6-H); $\delta_C$ (50 MHz) ($CDCl_3$) 25.6 ($CH_2CHBr\underline{C}H_3$), 46.6 ($\underline{C}H_2CHBrCH_3$), 51.3 ($CH_2\underline{C}HBrCH_3$), 115.6, 116.5, 122.0 (Ar-$\underline{C}H$), 131.9 (C-4), 142.1 and 143.2 (C-1 and C-2); *m/z* 232 ($M^+$,$^{81}Br$) (Found: $M^+$ 231.9923. $C_9H_{11}O_2{}^{81}Br$ requires 231.9923).

**2-methoxy-4-propylphenol (143)**

Eugenol (123) (1.0 g, 6.10 mmol) was dissolved in ethanol (30 $cm^3$) and the solution was hydrogenated in the presence of palladium on carbon catalyst (0.7 g, 10 % Pd /C) under 1 atmosphere of hydrogen. Uptake of hydrogen ceased after 4 h. The solution was filtered through Celite and the filtrate evaporated to give a dark oil. Kugelrohr distillation gave 2-methoxy-4-propylphenol (143) as a clear oil (0.92 g, Yield 91 %); $\nu_{max}$ (thin film) 2959, 2932, 1516, 1269, 1234 and 1207 cm$^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 0.92 (3H, t, *J* 7 Hz, $CH_2CH_2C\underline{H}_3$), 1.58 (2H, sextet, *J* 7 Hz $CH_2C\underline{H}_2CH_3$), 2.49 (2H, t, *J* 7 Hz $C\underline{H}_2CH_2CH_3$), 3.78 (3H, s, $OC\underline{H}_3$), 5.76 (1H, br.s, OH exch. with $D_2O$) and 6.61 - 6.84 (3H, m, Ar-H); $\delta_C$ (50 MHz) ($CDCl_3$) 13.6 ($CH_2CH_2C\underline{H}_3$), 24.7 ($CH_2C\underline{H}_2CH_3$), 37.6 ($C\underline{H}_2CH_2CH_3$), 110.9, 114.1, 120.8 (Ar-H), 136.2 (C-4), 143.3 and 146.2 (C-1 and C-2); *m/z* 166 ($M^+$) (Found: $M^+$ 166.0995. $C_{10}H_{14}O_2$ requires 166.0994).

**4-*n*-Propylpyrocatechol (124)**

2-Methoxy-4-propylphenol (143) (1.0 g, 6.02 mmol) was dissolved in dichloromethane (40 cm$^3$) and cooled to - 78 °C in an acetone / dry ice bath. To this solution was added boron tribromide (neat) (1ml, 9.2 mmol). The dry ice / acetone bath was removed and the mixture warmed to ambient temperature and stirred for 24 h. The solution was then cooled to 0 °C in an ice bath and ether (50 cm$^3$) was added with care. Water (50 cm$^3$) was added to the reaction mixture and two layers were formed. The upper organic layer was removed and washed with water (2 x 50 cm$^3$). The lower aqueous layer was washed with ether (3 x 50 cm$^3$). The combined organic layers were dried with sodium sulfate, filtered and the filtrate evaporated *in vacuo* to give a dark oil. Kugelrohr distillation gave the title compound as a white crystalline solid (0.82 g, Yield 90 %); m.p 59-60 °C (lit.,[144] 60 °C). $\nu_{max}$ (thin film) 3355, 2959, 2929, 2868, 1605, 1517, 1444, 1348 and 1279 cm$^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 0.90 (3H, t, *J* 7

Hz, $CH_2CH_2C\underline{H}_3$), 1.55 (2H, sextet, *J* 7 Hz, $CH_2C\underline{H}_2CH_3$), 2.43 (2H, t, *J* 7 Hz $C\underline{H}_2CH_2CH_3$), 6.59 (1H, d with fine splitting, *J* 8 Hz, 5-H), 6.69 (1H, s with fine splitting, 3-H) and 6.76 (1H, d, *J* 8 Hz, 6-H); $\delta_C$ (50 MHz) ($CDCl_3$) 13.7 ($CH_2CH_2C\underline{H}_3$), 24.6 ($CH_2C\underline{H}_2CH_3$), 37.2 ($C\underline{H}_2CH_2CH_3$), 115.4, 115.7, 121.0 (Ar-H), 136.1 (C-4), 141.0 and 143.0 (C-1 and C-2); *m/z* 152 ($M^+$) (Found: $M^+$ 152.0832. $C_9H_{12}O_2$ requires 152.0837).

**4-*n*-Butylpyrocatechol (126)**

90% Zinc powder (2 g, 27.0 mmol) was washed with dilute hydrochloric acid (20 $cm^3$) and covered with a solution of mercuric chloride (0.4 g, 1.5 mmol) in water (10 $cm^3$). Amalgamation was effected by occasional agitation during 30 min. The zinc-mercury amalgam was transferred to a 3 - neck flask (100 $cm^3$) fitted with a mechanical stirrer and reflux condenser. Water (10 $cm^3$), concentrated hydrochloric acid (10 $cm^3$, 37 % w/v) and a solution of *n*-butyrlpyrocatechol (127) (1.08 g, 6.0 mmol) in ethanol (5 $cm^3$) was added to the amalgam. The mixture was agitated vigorously and heated at reflux for 12 h. To the mixture was added toluene (12 $cm^3$) and stirring was continued for 2-3 min. The organic layer was removed, washed with water (3 x 50 $cm^3$) and evaporated *in vacuo* to give a dark brown oil. Kugelrohr distillation gave a clear oil which produced the title compound as a white crystalline solid upon standing (0.51 g, yield 51 %), m.p 39-41 °C (lit.,[151] 39-41 °C); $\nu_{max}$ (KBr disc) 3379, 2959, 2930, 1523 and 1284 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 0.89 (3H, t, *J* 7 Hz, $CH_2CH_2CH_2C\underline{H}_3$), 1.21 - 1.38 (2H, m, $CH_2CH_2C\underline{H}_2CH_3$), 1.43 - 1.57, (2H, m, $CH_2C\underline{H}_2CH_2CH_3$), 2.44 (2H, t, *J* 7 Hz,

$C\underline{H}_2CH_2CH_2CH_3$), 6.57 (1H, d with fine splitting, *J* 8 Hz, 5-H), 6.68 (1H, s with fine splitting, 3-H) and 6.73 (1H, d with fine splitting, *J* 8.6 Hz, 5-H); $\delta_C$ (50 MHz) ($CDCl_3$) 13.9 ($CH_2CH_2CH_2C\underline{H}_3$), 22.3 ($CH_2CH_2C\underline{H}_2CH_3$), 33.7 ($CH_2C\underline{H}_2CH_2CH_3$), 34.9 ($C\underline{H}_2CH_2CH_2CH_3$), 115.6, 115.8, 120.9 (Ar-H), 136.3 (C-4), 141.2 and 141.3 (C-1 and C-2); *m/z* 166 ($M^+$) (Found: $M^+$ 166.1004. $C_{10}H_{14}O_2$ requires 166.0994). (Found : C, 72.05; H, 8.4%. $C_{10}H_{14}O_2$ requires C, 72.3; H, 8.4%).

### 4-Ethylpyrocatechol (119)

A solution of sodium dihydrogen phosphate (1.5 g) in water (500 $cm^3$) was placed in a conical flask. To this solution was added potassium nitrosodisulfonate (Fremy's salt) (9.0 g, 34 mmol) and the mixture was shaken to dissolve the inorganic radical. A solution of 4-ethylphenol (140) (1.6 g, 11.8 mmol) in ether (35 $cm^3$) was added quickly to the purple solution and the mixture was stirred vigorously for 20 min. The colour of the solution changed to red - brown and the solution was extracted with chloroform (3 x 200 $cm^3$). The combined organic extracts were placed in a conical flask and potassium iodide (3 g, 18.1 mmol) in acetic acid (200 $cm^3$) was added to the dark red solution and the mixture stirred for 2 min. The resultant mixture was washed with sodium bisulfite solution (100 $cm^3$ of 5 % w/v). The chloroform layer was washed with water (500 $cm^3$), dried with sodium sulfate, filtered and evaporated under reduced pressure to give a dark red oil which was a mixture of the desired 4-ethylpyrocatechol (119) and 4-ethylphenol (140). The mixture was purified by column chromatography on basic alumina and elution with diethyl ether : light petrol (b.p. 40 - 60°C ) (1:5). The product (119) was obtained as a dark red oil which was Kugelrhor distilled to produce an off-white crystalline solid (0.6 g, yield 33 %), m.p 38-39 °C (lit.,[136] 39 °C); $\nu_{max}$ (KBr disc) 3384, 2965, 1606, 1525, 1446, 1350, 1283, 1193 and 1111 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.14 (3H, t, *J* 7 Hz, Me), 2.48 (2H, q, *J* 7 Hz, $C\underline{H}_2Me$), 6.36 (2H, br.s, OH), 6.60 (1H, d with fine splitting, *J* 8 Hz, 5-H), 6.70 (1H, s with fine splitting, 3-H) and 6.78 (1H, d, *J* 8 Hz, 6-H); $\delta_C$ (50 MHz) ($CDCl_3$) 15.6 (Me), 28.0 ($C\underline{H}_2Me$), 115.3, 115.7, 120.3 (Ar-H), 137.7 (C-4), 141.0 and 143.1 (C-1 and C-2); *m/z* 138 ($M^+$) (Found: $M^+$ 138.0675. $C_8H_{10}O_2$ requires 138.0681).

### 3.2.3 Chloro Substituted Aromatics

**Chlorovanillin (144)** [152]

Vanillin (129) (17 g, 0.11 mmol) was dissolved in glacial acetic acid (150 $cm^3$) and a stream of dry chlorine gas was bubbled through the solution with stirring at ambient temperature. An exotherm was observed during the addition. After 15 min, crystals began to separate from the solution and the passage of gas was discontinued and the slurry allowed to stand at ambient temperature for 6 h. The precipitate was isolated by vaccum filtration and recrystallised from glacial acetic acid. The recrystallised solid was isolated by filtration. The filtrate was allowed to stand for 2 h and produced a second crop of crystals. The title compound was obtained as a white solid, crystallizing in square plates (18.1 g, Yield 87 %), m.p 165 °C (lit.,[152] 165 °C); $\nu_{max}$ (KBr disc) 3300, 1676, 1591, 1504, 1427, 1296 and 1163 $cm^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2SO$] 3.94 (3H, s, Me), 7.41 (1H, s with fine splitting, Ar-H), 7.60 (1H, s with fine splitting, Ar-H) and

9.79 (1H, s, CHO); $\delta_C$ (50 MHz) [$(CD_3)_2SO$] 56.4 (Me), 109.1 (C-3), 120.1 (C-2), 125.8 (C-5), 148.9 and 149.0 (C-4 and C-6) and 185.0 ($\underline{C}$HO); *m/z* 188 ($M^+$, $^{37}Cl$), 186 ($M^+$, $^{35}Cl$); (Found: $M^+$ 188.0052 $C_8H_7O_3{}^{37}Cl$ requires 188.0159), (Found: $M^+$ 186.0065 $C_8H_7O_3{}^{35}Cl$ requires 186.0189).

**2-Chloro-6-methoxy-4-methylphenol (145)**

Chlorovanillin (144) (2.0 g, 10.7 mmol) was dissolved in ethanol (120 $cm^3$) containing a catalytic amount of hydrochloric acid in ethanol (0.2 $cm^3$, 1M, ca. 0.2 mmol HCl) was hydrogenated in the presence of palladium / carbon catalyst (174 mg, 10 % Pd/C) with stirring at room temperature for 1 h until sufficient hydrogen was absorbed. The mixture was filtered through Celite to remove the catalyst and the filtrate was neutralised with solid sodium bicarbonate. Ethanol was evaporated under reduced pressure and the residue was extracted with ether (200 $cm^3$), leaving a mass of inorganic salts. The ether layer was dried over sodium sulfate, filtered and evaporated to give the desired product (119) as a light coloured oil (0.94 g, yield 51 %); $\nu_{max}$ (thin film) 3435, 2938, 1610, 1595, 1504, 1462, 1414, 1362, 1285 and 1236 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 2.15 (3H, s, Me), 3.82 (3H, s, OMe), 5.87 (1H, br.s, OH), 6.55 (1H, s with fine splitting, 3-H) and 6.71 (1H, s with fine splitting, 5-H); $\delta_C$ (50 MHz) ($CDCl_3$) 20.7 (Me), 56.0 (OMe), 110.2 (C-3), 116.8 (C-2), 121.7 (C-5), 129.6 (C-4), 139.5 and 147.0 (C-1 and C-2); *m/z* 174 ($M^+$,$^{37}Cl$), 172 ($M^+$,$^{35}Cl$); (Found: $M^+$ 174.0254. $C_8H_9O_2{}^{37}Cl$ requires 174.0262), (Found: $M^+$ 172.0277 $C_8H_9O_2{}^{35}Cl$ requires 172.0291).

## 3-Chloro-5-methylpyrocatechol (128)

Anhydrous aluminium chloride (0.49 g, 3.64 mmol) was suspended in a solution of 4-methyl-2-chloro-6-methoxyphenol (145) (0.54 g, 3.29 mmol) in dry dichloromethane (50 cm$^3$), in an apparatus protected from atmospheric moisture. Pyridine (1.15 g, 14.5 mmol) was added slowly while the reaction mixture was cooled to maintain the temperature at 30 - 45 °C. The mixture was heated under reflux for 24 h. The solution was cooled to 25 °C and dilute (15-20 %) hydrochloric acid added until the mixture was acidic to Congo Red indicator. The two layers were separated and the aqueous phase was washed with ether. The organic layers were dried over sodium sulfate, filtered and evaporated *in vacuo*. The crude product was chromatographed on a silica column eluted with ether/ light petrol (b.p. 40-60 °C) (1:5) to give equal amounts of starting material (119) and the desired *3-chloro-5-methylpyrocatechol* (128) (0.10 g, yield 22 %), m.p 58-60 °C; $\nu_{max}$ (KBr disc) 3410, 1610, 1512, 1500 and 1288 cm$^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 2.21 (3H, s, Me), 5.36 (2H, br.s, OH), 6.67 (2H, m, Ar-H); $\delta_C$ (50 MHz) ($CDCl_3$) 20.6 (Me), 115.1 (Ar-CH), 119.4 (C-3), 120.8 (Ar-H), 131.2 (C-2), 137.0 (C-5), 144.2 (C-1); *m/z* 160 ($M^+$,$^{37}$Cl), 158 ($M^+$,$^{35}$Cl) (Found: $M^+$ 160.0118. $C_7H_7O_2{}^{37}Cl$ requires 160.0105), (Found: $M^+$ 158.0139 $C_7H_7O_2{}^{35}Cl$ requires 158.0135).

## 2-Chloro-4-methylphenol (146)[153]

To melted 4-methylphenol (28) (27 g, 0.25 mol) was added neat sulfuryl chloride (20 cm$^3$, 0.28 mmol) slowly. Hydrogen chloride and sulfur dioxide were immediately evolved. The reaction mixture was warmed on a water bath and then washed with cold aqueous sodium carbonate. The residue was dried over fused calcium chloride, filtered and distilled. The chlorocresol (146) compound was obtained as a colourless oil, (22 g, yield 62 %); $\nu_{max}$ (thin film) 3524, 3447, 1500, 1282, 1251, 1215, 1182 and 1053 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 2.20 (3H, s, Me), 6.87 - 7.06 (3H, m, Ar-<u>H</u>); $\delta_C$ (50 MHz) ($CDCl_3$) 20.12 (Me), 115.87 (C-3), 119.32 (C-2), 128.82 (C-5), 129.12 (C-6), 130.89 (C-4), 148.87 (C-1); *m/z* 144 ($M^+$,$^{37}Cl$), 142 ($M^+$,$^{35}Cl$); (Found: $M^+$ 144.0155. $C_7H_7O^{37}Cl$ requires 144.0156), (Found: $M^+$ 142.0186 $C_7H_7O^{35}Cl$ requires 142.0185).

**Potassium Nitrosodisulfonate - Fremy's Salt** [124]

Sodium nitrite (32.5 g, 0.5 mol) was dissolved in water (100 $cm^3$), placed in a 2 l beaker and cooled to 0 °C in an ice bath. Chopped ice (200 g) was added and the solution stirred steadily during the addition of fresh sodium metabisulfite (100 ml, 31.92 % w/v), followed by glacial acetic acid (20 $cm^3$). Momentary darkening in colour of the reaction mixture occcurred after 2-3 min, and a sample was removed. The sample failed to decolourise iodine solution indicating the end of the reaction.

Concentrated ammonia solution (25 $cm^3$, sp gr 0.88) was added and the mixture was again cooled to 0 °C in an ice bath. Fresh ice was added whenever necessary to keep some present in the reaction mixture throughout the next stage. Aqueous potassium permanganate (0.2 M, 400 $cm^3$) was added dropwise with continued stirring over 1 h.

The precipitated manganese dioxide was removed by filtration at 0 °C through a bed of Celite and the filtrate was warmed to ambient temperature. A portion of the filtrate (10-15 $cm^3$) was treated with an equal volume of saturated aqueous potassium chloride. A precipitate of Fremy's salt was formed for seeding the main batch. The remainder of the filtrate was stirred, while saturated potassium chloride solution (250 $cm^3$) was added dropwise over a period of about 45 min. Small portions of the previously prepared suspension were added at intervals during this period until the solid persisted and the precipitate formed was cooled in ice for a further 45 min. The orange solid was collected by filtration but was not sucked dry. The filter cake was washed with ammoniacal saturated potassium chloride solution (containing ca 5 % w/v 0.88 ammonium hydroxide) (200 $cm^3$), ammoniacal methanol (containing ca 5 % v/w 0.88 ammonium hydroxide) (2 x 200 $cm^3$) and finally with acetone (200 $cm^3$) but not sucked dry. The solid was spread on a watch glass and the acetone was allowed to evaporate for 10-15 min. The resultant orange crystals were stored in a desiccator over calcium oxide, in the presence of ammonium carbonate in a separate dish to provide an ammoniacal atmosphere. Yield (82 %) (based upon metabisulfite).

**CAUTION**: This salt must not be stored in a sealed vessel; decomposition may cause a pressure explosion.

### 3-Chloro-5-methylpyrocatechol (128)

A solution of sodium dihydrogen phosphate (1.5 g) in water (500 $cm^3$) was placed in a conical flask. To this solution was added potassium nitrosodisulfonate (Fremy's salt) (9.0 g, 34 mmol) and the mixture was shaken to dissolve the inorganic radical. A solution of 2-chloro-4-methylphenol (146) (1.68 g, 11.8 mmol) in ether (35 $cm^3$) was added quickly to the purple solution and the mixture was stirred vigorously for

20 min. The colour of the solution changed to red - brown and the solution was extracted with chloroform (3 x 200 cm$^3$). The combined organic extracts were placed in a conical flask and potassium iodide (3 g, 18.1 mmol) in acetic acid (200 cm$^3$) was added to the dark red solution and the mixture stirred for 2 min. The resultant mixture was washed with sodium bisulfite solution (100 cm$^3$ of 5 % w/v). The chloroform layer was washed with water (500 cm$^3$), dried with sodium sulfate, filtered and evaporated under reduced pressure to give a dark red oil which was a mixture of the desired product (128) and unreacted starting material (146). The mixture was purified by column chromatography on basic alumina and elution with diethyl ether : light petrol (b.p. 40 - 60 °C ) (1:5). The product (128) was obtained as a dark red oil which was Kugelrohr distilled to produce an off-white crystalline solid (0.63 g, yield 34 %), m.p 58-60 °C; $\nu_{max}$ (KBr disc) 3410, 1610, 1512, 1500 and 1288 cm$^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 2.21 (3H, s, Me), 5.36 (2H, br.s, OH), 6.67 (2H, m, Ar-H); $\delta_C$ (50 MHz) ($CDCl_3$) 20.6 (Me), 115.1 (Ar-$\underline{C}$H), 119.4 (C-3), 120.8 (Ar-H), 131.2 (C-2), 137.0 (C-5), 144.2 (C-1); *m/z* 160 ($M^+$,$^{37}Cl$), 158 ($M^+$,$^{35}Cl$) (Found: $M^+$ 160.0118. $C_7H_7O_2{}^{37}Cl$ requires 160.0105), (Found: $M^+$ 158.0139 $C_7H_7O_2{}^{35}Cl$ requires 158.0135).

### 2-Chloro-4-methyl-6-nitrophenol (155)

2-Chloro-4-methylphenol (146) (3.50 g, 0.025 mmol) was dissolved in acetic acid (30 cm$^3$) and cooled to 0 °C using an ice / salt bath. To this cooled and stirred solution nitric acid (4.5 cm$^3$) in acetic acid (15 cm$^3$) was added rapidly. The resultant mixture was diluted immediately with water and a yellow precipitate was formed. The solid was filtered and washed with water (200 $cm^3$). The title compound was obtained as a yellow crystalline solid (2.8 g, yield 60.8 %), m.p 68 °C (lit.,[180] 68 °C); $\nu_{max}$ (KBr disc) 3196, 1543, 1464, 1406, 1311, 1255, 1217, 1155 and 1101 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 2.36 (3H, s, Me), 7.48 (1H, s with fine splitting, 3-H), 7.80 (1H, s with fine splitting, 5-H) and 10.80 (1H, br.s, OH); $\delta_C$ (50 MHz) ($CDCl_3$) 20.2 (Me), 123.2 (Ar-H), 123.9, 129.8 and 133.9 (Ar-C), 138.5 (Ar-H) and 149.2 (Ar-C); *m/z* 189 ($M^+$,$^{37}Cl$), 187 ($M^+$,$^{35}Cl$); (Found: $M^+$ 189.0003. $C_7H_6O_3N^{37}Cl$ requires 189.0007), (Found: $M^+$ 187.0038 $C_7H_6O_3N^{35}Cl$ requires 187.0036).

**3-Chloro-5-methyl-1,2-benzoquinone (158)**

3-Chloro-5-methylpyrocatechol (128) (0.1 g, 0.53 mmol) was dissolved in dry ether (5 $cm^3$) and placed in a dry flask fitted with a silica gel guard tube. To this solution was added silver (I) oxide (0.85 g, 3.66 mmol) as a suspension in ether (10 $cm^3$) and anhydrous magnesium sulfate (0.85 g, 7.08 mmol). The mixture was stirred for 1 h in the dark. The solution was filtered through a bed of Celite and dry sodium sulfate. The filtrate was concentrated to ~ 20 $cm^3$ and refrigerated in the dark for 16 h. The solution appeared dark red / green and no crystals were observed. The solution was evaporated to dryness *in vacuo* without heating. The *quinone* (158) was produced as a dark red oil, which was unstable when stored at -5 °C. (0.04 g, Yield 40 %). $\delta_H$ (200 MHz) ($CDCl_3$) 2.20 (3H, s, with fine splitting, Me), 6.25 (1H, s with fine splitting, Ar-H), 7.05 (1H, s with fine splitting, Ar-H); $\delta_C$ (50 MHz) ($CDCl_3$) 23.1 (Me), 126.4 (Ar-CH), 139.6 (Ar-CH), 151.8 (C-5), 141.6 (C-5), 173.3 (C=O) and 177.9 (C=O).

## 3.2.4 Trifluoromethyl Substituted Pyrocatechol

### 2-Nitro-4-trifluoromethylphenol (149) [154]

2-Nitro-4-trifluoromethylchlorobenzene (148) (5.63 g, 25 mmol) was dissolved in dimethylsulfoxide (7.5 $cm^3$) and finely powdered sodium hydroxide (3.0 g, 75 mmol) was added with stirring over an 8 h period. The reaction mixture was maintained at 20 - 25 °C throughout the addition period. The reaction was left to stand for 16 h without stirring then poured into cold water (100 $cm^3$) and shaken to mix. The mixture was washed with ether (2 x 50 $cm^3$), separated and the aqueous layer was acidified to pH 1 with concentrated hydrochloric acid. The acidified solution was extracted with ether (3 x 50 $cm^3$), and the combined dark red ether layers were filtered through Decalite and the solvent removed from the filtrate under reduced pressure. The title compound was obtained as a dark red oil (4.8 g, yield 87 %); $\nu_{max}$ (KBr disc) 3263, 1637, 1589, 1556, 1500, 1435, 1319, 1263, 1176, 1128, 1087 and 1072 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 7.32 (1H, d, *J* 8.8 Hz, 6-H), 7.84 (1H, d with fine splitting, *J* 8.8 Hz, 5-H), 8.42 (1H, s with fine splitting, 3-H) and 10.81 (1H, br.s, OH); $\delta_C$ (50 MHz) ($CDCl_3$) 121.2 (C-6), 123.1 ($CF_3$), 123.2 (C-5), 123.5 (C-4), 133.0 (C-2) 133.7 (C-3) and 157.1 (C-1); *m/z* 207 ($M^+$) (Found: $M^+$ 207.0140. $C_7H_4O_3NF_3$ requires 207.0143).

### 2-Amino-4-trifluoromethylphenol (150)

Method A: [155]

A solution of 4-hydroxy-3-nitrobenzotrifluoride (149) (8.0 g, 38.6 mmol) in ethanol (100 $cm^3$) was hydrogenated in the presence of Raney nickel catalyst (0.5 g), using low pressure hydrogenation apparatus. The reaction mixture was filtered through a bed of Celite and evaporated *in vacuo* to give a mid-brown solid. The solid was recrystallised from toluene to afford off white crystals of the title compound (5.2 g, yield 76 %), m.p 120-121 °C (lit.,[155] 121-122 °C); $\nu_{max}$ (KBr disc) 3395, 1620, 1346, 1331, 1213, 1203, 1169 and 1107 $cm^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 6.76- 6.99 (3H, m, Ar-H); $\delta_C$ (50 MHz) [$(CD_3)_2CO$] 111.7 (C-3), 114.6 (C-6), 114.9 (C-5), 122.5 (C-5), 126.3 ($CF_3$), 137.8 (C-2) and 147.9 (C-1); *m/z* 177 ($M^+$) (Found: $M^+$ 177.0395. $C_7H_6ONF_3$ requires 177.0401).

Method B: [156]

4-Hydroxy-3-nitrobenzotrifluoride (149) (0.3 g, 1.5 mmol) in formic acid (98 %, 5 $cm^3$) was stirred with 10 % Palladium on carbon catalyst (10 % Pd, 0.89 g) and the mixture was heated to 100 °C on a water bath until effervesence ceased (15 min). The mixture was filtered through a Celite bed and the Celite bed washed with water. The filtrate was extracted with ether (3 x 50 $cm^3$). The combined ether layers were dried over sodium sulfate, filtered and the filtrate evaporated *in vacuo* to afford a brown solid. The solid residue recrystallised from toluene to give 2-amino-4-trifluoromethylphenol as white crystals (0.21 g, yield 82 %), m.p 120-121 °C (lit.,[156] 121-122 °C).

**4-Trifluoromethylpyrocatechol (121)**

A solution of 4-hydroxy-3-aminobenzotrifluoride (150) (0.65 g, 3.7 mmol) in acetic acid (300 $cm^3$) was added within 3 mins to a vigorously stirred solution of sodium metaperiodiate (10.0 g, 0.04 mol) in a dilute solution of hydrochloric acid (0.1 N, 700 $cm^3$) at ambient temperature.

Stirring was continued for 30 sec and the mixture was then extracted with chloroform (3 x 500 $cm^3$). The organic layer was washed with water (2 x 500 $cm^3$) and potassium iodide (3.0 g, 18 mmol) in acetic acid (200 $cm^3$) was added. The mixture was stirred for 2 min, and then washed with sodium bisulfite solution (100 $cm^3$, 5 % w/v). The organic layer was washed with hydrochloric acid (0.1 N, 500 $cm^3$) to remove any remaining starting material. The chloroform layer was washed with water (500 $cm^3$) and the combined aqueous extracts back-extracted once with chloroform (200 $cm^3$). The combined organic extracts were dried over sodium sulfate and filtered. Acetic acid (5 $cm^3$) was added to the filtrate and the solvent removed *in vacuo.* A dark brown oil remained and was Kugelrhor distilled to afford *4-trifluoromethylpyrocatechol (121)* as an off white oil which failed to crystallise on standing (0.11 g, yield 17 %); $\nu_{max}$ (KBr disc) 3318, 3025, 2989, 1232, 1163 and 1112 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 6.52 (2H, br.s, OH, exch. with $D_2O$) and 6.88-7.09 (3H, m, Ar-H); $\delta_C$ (50 MHz) ($CDCl_3$) 112.5 (C-3), 115.1 (C-6), 118.5 (C-3), 123.2 (C-4), 124.0 ($CF_3$), 143.5 and 146.6 (C-1 and C-2); *m/z* 178 ($M^+$) (Found: $M^+$ 178.0241. $C_7H_5O_3F_3$ requires 178.0242).

### 3.2.5 Methyl Substituted Metabolites

**4-Methyl-1,2-benzoquinone (59)**

4-Methylpyrocatechol (44) (1.0g, 8.1 mmol) was dissolved in dry ether (5 $cm^3$) and placed in a dry flask fitted with a silica gel guard tube. To this solution was added silver (I) oxide (8.5 g, 36.6 mmol) as a suspension in ether (10 $cm^3$) and anhydrous magnesium sulfate (8.5 g, 70.8 mmol). The mixture was stirred for 1 h in the dark. The solution was filtered through a bed of Celite and dry sodium sulfate. The filtrate was reduced under vacuum at 20 - 23 °C. The residual oily red-brown crystals were slurried in ether (15 $cm^3$) and collected by filtration. This gave the title compound as dark red crystals (0.7 g, yield 71%), m.p 82-83 °C (lit.,[181] 83-84 °C); $\delta_H$ (200 MHz) ($CDCl_3$) 2.10 (3H, s, Me), 6.15 (1H, s with fine splitting, 3-H), 6.26 (1H, d, *J* 10 Hz, 6-H) and 6.85 (1H, d, *J* 10 Hz with fine splitting, 5-H ); $\delta_C$ (50 MHz) ($CDCl_3$) 22.6 (Me), 127.4 (C-3), 129.2 (C-6), 143.2 (C-5), 151.8 (C-4), 178.9 (C=O) and 180.1 (C=O).

### Monoperphthalic Acid [167]

Phthalic anhydride (20 g, 0.1 mol) was heated at reflux in dry benzene (200 $cm^3$). The mixture was filtered while hot and the filtrate was cooled to ambient temperature. Light petroleum (200 $cm^3$) was added and crystallisation occurred. The crystallised phthalic anhydride (10.3 g, Yield 52 %) was isolated by filtration.

To a solution of sodium carbonate (6.2 g, 50 mmol) in water (25 $cm^3$), cooled to 0 °C with stirring was added 30% aqueous hydrogen peroxide (6.9 g, 6.3 ml, 60 mmol). To this mixture was added recrystallised phthalic anhydride (7.4 g, 50 mmol) and the resultant mixture was stirred at -5 °C to 0 °C for 30 min. At the end of this period a clear solution had formed, which was transferred to a separating funnel and shaken with ether (35 $cm^3$). A solution of concentrated sulfuric acid (10 M, 3 $cm^3$) in water (15 $cm^3$) was added slowly over 10 min. The organic layer was separated and the aqueous layer extracted with ether (2 x 15 $cm^3$). The combined ether extracts were washed with saturated ammonium sulfate (2 x 20 cm$^3$) and dried in a refrigerator over anhydrous magnesium sulfate. The peracid content of the ethereal solution was determined by adding 20% aqueous potassium iodide (30 cm$^3$) to the peracid solution (2 cm$^3$) and titrating the liberated iodine after 5 min with sodium thiosulphate (0.12 M). The yield is 6.3 g in of 65 cm$^3$ ether corresponding to a 64 % yield (based on phthalic anhydride).

### 3-Methylmuconic Anhydride (156) [168]

To a stirred ice cold solution of 4-methyl-1,2-benzoquinone (59) (0.6 g, 4.9 mmol) in ether (45 cm$^3$) was added an ether solution of monoperphthalic acid (2.5 g, 26 cm$^3$, 15.4 mmol) and the mixture was stirred at -5 °C to 0 °C for 4 h, then at room temperature for 18 h. The solution was filtered and the filtrate evaporated under reduced pressure to give (131) as a white solid which was recrystallised from light petrol (0.28 g, yield 41 %), m.p 72-73 °C (lit.,[168] 73-74 °C); $\nu_{max}$ (KBr disc) 1733, 1693, 1645, 1320 and 1182 cm$^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 1.55 (3H, s, Me) and 7.61 - 7.82 (3H, m, C-H); *m/z* 138 ($M^+$); (Found: $M^+$ 138.0313. $C_7H_6O_3$ requires 138.0317).

### Disodium 3-methyl-*cis-cis*-muconate

To a stirred ice cold solution of aqueous sodium hydroxide (2 mmol, 5 cm$^3$) was added 3-methylmuconic anhyride (156) (0.1 g, 0.725 mmol). This solution was stirred for 10 min, then evaporated to dryness to give the disodium 3-methyl-*cis-cis*-muconate as a white powder (0.12 g, yield 83 %), m.p 350 °C (Decomp); $\nu_{max}$ (KBr disc) 3426, 1621, 1568, 1400, 1345 and 1304 cm$^{-1}$; $\delta_H$ (200 MHz) ($D_2O$) 1.76 (3H, s, Me), 5.60 (1H, s with fine splitting, 2-H), 5.77 (1H, d with fine spltting, *J* 13.0 Hz) and 6.49 (1H, d, *J* 13.0 Hz, 5-H); $\delta_C$ (50 MHz) ($D_2O$) 21.5 (Me), 127.7, 128.6, 129.3 (C-H), 142.3 (C-4), 176.9 (C=O) and 178.6 (C=O).

### 3-Methyl-2-*cis*-4-*trans*-muconic Acid (157) [168]

To a stirred ice cold aqueous solution of sodium hydrogen carbonate (0.121 g, 1.45 mmol, 5 cm$^3$) was added 3-methylmuconic anhyride (156) (0.1 g, 0.725 mmol). The solution was stirred for 1 h then evaporated to dryness to give the 3-methyl-2-*cis*-4-*trans*-muconic acid (157) as a white solid, which was recrystallised from ethanol (0.06 g, yield 53 %), m.p 178 -179 °C (lit.,[168] 177-178 °C); $\nu_{max}$ (KBr disc) 2966, 1690, 1628, 1598, 1314, 1266 and 1191 cm$^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2SO$] 1.99 (3H, s, Me), 5.96 (1H, s, 2-H), 6.15 (1H, d, *J* 16 Hz, 4-H) and 8.45 (1H, d, *J* 16 Hz, 5-H); $\delta_C$ (50 MHz) [$(CD_3)_2SO$] 19.9 (Me), 124.1 (C-H), 124.9 (C-H), 139.5 (C-H), 146.7 (C-3), 166.4 (C=O) and 167.3 (C=O).

### (±)-3-Methylmuconolactone (51) [169]

3-Methyl-2-*cis*-4-*trans*-muconic acid (157) (55 mg, 0.35 mmol) was heated at reflux for 12 h with water (20 cm$^3$) containing hydrochloric acid (0.2 cm$^3$, 10 M). The solution was evaporated to dryness and (±)-3-methylmuconolactone (51) was obtained as a white solid which was recrystallised from ethyl acetate (47 mg, yield 85 %), m.p 127-128 °C (lit.,[169] 127-128 °C); $\nu_{max}$ (KBr disc) 1732, 1693, 1645, 1320, 1179 and 1157 cm$^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 2.13 (3H, s, Me), 2.52 (1H, dd, *J*

16.4 and 8.5 Hz, 5-H), 3.01 (1H, dd, *J* 16.4 and 3.8 Hz, 5-H), 5.25 - 5.31 (1H, m, 4-H) and 5.81 (1H, s with fine splitting, 2-H); $\delta_C$ (50 MHz) [$(CD_3)_2CO$] 14.1 (Me), 37.6 (C-5), 81.9 (C-4), 117.7 (C-2), 170.0 (C-3), 171.4 (C=O) and 173.3 (C=O); *m/z* 156 ($M^+$); (Found: $M^+$ 156.0422. $C_7H_8O_4$ requires 156.0422).

**(±) -1-Methyl-8-bromomuconodilactone (158)**

To a solution of (±)-3-methylmuconolactone (51) (40 mg, 0.26 mmol) was added sodium hydrogen carbonate (25 mM, 5 $cm^3$). To the resultant solution was added bromine (0.04 g, 0.25 mmol) in dichloromethane (10 $cm^3$) at 0 °C with stirring. The mixture was warmed to ambient temperature and was stirred for 7 h. The resultant mixture was washed with aqueous sodium thiosulfate (2.0 M, 20 $cm^3$). The mixture was extracted with dichloromethane (3 x 25 $cm^3$) and the extracts dried with magnesium sulfate and filtered. Evaporation of the solvents gave the bromodilactone (158) as a white solid, which crystallised from chloroform - hexane (53 mg, yield 88 %), m.p 89-90 °C (lit.,[172] 88-89 °C); $\nu_{max}$ (KBr disc) 1285, 1242, 1187, 1113, 1077 and 1028 $cm^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 1.78 (3H, s, Me), 2.92 (1H, d, *J* 18.8 Hz, 4-H), 3.37 (1H, dd, *J* 18.8 and 4.9 Hz, 4-H), 4.86 (1H, s with fine splitting, 8-H) and 5.32 (1H, d, *J* 4.9 Hz, 5-H); $\delta_C$ (50 MHz) [$(CD_3)_2CO$] 19.8 (Me), 35.0 (C-4), 43.5 (C-8), 82.6 (C-5), 89.5 (C-1), 171.0 (C=O) and 172.7 (C=O); *m/z* 234 ($M^+$, $^{79}Br$) (Found: $M^+$ 233.9523. $C_9H_{11}O_4{}^{79}Br$ requires 233.9528).

**(±)-1-Methylmuconodilactone (159)**

To a suspension of the bromodilactone (158) (50 mg, 0.2 mmol) in dry benzene (2 $cm^3$) was added azoisobutyronitrile (3 mg, 0.1 mmol) under nitrogen. Tributyltin hydride (0.12 $cm^3$, ca. 0.3 mmol) was added to this mixture *via* syringe, and the resultant mixture then warmed to 30 - 40 °C to give a clear solution. The mixture was heated for another 15 min and then cooled to room temperature over 1 h with stirring. Hexane (3 $cm^3$) was added and a white precipitate formed, which was isolated by filtration to give a white solid (159) which was recrystallised from chloroform-hexane (21 mg, yield 63 %), m.p 87-88 °C (lit.,[172] 90-91 °C); $\nu_{max}$ (KBr disc) 1780, 1306, 1258, 1231, 1212, 1200, 1125, 1089 and 1043 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.58 (3H, s, Me), 2.68 - 3.04 (4H, m, 4-$H_2$ and 8-$H_2$) and 4.86 (1H, d with fine splitting, *J* 4.8 Hz, 5-H); $\delta_C$ (50 MHz) ($CDCl_3$) 21.9 (Me), 34.8 (C-4), 40.2 (C-8), 82.1 (C-5), 87.5 (C-1), 172.49 (C=O) and 172.69 (C=O); *m/z* 156 ($M^+$) (Found: $M^+$ 156.0422. $C_7H_8O_4$ requires 156.0422.)

## 3.3 METABOLISM

### 3.3.1 *Pseudomonas putida*

**Maintenance:**

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

**Chemicals:**

4-Methylpyrocatechol and 4-*t*-butylpyrocatechol were purchased from BDH company. All other substrates were prepared by the author.

**Culture Conditions:**

The nutrient medium was prepared as described by Ornston and Stanier with some modifications.

| | |
|---|---|
| Nitriloacetic acid | 220 mg |
| Magnesium sulfate heptahydrate | 580 mg |
| Calcium chloride dihydrate | 88.5 mg |
| Ferrous sulfate | 7 mg |
| Ammonium sulfate | 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 $cm^3$ |
| Deionised water | 1 litre |

* Hunter' metal solution '44' Solution for Ornston and Stanier medium

| | |
|---|---|
| Ethylenediamine Tetraacetate, Disodium Salt<br>(E.D.T.A. $Na_2.2H_2O$) | 318 mg |
| Zinc Sulfate Hydrate | 1095 mg |
| Manganese Sulfate Hydrate | 154 mg |
| Copper Sulfate Hydrate | 39.2 mg |
| Cobalt Nitrate Hydrate | 24.8 mg |
| Sodium Tetraborate | 17.7 mg |
| Ammonium Molybdate | 20 mg |
| Deionised water | 100 $cm^3$ |

**Procedures:**

The *Pseudomonas putida* (ATCC 12633) slants were maintained on 1 % yeast extract agar for 3 days at 25 °C. A water suspension of the bacteria was prepared by adding sterile water (2 $cm^3$) to the slope and scratching the surface gently to form a suspension. The suspension was used to inoculate 3 conical flasks each containing 100 $cm^3$ of the Ornston and Stainer medium. The flasks were 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 these 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 for 18 h. The ferric chloride test was again negative after this second addition, and a third batch of pyrocatechol in water was fed. After a further 24 h, the cultures were combined, the pH adjusted to pH 7.5 with aqueous sodium hydroxide and the mixture extracted with ether to remove non-acidic material. The pH of the culture medium was then adjusted to pH 2.5 with orthophosphoric acid, saturated with sodium chloride, and extracted with ether to obtain the 4-substituted muconolactone. The remaining aqueous extract was then continuously extracted with ether for 48 h to remove any remaining metabolites. The ether extracts were filtered through Celite, dried over sodium sulfate and evaporated *in vacuo.*

**Metabolism of 4-methylpyrocatechol (44)**

This pyrocatechol was used as a control each time. Examination of the ether extracts of the medium at pH 7.5 yielded any unmetabolised 4-methylpyrocatechol (44). Ether extracts of the medium at pH 2.5 yielded an oily solid which by $^1$H NMR spectroscopy of the total extract in [$(CD_3)_2SO$] was shown to contain 4-methylmuconolactone (50) as the major metabolite, with 3-methylmuconolactone (51) and 3-methyl-2-*cis*-4-*trans*-muconic acid (157) also being present. The 3-methyl-2-*cis*-4-*trans*-muconic-acid (132) was separated by centrifrugation with acetone. Preparative TLC on silica plates developed with diisopropyl ether - formic acid - water (200 : 7 : 3 ) successfully separated the remaining oil to give (S)-(+)-4-methylmuconolactone (50), $R_F$ 0.4, and (±)-3-methylmuconolactone (51), $R_F$ 0.2. The feeding data for these experiments are contained in Tables 1 to 5 on pages 177 to 181.

### (*S*)-(+)-4-Methylmuconolactone (50)

Preparative TLC gave the (S)-(+)-4-methylmuconolactone (50) as an oil which failed to crystallise, $[\alpha]_D$ + 31 ° (*c*, 0.9 in methanol) (lit.,[175] + 31 °); $\nu_{max}$ (KBr disc) 3111, 2984, 2934, 1743, 1176 and 1107 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.52 (3H, s, Me), 2.69 and 3.04 (2H, ABq, *J* 15.9 Hz, 5-$H_2$), 6.03 (1H, d, *J* 5.7 Hz, 2-H) and 7.65 (1H, d, 5.7 Hz, 3-H); $\delta_C$ (50 MHz) ($CDCl_3$) 23.7 (Me), 42.7 (C-5), 85.9 (C-4), 120.4 (C-2), 160.0 (C-3), 172.4 (C=O) and 173.0 (C=O); *m*/*z* 156 ($M^+$) (Found: $M^+$ 156.0415. $C_7H_8O_4$ requires 156.0422).

### (±)-3-Methylmuconolactone (51)

Preparative TLC of the ether extract of the medium at pH 2.5 gave (±)-3-methylmuconolactone (51) as an oil which failed to crystallise; $\nu_{max}$ (KBr disc) 3111, 3047, 2990, 1736, 1645, 1414, 1398, 1319 and 1186 $cm^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 2.02 (3H, s, Me), 2.52 (1H, dd, *J* 16.3 and 8.5 Hz, 5-H), 3.00 (1H, dd, *J* 16.3 and 3.8 Hz, 5-H), 5.13 - 5.19 (1H, m, 4-H) and 5.94 (1H, s with fine splitting, 2-H); $\delta_C$ (50 MHz) [$(CD_3)_2CO$] 13.7 (Me), 37.5 (C-5), 81.6 (C-4), 117.4 (C-2), 169.6 (C-3), 171.2 (C=O) and 172.8 (C=O); *m*/*z* 156 ($M^+$) (Found: $M^+$ 156.0437. $C_7H_8O_4$ requires 156.0422).

### 3-Methyl-2-*cis*-4-*trans*-muconic Acid (157)

Separation of 3-methyl-2-*cis*-4-*trans*-muconic acid (157) from the total extract of the medium at pH 2.5 was achieved by centrifugation to give a white crystalline solid with m.p 176-177 °C (lit.,[168] 177-178 °C); $\nu_{max}$

(KBr disc) 2966, 1690, 1628, 1598, 1314, 1266 and 1191 cm$^{-1}$; $\delta_H$(200 MHz) [$(CD_3)_2SO$] 1.99 (3H, s, Me), 5.96 (1H, s, 2-H), 6.15 (1H, d, *J* 16 Hz, 5-H) and 8.45 (1H, d, *J* 16 Hz, 4-H); $\delta_C$(50 MHz) [$(CD_3)_2SO$] 19.9 (Me), 124.1 (CH), 124.9 (CH), 139.5 (CH), 146.7 (C-3), 166.4 (C=O) and 167.3 (C=O).

**(-)-1-Methyl-4-bromomuconodilactone (170)**

(*S*)-(+)-4-Methylmuconolactone (50) (78 mg, 0.5 mmol) was brominated using the standard procedure to give (-)-1-methyl-4-bromomuconodilactone (170) (97 mg, yield 83 %) as a white crystalline solid, m.p 123-124 °C, $[\alpha]_D$ - 68 ° (*c*, 0.9 in methanol); $\nu_{max}$(KBr disc) 1785, 1293, 1265, 1202, 1117, 1085 and 1015 cm$^{-1}$; $\delta_H$(360 MHz) ($CDCl_3$) 1.88 (3H, s, Me), 2.78 and 3.06 (1H, ABq, *J* 18.8 Hz, 8-$H_2$), 4.47 (1H, s, 5-H) and 4.97 (1H, s, 4-H); $\delta_C$(50 MHz) ($CDCl_3$) 22.7 (Me), 36.8 (C-4), 40.5 (C-8), 86.2 (C-5), 87.7 (C-1), 169.3 (C=O) and 171.0 (C=O); *m/z* 234 ($M^+$,$^{79}$Br); (Found: $M^+$ 233.9587. $C_7H_7{}^{79}Br\,O_4$ requires 233.9528).

**(-)-1-Methylmuconodilactone (54)**

(-)-1-Methyl-4-bromomuconodilactone (170) (59mg, 0.25 mmol) was debrominated by the standard procedure to give (-)-1-methylmuconodilactone (54) (21 mg, yield 53 %) as a white crystalline solid, m.p. 108-109 °C (lit.,[175] 108 °C), $[\alpha]_D$ - 132 ° (*c*, 0.2 in methanol) (lit.,[163] - 131 °); $\nu_{max}$(KBr disc) 1784, 1296, 1259, 1199, 1127, 1090 and 1033 cm$^{-1}$; $\delta_H$(200 MHz) ($CDCl_3$) 1.55 (3H, s, Me), 2.68 - 2.99 (4H, m,

$4\text{-}H_2$ and $8\text{-}H_2$) and 4.85 (1H, d, *J* 4.4 Hz, 5-H); $\delta_C$ (50 MHz) ($CDCl_3$) 21.5 ($CH_3$), 34.6 ($C\text{-}H_2$), 40.0 ($C\text{-}H_2$), 82.0 (C-5), 87.5 (C-1), 172.6 (C=O) and 172.8 (C=O); *m/z* 156 ($M^+$) (Found: $M^+$ 156.0419. $C_7H_8O_4$ requires 156.0422.)

**Metabolism of 4-isopropylpyrocatechol (120)**

Examination of the ether extracts at pH 7.5 recovered unmetabolised 4-isopropylpyrocatechol (120). At pH 2.5 evaporation of the ether extracts produced a dark red gum which when examined by $^1H$ NMR spectroscopy showed the presence of 4-isopropylmuconolactone (165) as the major metabolite with 3-isopropylmuconolactone (152) and 3-isopropyl-2-pyrone-5-carboxylic acid (166) also present. Preparative TLC on silica plates with diisopropyl ether - formic acid - water (200 : 7 : 3) as eluent, successfully separated (165) and (152) with $R_F$ 0.4 and 0.2 respectively. 3-Isopropyl-2-pyrone-5-carboxylic acid (166) was not recovered. The feeding data for these experiments are contained in Table 1 on page 177.

**(+)-4-Isopropylmuconolactone (165)**

Preparative TLC gave the *(+)-4-isopropylmuconolactone* (165) as an oil which failed to crystallise, $[\alpha]_D$ + 21 ° (*c*, 0.7 in methanol); $\nu_{max}$ (KBr disc) 3431, 3200, 3096, 2974, 2941, 2885, 1745, 1736, 1701 and 1390 $cm^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 0.92 (3H, d, *J* 6.9 Hz, Me), 0.96 (3H, d, *J* 6.9 Hz, Me), 2.22 (1H, m, $C\underline{H}Me_2$), 2.79 and 2.97 (2H, ABq, *J* 15.9 Hz, $5\text{-}H_2$), 6.12 (1H, d, *J* 5.7 Hz, 2-H), 7.76 (1H, d, *J* 5.8 Hz, 3-H); $\delta_C$ (50 MHz) [$(CD_3)_2CO$] 17.0 (Me),17.5 (Me), 34.7 ($\underline{C}HMe_2$), 40.1 (C-5), 90.9 (C-4), 122.0 (C-2), 159.3 (C-3), 170.5 (C=O), 172.5 (C=O); *m/z* 184 ($M^+$); (Found: $M^+$ 184.0751 $C_9H_{12}O_4$ requires 184.0736).

### (±)-3-Isopropylmuconolactone (152)

Preparative TLC recovered *(±)-3-isopropylmuconolactone* (152) as an oil which failed to crystallise; $\nu_{max}$ ($CHCl_3$) 3010, 3000, 1800, 1760, 1720, 1240, 1180 and 1100 $cm^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 1.17 (3H, d, *J* 6.7 Hz, Me), 1.21 (3H, d, *J* 6.7 Hz, Me), 2.25 (1H, m, C$\underline{H}$Me$_2$), 2.45 - 3.12 (2H, m, 5-$H_2$), 5.35 - 5.45 (1H, m, 4-H) and 5.86 (1H, s with fine splitting, 2-H); $\delta_C$ (50 MHz) [$(CD_3)_2CO$] 20.7 and 22.0 $(Me)_2$, 28.0 ($CHMe_2$), 37.6 (C-5), 80.1 (C-4), 114.8 (C-2), 171.0 (C=O), 172.8 (C=O) and 179.2 (C-3); *m/z* 184 ($M^+$) (Found: $M^+$ 184.0722. $C_9H_{12}O_4$ requires 184.0736).

### (-)-1-Isopropyl-4-bromomuconodilactone (169)

(+)-4-Isopropylmuconolactone (165) (100 mg, 0.54 mmol) was brominated using the standard procedure to give *(-)-1-isopropyl-4-bromomuconodilactone* (169) (131 mg, yield 91 %) as an oil which failed to crystallise, $[\alpha]_D$ - 49 ° (*c*, 0.6 in methanol); $\nu_{max}$ ($CHCl_3$) 3317, 3029, 2984, 2406, 2355 and 1798 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.10 (3H, d, *J* 6.8 Hz, Me), 1.20 (3H, d, *J* 6.8 Hz, Me), 2.41 (1H, septet, *J* 6.8 Hz, C$\underline{H}$Me$_2$), 2.72 - 3.00 (2H, m, 8-$H_2$), 4.49 (1H, s, 5-H) and 5.08 (1H, s, 4-H); $\delta_C$ (50 MHz) ($CDCl_3$) 17.7 and 17.9 ($Me_2$), 33.2 ($\underline{C}$HMe$_2$), 34.4 (C-8), 37.3 (C-4), 84.1 (C-5), 94.3 (C-1), 171.0 (C=O) and 173.0 (C=O); *m/z* 264 ($M^+$,$^{81}Br$), 262 ($M^+$,$^{79}Br$); (Found: $M^+$ 263.9847. $C_9H_{11}O_4\,^{81}Br$

requires 263.9821), (Found: M$^+$ 261.9842. $C_9H_{11}O_4\,^{79}Br$ requires 261.9841).

**(-)-1-Isopropylmuconodilactone (168)**

(-)-1-Isopropyl-4-bromomuconodilactone (169) (120 mg, 0.45 mmol) was debrominated by the standard procedure to give *(-)-1-isopropylmuconodilactone* (168) (29 mg, yield 41 %) as an oil which failed to crystallise, $[\alpha]_D$ - 117 ° (*c*, 0.3 in methanol); $\nu_{max}$ ($CHCl_3$) 3316, 3022, 2983, 2402, 2352, 1788, 1632 and 1394 cm$^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 0.99 (3H, d, *J* 6.8 Hz, Me), 1.07 (3H, d, *J* 6.8 Hz, Me), 2.24 (1H, septet, $C\underline{H}Me_2$), 2.72 - 3.22 (4H, m, 4-$H_2$ and 8-$H_2$), 5.23 (1H, br.d, *J* 5.8 Hz, 5-H); $\delta_C$ (50 MHz) [$(CD_3)_2CO$] 16.4 and 17.7 $Me_2$), 34.2 ($\underline{C}HMe_2$), 34.2 (C-9), 36.2 and 37.7 (C-4 and C-8), 81.2 (C-5), 94.2 (C-1), 173.8 (C=O) and 174.0 (C=O); *m/z* 184 (M$^+$) (Found: M$^+$ 184.0746. $C_9H_{12}O_4$ requires 184.0736).

**Metabolism of 4-Ethylpyrocatechol (119)**

4-Ethylpyrocatechol (119) was recovered in small quantities by manual extraction of the aqueous broth with ether at pH 7.5. Examination of the ether extracts at pH 2.5 by $^1H$ NMR confirmed the presence of 4-ethylmuconolactone (171) as the major metabolite, with 3-ethylmuconolactone (172) and 3-ethyl-2-*cis*-4-*trans*-muconic acid (173) also present as minor metabolites. Centrifugation of the total extract at pH 2.5 was successful in separating 3-ethyl-2-*cis*-4-*trans*-muconic acid (173); however the extract was contaminated with the 4-ethyl and 3-ethylmuconolactones. The $^1H$ NMR spectrum of the remaining extract displayed all the proton signals of both 4-ethylmuconolactone (171) and 3-ethylmuconolactone (172). The feeding data for this experiment are shown in Table 4 on page 180.

**4-Ethylmuconolactone (171)**

Examination of the extract at pH 2.5 by $^1$H NMR spectroscopy showed the presence of 4-ethylmuconolactone (171); $\delta_H$ (200 MHz) ($CDCl_3$) 0.77 (3H, t, *J* 7.34 Hz, $CH_2C\underline{H}_3$), 1.86 (2H, q, *J* 7.34 Hz, $C\underline{H}_2CH_3$), 2.68 and 2.89 (2H, ABq, *J* 15.7 Hz, 5-$H_2$), 6.05 (1H, d, *J* 5.7 Hz, 2-H) and 7.56 (1H, d, *J* 5.7 Hz, 3-H); $\delta_C$ (50 MHz) ($CDCl_3$) 7.3 ($CH_2C\underline{H}_3$), 29.1 ($C\underline{H}_2CH_3$), 41.1 (C-5), 88.4 (C-4), 121.3 (C-2), 158.8 (C-3), 172.3 (C=O) and 173.2 (C=O).

**3-Ethylmuconolactone (172)**

Examination of the extract of the medium at pH 2.5 by $^1$H NMR spectroscopy confirmed the presence of 3-ethylmuconolactone (172); $\delta_H$ (200 MHz) ($CDCl_3$) 1.14 (3H, t, *J* 8 Hz, $CH_2C\underline{H}_3$), 1.86 (2H, q, *J* 8 Hz, $C\underline{H}_2CH_3$), 2.60 (1H, dd, *J* 16.5 and 8.4 Hz, 5-H), 2.94 (1H, dd, *J* 16.5 and 4.1 Hz, 5-H), 5.19 - 5.25 (1H, m, 4-H) and 5.80 (1H, s with fine splitting, 2-H); $\delta_C$ (50 MHz) ($CDCl_3$) 10.9 ($CH_2C\underline{H}_3$), 21.1 ($C\underline{H}_2CH_3$), 36.7 (C-5), 79.9 (C-4), 115.1 (C-1), 172.7 (C-3), 173.6 (C=O) and 174.1 (C=O).

**Metabolism of 4-Allylpyrocatechol (125)**

Examination of the ether extracts of the medium at pH 7.5 showed the presence of unmetabolised 4-allylpyrocatechol (125). The total extract of the medium at pH 2.5 was examined by $^1$H NMR spectroscopy to show a complex mixture of metabolites. 4-Allylmuconolactone (174), and 3-allyl-2-*cis*-4-*trans*-muconic acid (175) were found to be present in approximately equal quantities. This mixture was subjected to preparative TLC on silica plates with diisopropyl ether - formic acid - water (200 : 7 : 3) as eluent, which recovered (+)-4-allylmuconolactone (174) with $R_F$ 0.4. (±)-3-Allylmuconolactone and 3-allyl-2-*cis*-4-*trans*-muconic acid (175) were not isolated. The feeding data for these experiments are contained in Table 2 on page 178.

**(+)-4-Allylmuconolactone (174)**

Preparative TLC yielded *(+)-4-allylmuconolactone* (174) as an oil which failed to crystallise, $[\alpha]_D$ + 10 ° (*c*, 0.5 in methanol); $\nu_{max}$ ($CHCl_3$) 3005, 1760, 1700, 1235, 1225, 1110 and 1100 cm$^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 2.58 - 2.64 (2H, m, $C\underline{H_2}$-CH=$CH_2$), 2.69 - 3.01 (2H, m, 5-$H_2$), 5.11 - 5.27 (2H, m, $CH_2$-CH=$C\underline{H_2}$), 5.55 - 5.75 (1H, m, $CH_2$-C$\underline{H}$=$CH_2$), 6.12 (1H, d, *J* 5.5 Hz, 2-H), 7.61 (1H, d, *J* 5.6 Hz, 3-H) and 9.97 (1H, br.s, OH); $\delta_C$ (50 MHz) ($CDCl_3$) 40.1 (C-5 and C-7), 87.2 (C-4), 121.8 ($CH_2$-CH=$\underline{C}H_2$), 121.9 (C-2), 130.0 (C-8), 158.4 (C-3), 172.0 (C=O) and 174.1 (C=O); *m/z* 182 ($M^+$) (Found: $M^+$ 182.0575. $C_9H_{10}O_4$ requires 182.0579).

**(-)-1-Allylmuconodilactone (176)**

(+)-4-Allylmuconolactone (174) cyclised upon storage at ambient temperature to give *(-)-1-allylmuconodilactone* as a white crystalline compound, m.p. 114-115 °C, $[\alpha]_D$ - 82 ° (*c*, 0.3 in methanol); $\nu_{max}$ (KBr disc) 3441, 1783, 1257 and 1210 cm$^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 2.73 -

2.77 (2H, m, $C\underline{H}_2$-CH=$CH_2$), 2.89 - 3.25 (4H, m, 4-$H_2$ and 8-$H_2$), 5.16 - 5.18 (1H, br.d, *J* 5.6 Hz, 5-H), 5.20 - 5.34 (2H, m, $CH_2$-CH=$C\underline{H}_2$) and 5.78 - 6.0 (1H, m, $CH_2$-C$\underline{H}$=$CH_2$); $\delta_C$ (50 MHz) [$(CD_3)_2CO$] 35.1 ($CH_2$-CH=$\underline{C}H_2$) 38.5 and 39.4 (C-4 and C-8), 81.2 (C-5), 89.8 (C-1), 120.3 ($CH_2$-CH=$\underline{C}H_2$), 131.2 ($CH_2$-$\underline{C}$H=$CH_2$), 173.0 (C=O), 173.1 (C=O); *m/z* 182 ($M^+$) (Found: $M^+$ 182.0574. $C_9H_{10}O_4$ requires 182.0579).

**Metabolism of 4-Propylpyrocatechol (124)**

Examination of the ether extracts at pH 7.5 showed the presence of unmetabolised 4-propylpyrocatechol (124).The ether extracts at pH 2.5, when examined by $^1$H NMR, showed 4-propylmuconolactone (177), 3-propylmuconolactone (178) and 3-propyl-2-*cis*-4-*trans*- muconic acid (179) present in approximately equal quantities. The total extract was separated by preparative TLC on silica plates (diisopropyl ether - formic acid - water (200 : 7 : 3) as eluent. (+)-4-Propylmuconolactone (177) and (±)-3-propylmuconolactone (178) with $R_F$ 0.4 and 0.2 respectively were recovered. 3-Propyl-2-*cis*-4-*trans*-muconic acid (179) was not recovered. The feeding data for these experiments are contained in Table 3 on page 179.

**(+)-4-Propylmuconolactone (177)**

Preparative TLC recovered the target lactone *(+)-4-propylmuconolactone* (177) as an oil which failed to crystallise. $[\alpha]_D$ + 11 ° (*c*, 0.7 in methanol); $\nu_{max}$ ($CHCl_3$) 3020, 2960, 2900, 1750, 1700, 1360, 1350, 1310, 1120 and 1100 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 0.90 (3H, t, *J* 7.3 Hz, $CH_2CH_2C\underline{H}_3$), 1.19 - 1.35 (2H, m, $CH_2C\underline{H}_2CH_3$), 1.71 - 1.94

($C\underline{H}_2CH_2CH_3$), 2.16 and 3.04 (2H, ABq *J* 15.8 Hz, 5-$H_2$), 6.10 (1H, d, *J* 5.7 Hz, 2-H), 7.60 (1H, d, J 5.7 Hz, 3-H); $\delta_C$ (50 MHz) ($CDCl_3$) 13.9 ($CH_2CH_2\underline{C}H_3$), 16.6 ($CH_2\underline{C}H_2CH_3$), 38.4 ($\underline{C}H_2CH_2CH_3$), 41.9 (C-5), 88.0 (C-4), 121.5 (C-2), 158.6 (C-3), 172.2 (C=O) and 174.2 (C=O); *m/z* 184 ($M^+$) (Found: $M^+$ 184.0745. $C_9H_{12}O_4$ requires 184.0735).

### (±)-3-Propylmuconolactone (178)

Preparative TLC afforded *(±)-3-propylmuconolactone* (178) as an oil which failed to crystallise. $\nu_{max}$ (thin film) 2963, 2932, 2876, 1738, 1635 and 1178 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 0.98 (3H, t, *J* 7.2 Hz, $CH_2CH_2C\underline{H}_3$), 1.53 - 1.72 (2H, m, $CH_2C\underline{H}_2CH_3$), 2.25 - 2.33 (2H, m, $C\underline{H}_2CH_2CH_3$), 2.48 - 2.92 (2H, m, 5-$H_2$), 5.25 - 5.27 (1H, m, 4-H), 5.84 (1H, br.s, 2-H); $\delta_C$ (50 MHz) ($CDCl_3$) 13.7 ($CH_2CH_2\underline{C}H_3$), 20.3 ($CH_2\underline{C}H_2CH_3$), 29.9 ($\underline{C}H_2CH_2CH_3$), 37.2 (C-5), 80.0 (C-4), 116.1 (C-2), 172.3 (C=O), 173.0 (C=O) and 174.1 (C-3); *m/z* 184 ($M^+$) (Found: $M^+$ 184.0726. $C_9H_{12}O_4$ requires 184.0735).

### (-)-1-Propylmuconodilactone (180)

(+)-4-Propylmuconolactone (177) cyclised upon storage at ambient temperature to give *(-)-1-propylmuconodilactone* (180) as an oil which failed to crystallise, $[\alpha]_D$ - 98 ° (*c*, 0.5 in methanol); $\nu_{max}$ ($CHCl_3$) 3026, 1968, 1789, 1225, 1203 and 1110 $cm^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 0.97 (3H, t, *J* 7.3 Hz, $CH_2CH_2C\underline{H}_3$), 1.50 (2H, sextet, *J* 7.3 Hz, $CH_2C\underline{H}_2CH_3$), 1.91 (2H, t *J* 7.3 Hz, $C\underline{H}_2CH_2CH_3$), 2.72 - 3.25 (4H, m, 4-$H_2$ and 8-$H_2$) and 5.12 (1H, d *J* 5.5 Hz, 5-H); $\delta_C$ (50 MHz) [$(CD_3)_2CO$] 14.3

($CH_2CH_2\underline{C}H_3$), 18.0 ($CH_2\underline{C}H_2CH_3$), 35.6 ($\underline{C}H_2CH_2CH_3$), 38.1 and 39.2 (C-4 and C-8), 82.5 (C-5), 91.4 (C-1), 173.1 (C=O) and 174.0 (C=O); *m/z* 184 ($M^+$) (Found: $M^+$ 184.0728. $C_9H_{12}O_4$ requires 184.0735).

### Metabolism of 4-*n*-butylpyrocatechol (126)

4-*n*-Butylpyrocatechol (126) was recovered by manual extraction with ether at pH 7.5. Examination of the total ether extract at pH 2.5 by $^1H$ NMR spectroscopy showed that the major components present were 3-*n*-butylmuconolactone (181) and 3-*n*-butyl-2-*cis*-4-*trans*-muconic acid (182). Also present were trace levels of 4-*n*-butylmuconlactone (183). This extract was not purified but approximate quantities of metabolites produced were determined by $^1H$ NMR spectroscopy. The feeding data for this experiment are contained in Table 4 on page 180.

### Metabolism of 4-*t*-butylpyrocatechol (161)

Examination of the ether extracts at pH 7.5 showed the presence of 4-t-butylpyrocatechol. Examination by $^1H$ NMR of the total ether extract at pH 2.5 showed that few metabolites were present. Preparative TLC on silica plates (diisopropyl ether/ formic acid/water at 200 : 7 : 3, v/v), allowed successful recovery of 3-*t*-butyl-2-pyrone-5-carboxylic acid (184) with $R_F$ 0.25. The feeding data for these experiments are contained in Table 4 on page 180.

### 3-*t*-Butyl-2-pyrone-5-carboxylic acid (184)

Preparative TLC gave *3-t-butyl-2-pyrone-5-carboxylic acid* (184) as an oil which failed to crystallise. $\nu_{max}$ ($CHCl_3$) 2970, 1724, 1252, 1123 and

1091 cm$^{-1}$; $\delta_H$ (200 MHz) [$(CD_3)_2CO$] 1.29 (9H, s, $Me_3$), 6.33 (1H, s with fine splitting, *J* 1.7 Hz, 4-H) and 7.24 (1H, s with fine splittting *J* 1.7 Hz, 2-H); $\delta_C$ (50 MHz) [$(CD_3)_2CO$] 28.7 ($Me_3$), 110.1 (C4-H), 114.2 (C2-H), 149.9 ($\underline{C}Me_3$), 161.0 (C=O), 161.4 (C=O), 166.8 (C-3) and 194.5 (C-5); *m*/*z* 196 ($M^+$) (Found: $M^+$ 196.0744. $C_{10}H_{12}O_4$ requires 196.0736).

**Metabolism of 4-trifluoromethylpyrocatechol (121)**

Examination of the ether extracts at pH 7.5 showed the presence of unmetabolised 4-trifluoromethylpyrocatechol (121). Examination of the total ether extract at pH 2.5 by $^1$H NMR showed few metabolites were present. Preparative TLC on silica plates (diisopropyl ether - formic acid - water (200 : 7 : 3)), did not enable recovery of any metabolites. The feeding data for this experiment are contained in Table 5 on page 181.

**Metabolism of 3-chloro-5-methylpyrocatechol (128)**

3-Chloro-5-methylpyrocatechol (128) was recovered by manual extraction with ether at pH 7.5. Examination by of the total ether extract at pH 2.5 by $^1$H NMR spectroscopy showed that few metabolites were present. Preparative TLC on silica plates (diisopropyl ether - formic acid - water (200 : 7 : 3)) did not enable recovery of any metabolites. The feeding data for these experiments are contained in Table 5 on page 181.

Table 1: Feedings of 4-isopropylpyrocatechol (120) to cultures of *Pseudomonas putida*

| Experiment | 1 | Methyl Control[b] | 2[c] | Methyl Control[a,c] | 3 | Methyl Control[c] | 4 | Methyl Control[c] |
|---|---|---|---|---|---|---|---|---|
| Total number of flasks used[a] | 15 | 8 | 16 | 8 | 14 | 6 | 14 | 6 |
| Amount (mg) of pyrocatechol fed | 375 | 200 | 400 | 200 | 350 | 150 | 350 | 150 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 91<br>(23%) | - | 90<br>(23%) | 5<br>(3%) | 72<br>(20%) | 2<br>(1%) | 104<br>(30%) | 6<br>(4%) |
| Total metabolites (mg) extracted at pH 2.5 | 245 | 181 | 185 | 185 | 204 | 125 | 153 | 110 |
| Yield (mg) of 4-alkyl-muconolactone | 160<br>(33%) | 122<br>(48.3%) | 149<br>(31%) | 115<br>(46%) | 132<br>(31%) | 71<br>(38%) | 111<br>(26%) | 64<br>(34%) |
| Yield (mg) of 3-alkyl-muconolactone | 34<br>(5%) | 18<br>(7%) | 26<br>(5%) | 15<br>(6%) | 16<br>(4%) | 18<br>(10%) | 8<br>(2%) | 14<br>(8%) |
| Yield (mg) of 3-alkyl-*cis-trans* muconic acid | - | 16<br>(6%) | - | 38<br>(15%) | - | 12<br>(7%) | - | 18<br>(10%) |
| Yield (mg) of 3-alkyl-pyrone acid | - | - | 22<br>(5%) | - | - | - | - | - |

[a] Each incubation flask contained 100 $cm^3$ medium.

[b] 4-Methylpyrocatechol was fed in parallel

[c] The figures for metabolite yield have been measured from quantitative $^1$H NMR spectroscopy.

**Table 2: Feedings of 4-allylpyrocatechol (125) to cultures of *Pseudomonas putida***

| Experiment | 1 | Methyl Control[b] | 2 | Methyl Control[b] | 3 | Methyl Control[b] | 4 | Methyl Control[b] |
|---|---|---|---|---|---|---|---|---|
| Total number of flasks used[a] | 15 | 8 | 15 | 8 | 17 | 6 | 15 | 8 |
| Amount (mg) of pyrocatechol fed | 375 | 200 | 375 | 200 | 425 | 150 | 375 | 200 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 40<br>(11%) | - | 20<br>(5%) | 4<br>(2%) | 31<br>(7%) | - | 57<br>(15%) | 3<br>(1%) |
| Total metabolites (mg) extracted at pH 2.5 | 150 | 181 | 196 | 175 | 280 | 143 | 130 | 147 |
| Yield (mg) of 4-alkyl-muconolactone | 48<br>(11%) | 122<br>(48.3%) | 43<br>(9%) | 108<br>(43%) | 61<br>(12%) | 115<br>(61%) | 36<br>(8%) | 80<br>(32%) |
| Yield (mg) of 3-alkyl-muconolactone | - | 18<br>(7%) | - | 11<br>(4%) | - | 16<br>(9%) | - | 19<br>(7%) |
| Yield (mg) of 3-alkyl-*cis-trans* muconic acid | 40[c]<br>(9%) | 16<br>(6%) | - | 15<br>(6%) | 45[c]<br>(9%) | 10<br>(5%) | - | 22<br>(9%) |

[a] Each incubation flask contained 100 cm$^3$ medium.

[b] 4-Methylpyrocatechol was fed in parallel

[c] The figures for metabolite yield have been measured from $^1$H NMR spectroscopy.

**Table 3: Feedings of 4-n-propylpyrocatechol (124) to cultures of *Pseudomonas putida***

| Experiment | 1 | Methyl Control[b] | 2 | Methyl Control[b] | 3 | Methyl Control[b] | 4 | Methyl Control[b] |
|---|---|---|---|---|---|---|---|---|
| Total number of flasks used[a] | 15 | 8 | 17 | 6 | 14 | 6 | 14 | 6 |
| Amount (mg) of pyrocatechol fed | 375 | 200 | 425 | 150 | 350 | 150 | 350 | 150 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 72<br>(20%) | 3<br>(1%) | 84<br>(20%) | - | 86<br>(25%) | - | 105<br>(30%) | 5<br>(4%) |
| Total metabolites (mg) extracted at pH 2.5 | 274 | 147 | 280 | 143 | 261 | 103 | 247 | 89 |
| Yield (mg) of 4-alkyl-muconolactone | 72<br>(16%) | 80<br>(32%) | 69<br>(13%) | 115<br>(61%) | 74<br>(16%) | 94<br>(50%) | 52<br>(12%) | 69<br>(37%) |
| Yield (mg) of 3-alkyl-muconolactone | 66<br>(15%) | 19<br>(7%) | 62<br>(12%) | 16<br>(9%) | 62<br>(15%) | 16<br>(9%) | 48<br>(11%) | 21<br>(11%) |
| Yield (mg) of 3-alkyl-*cis-trans* muconic acid | 72<br>(16%)[c] | 22<br>(9%) | - | 10<br>(5%) | - | 8<br>(4%) | - | 13.6<br>(7%) |

[a] Each incubation flask contained 100 $cm^3$ medium.

[b] 4-Methylpyrocatechol (44) was fed in parallel

[c] The figures for metabolite yield have been measured from $^1H$ NMR spectroscopy.

Table 4: Feedings of other 4-Alkylpyrocatechol to cultures of *Pseudomonas putida*

| Experiment | 4-Ethyl pyrocatechol (119)[c] | Methyl Control[b] | 4-t-Butyl-pyrocatechol (161) | Methyl Control[b] | 4-n-Butyl-pyrocatechol (126) | Methyl Control[b] |
|---|---|---|---|---|---|---|
| Total number of flasks used[a] | 16 | 8 | 15 | 8 | 15 | 7 |
| Amount (mg) of pyrocatechol fed | 400 | 200 | 375 | 200 | 375 | 175 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 2<br>(1%) | 5<br>(3%) | 238<br>(68 %) | 10.2<br>(5%) | 242<br>(64 %) | 6<br>(2%) |
| Total metabolites (mg) extracted at pH 2.5 | 382 | 185 | 86 | 155 | 108 | 117 |
| Yield (mg) of 4-alkyl-muconolactone | 224<br>(46%) | 115<br>(46%) | - | 107<br>(43%) | 1[c] | 91<br>(41%) |
| Yield (mg) of 3-alkyl-muconolactone | 36<br>(7%) | 15<br>(6%) | - | 10<br>(4%) | 3[c] | 3<br>(2%) |
| Yield (mg) of 3-alkyl-*cis-trans* muconic acid | 28<br>(6%) | 38<br>(15%) | - | 16<br>(6%) | 3[c] | 10<br>(4%) |
| Yield (mg) of 3-alkyl pyrone acid | - | 38<br>(15%) | 11<br>(3%) | - | - | - |

[a] Each incubation flask contained 100 $cm^3$ medium.

[b] 4-Methylpyrocatechol was fed in parallel

[c] Ratio of metabolities calculated from $^1H$ NMR spectroscopy (1:3:3).

**Table 5: Feedings of other 4-Alkylpyrocatechol to cultures of *Pseudomonas putida***

| Experiment | 2-Chloro-5-methyl pyrocatechol (128) | Methyl control[b] | 4-Trifluromethyl-pyrocatechol (121) | Methyl Control[b] |
|---|---|---|---|---|
| Total number of flasks used[a] | 15 | 8 | 15 | 7 |
| Amount (mg) of pyrocatechol fed | 375 | 200 | 180[c] | 175 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 50<br>(13%) | 10<br>(5%) | 31<br>(17%) | 6<br>(2%) |
| Total metabolities (mg) extracted at pH 2.5 | 185 | 155 | 26 | 117 |
| Yield (mg) of 4-alkyl-muconolactone | - | 107<br>(43%) | - | 91<br>(41%) |
| Yield (mg) of 3-alkyl-muconolactone | - | 10<br>(4%) | - | 3<br>(2%) |
| Yield (mg) of 3-alkyl-*cis-trans* muconic acid | - | 16<br>(6%) | - | 10<br>(4%) |

[a] Each incubation flask contained 100 $cm^3$ medium.

[b] 4-Methylpyrocatechol was fed in parallel.

[c] Quantity of 4-Trifluromethylpyrocatechol (121) that was available.

### 3.3.2 *ASPERGILLUS NIGER*

**Maintenance:**

*Aspergillus niger* EM 32 mutant was orginally obtained from University of Newcastle-Upon-Tyne. It was maintained at 4 °C on *Difco bact* potato dextrose slopes in 10 ml 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. All other substrates were prepared by the author.

**Culture Conditions:**

Fungi were grown in liquid culture in a basal salts medium containing

| | |
|---|---|
| Ammonium sulfate | 0.5 g |
| Potassium dihydrogen orthophosphate | 1.0 g |
| Magnesium sulfate heptahydrate | 50 mg |
| Trace elements solution * | 1 $cm^3$ |
| Deionised water | 1 litre |
| Glucose | 1.8 g |

* Trace elements solution:

| | |
|---|---|
| Ferrous sulfate heptahydrate | 54 mg |
| Manganous sulfate tetrahydrate | 40 mg |
| Zinc sulfate heptahydrate | 20 mg |
| Copper sulfate pentahydrate | 4 mg |
| Cobaltous chloride hexahydrate | 4 mg |
| Potassium iodide | 30 mg |
| Sodium molybdate dihydrate | 5 mg |
| Calcium chloride dihydrate | 0.662 g |
| Sodium chloride | 1.0 g |
| Deionised water | 100 $cm^3$ |

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 15 $lb/in^2$, 120 °C.

**Procedures:**

Fresh conidia were obtained by inoculating slope cultures stored onto the surface of the fresh potato dextrose agar at 21 °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 1.0 $cm^3$ inoculum was put into each flask which contained 100 $cm^3$ of the medium. The flasks were incubated at 30 °C for 46 h with a shaking speed of 160 r.p.m. The utilization of glucose was monitored by U.V. spectrometry. When the glucose was almost completely utilized, the first batch of pyrocatechol was fed. The pH of the broth was maintained at pH

5.5 by the addition of sterile dipotassium hydrogen orthophosphate. The flasks were kept at 30 °C for 18 h with the same shaking speed. A ferric chloride test showed that all the pyrocatechol had been metabolised. A second batch of the pyrocatechol was fed and the pH adjusted to pH 5.5 as before and left shaking for further 24 h. The cultures were combined and the pH of the combined mixture adjusted to pH 7.2 with saturated sodium bicarbonate and extracted with ether to remove non-acidic material. The pH of the cultures was adjusted to pH 2.0 with orthophosphoric acid, saturated with sodium chloride, and extracted with ether to obtain the target lactone. The remaining aqueous extract was then continuously extracted with ether for 48 h to remove any remaining metabolites. The ether extracts were filtered through Celite, dried over sodium sulfate and concentrated *in vacuo.*

### Metabolism of 4-methylpyrocatechol (44)

Feeding of 4-methylpyrocatechol (44) was used as a control each time. Examination of the ether extracts at pH 7.2 showed evidence of unmetabolised 4-methylpyrocatechol (44). Ether extracts at pH 2.0 and continuous extraction gave (-)-(*S*)-3-methylmuconolactone (48). This extract was purified by preparative TLC on silica plates (diisopropyl ether - formic acid - water (200 : 7 : 3)), to give the target lactone (48) $R_F$ 0.2. The feeding data for this experiment is contained in Table 6 on page 188.

### (*S*)-(-)-3-Methylmuconolactone (48)

Preparative TLC gave the title lactone (*S*)-(-)-3-methylmuconolactone (48) as an oil which failed to crystallise, $[\alpha]_D$ - 26 ° (*c*, 0.8 in methanol) (lit.,[177] - 35.8 °); $\nu_{max}$ (KBr disc) 1732, 1693, 1645, 1320 and 1179 $cm^{-1}$; $\delta_H$ (200 MHz) ($D_2O$, t-butanol as int.ref std) 2.17 (3H, s, Me), 2.63 (1H, dd, *J* 16.7 and 8.5 Hz, 5-H), 3.16 (1H, dd, *J* 16.7 and 3.7 Hz, 5-H), 5.42 - 5.48 (1H, m, 4-H) and 6.00 - 6.01 (1H, m, 2-H); $\delta_C$ (50 MHz) ($D_2O$) 13.9 (Me), 36.9 (C-5), 82.9 (C-4), 116.7 (C-2), 172.7 (C-3), 174.5 (C=O) and 177.2 (C=O); *m*/*z* 156 ($M^+$) (Found: $M^+$ 156.0419. $C_7H_8O_4$ requires 156.0422).

### (-)-1-Methyl-8-bromomuconodilactone (53)

(*S*)-(-)-3-Methylmuconolactone (48) (30 mg, 0.2 mmol) was brominated using the standard procedure to give (-)-1-methyl-8-bromomuconodilactone (53) (34 mg, yield 80 %) as a white crystalline solid, m.p 83-85 °C (lit.,[79] 84.5-86 °C), $[\alpha]_D$ - 82° (*c*, 0.3 in methanol) (lit.,[80] - 86.5 °); $\nu_{max}$ (KBr disc) 1796, 1778, 1284, 1187, 1112, 1076 and 1029 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.68 (3H, s, Me), 2.85 (1H, d, *J* 18.8 Hz, 4-H), 3.00 (1H, dd, *J* 18.8 and 4.4 Hz, 4-H), 4.46 (1H, s, 8-H) and 4.99 - 5.01 (1H, d, *J* 3.8 Hz, 5-H); $\delta_C$ (50 MHz) ($CDCl_3$) 19.7 (Me), 34.3 (C-4), 41.9 (C-8), 81.2 (C-5), 88.7 (C-1), 169.7 (C=O), 171.4 (C=O); *m*/*z* 236 ($M^+$,$^{81}Br$), 234 ($M^+$,$^{79}Br$); (Found: $M^+$ 235.9525. $C_7H_7O_4{}^{81}Br$ requires 235.9508).(Found: $M^+$ 233.9521. $C_7H_7O_4{}^{79}Br$ requires 233.9528).

### (-)-1-Methylmuconodilactone (54)

(-)-1-Methyl-8-bromomuconodilactone (53) (30 mg, 0.13 mmol) was debrominated by the standard procedure to give (-)-1-methylmuconodilactone (54) as a white crystalline solid (12 mg, yield 60 %), m.p. 107 °C (lit.,[175] 129 °C), $[\alpha]_D$ - 130 ° (*c*, 1.2 in methanol) (lit.,[163] - 131 °); $\nu_{max}$ (KBr disc) 1781, 1306, 1258, 1231, 1200, 1134, 1125, 1089 and 1043 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.59 (3H, s, Me), 2.69 - 3.06 (4H, m, 4-$H_2$ and 8-$H_2$) and 4.85 (1H, d with fine splitting, *J* 4.7 Hz, 5-H); $\delta_C$ (50 MHz) ($CDCl_3$) 21.9 (Me), 34.8 (C-4), 40.3 (C-8), 82.1 (C-5), 87.5 (C-1), 172.5 (C=O) and 172.7 (C=O); *m/z* 156 ($M^+$) (Found: $M^+$ 156.0416. $C_7H_8O_4$ requires 156.0422.)

### Metabolism of 4-isopropylpyrocatechol (120)

Examination of the ether extracts at pH 7.2 showed the presence of unmetabolised 4-isopropylpyrocatechol (120). Ether extracts at pH 2.0 and continuous extraction gave the 3-isopropylmuconolactone (163). This extract was purified by preparative TLC on silica plates (diisopropyl ether - formic acid - water (200 : 7 : 3 )), to give the title lactone with $R_F$ 0.2. The feeding data for this experiment are shown in Table 6 on page 188.

### (-)-(*S*)-3-Isopropylmuconolactone (189)

Preparative TLC gave the title lactone 3-isopropylmuconolactone (189) as an oil which failed to crystallise: $\nu_{max}$ (thin film) 3027, 2975, 1757, 1723, 1226 and 1092 $cm^{-1}$; $\delta_H$ (200 MHz) ($CDCl_3$) 1.18 (3H, d, *J* 7.0 Hz, Me), 1.25 (3H, d, *J* 7.0 Hz, Me), 2.00 (1H, m, C<u>H</u>$Me_2$), 2.57 (1H, dd, *J* 16.5 and 8.5 Hz, 5-H), ), 3.00 (1H, dd, *J* 16.4 and 4.0 Hz, 5-H), 5.36

(1H, ddd, *J* 8.5, 3.9 and 1.6 Hz, 4-H) and 5.85 (1H, s with fine splitting, 2-H); *m*/*z* 184 ($M^+$) (Found: $M^+$ 184.0727. $C_9H_{12}O_4$ requires 184.0736).

### Metabolism of 4-*n*-propylpyrocatechol (124)

4-*n*-Propylpyrocatechol (124) was recovered by manual ether extraction of the aqueous broth at pH 7.2. Examination by $^1H$ NMR spectroscopy of both the manual and continuous extractions with ether at pH 2.0 failed to show any metabolites. The feeding data for this experiment are shown in Table 6 on page 188.

### Metabolism of 3-chloro-5-methylpyrocatechol (128)

Examination of the ether extracts at pH 7.2 showed the presence of 3-chloro-5-methylpyrocatechol (128). Examination of both manual and continuous ether extracts at pH 2.0 by $^1H$ NMR spectroscopy did not show any metabolites having been produced. The feeding data for this experiment are shown in Table 6 on page 188.

Table 6: Feedings of 4-alkylpyrocatechols to cultures of *Aspergillus niger*.

| Experiment | 2-chloro-5-methyl pyrocatechol (128) | Methyl Control[b] | 4-isopropyl-pyrocatechol (120) | Methyl Control[b] | 4-n-propyl-pyrocatechol (124) | Methyl Control[b] |
|---|---|---|---|---|---|---|
| Total number of flasks used[a] | 19 | 9 | 24 | 12 | 18 | 18 |
| Amount (mg) of pyrocatechol fed | 475 | 225 | 300 | 300 | 200 | 450 |
| Yield (mg) of 4-alkylpyrocatechol recovered at pH 7.5 | 228<br>(48%) | 32<br>(14%) | 217<br>(72%) | 49<br>(16%) | 121<br>(61%) | 24<br>(5%) |
| Total metabolites (mg) extracted at pH 2.5 | 63 | 56 | 65 | 63 | 69 | 42 |
| Yield (mg) of 4-alkyl-muconolactone | - | 32<br>(11%) | 16<br>(4%) | 39<br>(10%) | - | 39<br>(7%) |

[a] Each incubation flask contained 100 $cm^3$ medium.

[b] 4-Methylpyrocatechol was fed in parallel.

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