SELECTIVE INHIBITION OF THE BIOSYNTHESIS OF

PENICILLIC ACID

A thesis presented to the University of Surrey for the degree of Doctor of Philosophy in the Faculty of Biological and Chemical Sciences.

by

Jalil Lari

Joseph Kenyon Research Laboratories,
Department of Chemistry,
University of Surrey.

February 1980
Summary

In the present study the mould *Penicillium-cyclopium* was chosen as a model system, with a view to exploring the possibility of selectively inhibiting the interconversion of its metabolites, orsellinic acid and penicillic acid. This sequence necessitates a ring fission, involving cleavage of the bond linking carbon atoms 4 and 5 of orsellinic acid.

This thesis is divided into three chapters:

1. A general introduction consisting of a survey of the background literature relating to the biosynthesis of penicillic acid.

2. This describes the syntheses of potential inhibitors including some derivatives of orsellinic acid. The established pathway for the preparation of orsellinic acid from ethyl acetoacetate and ethyl crotonate via bromination, hydrolysis and debromination is also evaluated, and the existing confusion in the literature concerning the bromination stage of this procedure is discussed.

3. This chapter is divided into two parts:
   Part one concerns an investigation of various aspects of the course of the fermentation of *P. cyclopium* and its relationship to the biosynthesis of penicillic acid. In addition, the recovery of the culture filtrate metabolites 3-methoxy-2,5-toluquinol and the corresponding quinone is described.
Part two is devoted to a study of the feasibility of selectively inhibiting penicillic acid biosynthesis, using strategically-designed derivatives of advanced intermediates such as 5-chloroorsellinic acid. The inhibitory effect of low concentrations of this inhibitor was demonstrated through a series of feeding and autoradiographic experiments, which also led to the observation of the concomitant accumulation of other metabolites, two of which were shown to be precursors of penicillic acid.
Acknowledgements

My sincere thanks are due to Professor R. Thomas for his continuous help, advice and encouragement throughout this work.

I am also greatly thankful to all my colleagues and friends in the Chemistry Department of the University of Surrey.

It is a pleasure to record my gratitude in acknowledging the financial support of the University of Kerman, Iran.

Finally, I would like to thank Mr. E. Ah-Sing for his patience and care in typing the manuscript.
TO MY PARENTS
CONTENTS

Summary ................................................................. i
Acknowledgements ..................................................... iii
Dedication ................................................................. iv

Chapter 1
General Introduction .................................................. 1

Chapter 2
Synthesis of Potential Inhibitors of Penicillic Acid Biosynthesis .......... 45

Chapter 3
Effects of Inhibitors on the Biosynthesis of Penicillic Acid ................. 87

List of Schemes, Figures and Tables ................................. 160

Reference ................................................................. 168
Chapter 1: General Introduction

1.1 The nature of inhibition studies ............... 1
1.2 The classification of primary and secondary metabolites .................. 3
1.3 Polyketides ........................................ 6
1.3.1 Biosynthesis of polyketides .................. 9
1.3.2 Biosynthesis of fatty acids and secondary metabolites in cell-free systems .......... 23
1.4 Tetronic acids including penicillic acid .... 28
1.4.1 Chemistry and natural occurrence of penicillic acid .................. 33
1.4.2 Biosynthesis of penicillic acid ............... 38
1.1 The nature of inhibition studies

Living cells are characterised by complex organised patterns of chemical reactions, which are catalysed and directed by highly integrated enzyme systems. These reactions provide the necessary energy for the various functions of the cell, and support the synthesis of material upon which the cell depends for maintenance, growth and duplication. The metabolic activity of a living cell can be affected by many diverse environmental factors such as pH, X-ray irradiations, or high pressure. Since these factors have no specific role, it is difficult to use them to probe precise metabolic events. Studies of the effect of enzyme inhibition on cellular metabolism provide a more selective approach to the exploration of the nature of living material and its associated bio-transformations.

The introduction of a chemical substance into a cell can cause distortion of the normal biosynthetic pathways, through the inhibition of selected enzymes, thereby blocking or diverting a main sequence into an alternative channel. The nature of inhibition processes can sometimes be rationalised in mechanistic terms, for example when an individual chemical substance specifically blocks a particular reaction or step in a metabolic sequence. It is sometimes possible to isolate the responsible enzyme or enzyme system in order to study the mechanism or interference. This can lead to a
more precise characterisation of the processes occurring within the cell in which the inhibitor exerts its effect.

Metabolic inhibitors are often categorised as:

1. Competitive inhibitors. These combine reversibly with an enzyme at the same site as that involved in the formation of a normal enzyme-substrate complex. In this case, the degree of inhibition can be determined by the ratio of the relative concentrations of the substrate and the competitive inhibitor.

2. Non-competitive inhibitors. These bind irreversibly at the active site of an enzyme and may exhibit varying degrees of specificity. A selective inhibitor is one which blocks the action of an enzyme responsible for the catalysis of a specific individual step in a metabolic sequence.

3. Negative feed-back control of enzyme action. This effect is frequently observed and is a consequence of the ability of a metabolic product to inhibit one or more steps in its own biosynthesis.

The present investigation has been directed to the design of potential selective inhibitors based on the preparation of close structural analogues of essential intermediates involved in secondary biosynthetic pathways with particular reference to penicillic acid. As part of an initial study of the feasibility of this approach, the mould *Penicillium cyclopium* was chosen as a model system,
with a view to exploring the possibility of selectively inhibiting the interconversion of its metabolites, orsellinic acid (1) and penicillic acid (2). This sequence necessitates a ring fission, involving cleavage of the bond joining carbon atoms 4 and 5 of orsellinic acid, as indicated in scheme 1.1. As will be described, it has proved possible to demonstrate that this process can be effectively blocked by introducing suitable substituents at either carbon.

Scheme 1.1 C(4)-C(5) cleavage of orsellinic acid, leading to the biosynthesis of penicillic acid.\(^1,2\) ( O = oxidative step, C\(_1\) = formate or methionine-derived methyl group and \(\sim\) indicates C-C cleavage).

1.2 The classification of primary and secondary metabolites

Primary metabolites may be defined as those metabolic products resulting from a series of interrelated enzyme-catalysed reactions, which provide the organism with its energy, synthetic intermediates and key macromolecules such
as proteins and DNA. They are basically the same for all living systems, whether plants, animals or microorganisms. Some examples of primary metabolites are amino acids, acetyl-coenzyme A, monosaccharides, mevalonic acid and nucleotides. On the other hand, secondary metabolites are the products of specialised biosynthetic pathways and are derived from primary metabolites (Table 1.1). These products have no obvious role in the economy of the corresponding organism. Secondary metabolites are found mostly in plants and microorganisms, and they have restricted taxonomic distribution.

Organic chemists have been responsible for the majority of studies of the structure and biosynthesis of those natural products categorised as secondary metabolites, which appear to express the individuality of species in chemical terms.

One of the earliest systematic studies of secondary metabolites began with the work of Raistrick et al., who were particularly concerned with fungal products. The chemistry and biosynthesis of these compounds has been discussed in several reviews.
Table 1.1 Biosynthetic Classification of Secondary Metabolites

<table>
<thead>
<tr>
<th>Primary precursors</th>
<th>Secondary metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>Alkaloids, peptide derivatives (e.g. oligopeptides, diketopiperazines and penicillins), non-nitrogenous products (e.g. cinnamic acid derivatives).</td>
</tr>
<tr>
<td>Mevalonic acid</td>
<td>Terpenes, sterols, carotenoids and general polyisoprenoids.</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glycosides, modified sugars (e.g. kojic acid) and oligosaccharides (e.g. streptomycin).</td>
</tr>
<tr>
<td>Acetyl-CoA and propionyl-CoA</td>
<td>Polyketides: fatty acid derivatives polyacetylenes, and numerous phenols.</td>
</tr>
<tr>
<td>$C_1$ units (ex formate and methionine)</td>
<td>-OMe, -SMe, &gt;NMe, &gt;CMe, &gt;OCH$_2$OH groups, etc.</td>
</tr>
</tbody>
</table>
1.3 Polyketides

These are a large group of natural products, which are related to each other by their common biogenetic origins. A polyketide is a general term, which was first used by Collie, to describe compounds derived from poly-\(\beta\)-keto intermediates. They include fatty acids, polyacetylenes and cyclic compounds which are mostly aromatic. These latter include mono and polycyclic phenols, e.g. orsellinic acid and the tetracycline antibiotics. Fatty acids are found in all organisms, and Shaw has compiled a list of the fatty acids of fungi. Polyacetylenes are obtained from plants and fungi, and consist of linear carbon chains containing conjugated acetylenic or acetylenic-ethylenic systems, which show a structural resemblance to fatty acids. The biosynthesis of the polyacetylenes has been frequently reviewed.

Aromatic polyketides, which comprise a major group of the polyketides, are produced by microorganisms and higher plants. Simple phenolic polyketides include 6-methyl salicylic acid (3) and gentisic acid (4), which have been isolated from the culture filtrates of *Penicillium griseofulvum.*

Recognition of the biogenetic origin of aromatic polyketides is primarily due to the work of Birch who carried out an extensive series of tracer incorporation experiments.
On the basis of an earlier observation of Oppenheim\textsuperscript{17} in which orcinol (5) was formed on heating "dehydracetic acid" (6) with baryta, Collie\textsuperscript{8,18} noted that treatment of dehydracetic acid with hot concentrated sodium hydroxide also gave compound (5). He also found that dehydracetic acid with hot concentrated hydrochloric acid produced 2,6-dimethyl-4-pyrone (7). The latter compound can be transformed into diacetylacetone (8), which also gives orcinol in acidic or strongly basic solutions (Scheme 1.2). Furthermore the conversion of appropriate pyrones to secondary metabolites such as orsellinic acid have also been studied.\textsuperscript{19}

Nearly thirty years ago Robinson\textsuperscript{20} and Birch\textsuperscript{15} independently recalled Collie's work and suggested that phenols could be obtained by head-to-tail condensation of acetate units. Robinson's hypothesis suggested that orsellinic acid\textsuperscript{[1]} could be produced from a C\textsubscript{4} unit corresponding to acetoacetic acid (9); Scheme 1.3.
Scheme 1.2 Alternative synthetic routes to orcinol (Collie)."
1.3.1 Biosynthesis of polyketides

Birch and Donovan\textsuperscript{15} showed that the correlation of the structures of some natural products, for example, those containing orcinol or phloroglucinol nuclei, supports the hypothesis that these molecules are elaborated, at least in part, by the head-to-tail linkage of acetate units. Birch has confirmed his general hypothesis through a number of \textsuperscript{14}C-acetate incorporation experiments, such as those which established the biosynthesis of palitantin (10), and cyclopaldic acid (11) (from \textit{P. cyclopium})\textsuperscript{21}, 6-methyl salicyclic acid (3) and griseofulvin (13) (from \textit{P. griseofulvum})\textsuperscript{22,23}

\begin{itemize}
  \item Birch\textsuperscript{24} also suggested that a \(\beta\)-polyketo acid chain can undergo intramolecular cyclisation via aldol condensation or Claisen condensation. Aldol condensation leads to the production of substituted orcinols, whereas Claisen condensation yields a phloroglucinol (12), as follows:
\end{itemize}
As indicated in Scheme 1.4, a polyketide chain containing 8 or more carbon atoms can undergo reduction and dehydration reactions to form a variety of phenolic products. The acetate hypothesis received further support from early studies by Thomas\textsuperscript{25-28}, demonstrating that seven acetate units are involved in the biosynthesis of palitantin (10), alternariol (14) (Scheme 1.5) and the phenalenone nucleus of norherqueic- none (17).
Scheme 1.4 Biosynthesis of modified phenolic polyketides, 
R = CH₃- or other suitable group e.g. C₆H₅-CH=CH-

Since the fatty acids are biogenetically related to 
many phenolic polyketides, a brief discussion of their bio-
synthetic pathway is relevant. From the discovery of β-oxida-
tion of fatty acids until 1958, it was thought that fatty 
acids could be derived from acetate units by the reverse of 
the degradative pathway involving β-oxidation breakdown. In 
1959, Lynen suggested that malonyl coenzyme A might be a key 
intermediate in fatty acid biosynthesis. Malonylcoenzyme A 
was first obtained in the course of studies of yeast enzymes.
Scheme 1.5  Head-to-tail linkage of seven acetate units leading to the formation of alternariol (14) and griseofulvin (13).

Lynen\textsuperscript{32} also found that acetyl-coenzyme A serves as the primer of the process and that its $C_2$ unit is only incorporated into the carbon-methyl moiety of the fatty acid produced. Tanenbaum and Bassett\textsuperscript{33} in their studies of the biosynthesis of 6-methyl salicylic acid, showed that the acetate and malonate precursors are incorporated as their CoA esters. They also observed that in the absence of added malonate, biotin-dependent carboxylation of acetyl-coenzyme A to malonyl-coenzyme A takes place.
Gatenbeck and Mosbach\textsuperscript{34} provided further evidence for
the involvement of a $\beta$-polyketo acid-type intermediate in
the biosynthesis of orsellinic acid. They introduced
\textsuperscript{18}O-labelled sodium acetate to growing cultures of Chaetomium
cochliodes and observed radioactive orsellinic acid (1) with
\textsuperscript{18}O in both hydroxyl and carboxyl groups (Scheme 1.6).

\begin{align*}
4 \text{Me-C\textsuperscript{18}O}_2\text{H} & \rightarrow 4 \text{MeC\textsuperscript{18}O}_\text{SCoA} & \rightarrow \text{MeC\textsuperscript{18}O-}(\text{CH}_2-\text{C\textsuperscript{18}O})_2-\text{CH}_2-\text{C\textsuperscript{18}O-S-} \\
& \rightarrow \rightarrow \rightarrow \\
& \text{Me} \\
& \text{C\textsuperscript{18}OOH} \\
& \text{H\textsuperscript{18}O} \\
& \text{18}OH
\end{align*}

\textbf{Scheme 1.6} Biosynthesis of orsellinic acid from \textsuperscript{18}O-labelled acetate.

Enzymes capable of decarboxylating malonyl-coenzyme A
to acetyl-coenzyme A were extracted from cells of \textit{Pseudomonas}
species by Hayaishi.\textsuperscript{35} This reaction does not apparently
occur in \textit{P.urticae},\textsuperscript{36} or \textit{P.cyclopium},\textsuperscript{37} although it does
seem to take place to a significant extent in other penicillia
species.\textsuperscript{38,39,40} Bentley and Keil in their study of \textit{P.cyclopium}
showed that acetate and malonate units, in the ratio
of one to three, must be present for the biosynthesis of
penicillic acid (2) via orsellinic acid (1) (Scheme 1.7).
Scheme 1.7 Pathway of penicillic acid formation from one acetate and three malonate units (Bentley and Keil).

- acetate methyl
- acetate carboxyl
- acetate methyl or malonate methylene
- acetate or malonate carboxyl

It has been shown that 6-methyl salicylic acid, 
orsellinic acid, and alternariol are in fact each synthesised from one primer molecule of acetyl-coenzyme A, the remaining carbon atoms being derived from malonyl-coenzyme A. These metabolites possess a C-methyl group which corresponds to the acetyl starter unit. It is generally considered that the cyclisation of a growing chain involves a combination of Claisen and aldol condensations (path a, Scheme 1.9). Thomas, however, has suggested an alternative
mechanism (path b, Scheme 1.9) which could generate phenols without the mediation of ketonic intermediates. This involves the formation of an enzyme-stabilised poly-β-enolate (15), with the appropriate geometry required to allow it to undergo an electrocyclic rearrangement to a carboxylic intermediate from which orsellinic acid is formed by a simple elimination process. The poly-β-enolate mechanism is of particular interest in respect to the formation of polycyclic phenolics as illustrated by the possible derivation of phenalenones (based on 17) from the precyclisation intermediate (16) through a single concerted cyclisation step (Scheme 1.10). The actual mode of cyclisation of any poly-β-enolate, would be predetermined by the sequence of cis and trans double bonds, the formation of which may be dependent upon the stereospecificity of the elimination of the thioester group from the polyketide intermediates as shown in Scheme 1.8.
Most of the acetate-derived phenolic metabolites investigated, belong to a family of compounds whose structures point to a one-chain-polyketo origin. There are however, a few compounds whose structures appear to be derived from two polyketo chains e.g. citromycetin (19). The result of the chemical degradation of citromycetin (19) derived from (2\(^{14}\)C)-malonate, led Gatenbeck and Mosbach\(^{45}\) to suggest that two acetate "starter" units are involved in the biosyn-
1 acetate + 3 malonate

(a) via poly-β-ketointermediates
(b) via poly-β-enolate intermediates

Scheme 1.9 Alternative pathways for the cyclisation of polyketides.

Scheme 1.10 Cyclisation of the phenalenone precursor (Thomas.)
Scheme 1.11 Alternative biosynthetic pathways of citromycetin via
(a) a linear polyketide chain, (b) branched chain intermediate.

thesis of compound (19) as demonstrated in Scheme 1.11, path b). Their findings apparently excluded the mediation of a linear polyketide-derived bicyclic intermediate such as (18) which could yield the carbon skeleton of citromycetin following C(10)-C(11) ring cleavage (Scheme 1.11, path a). Actually
Gatenbeck and Mosbach in their paper describing this work represented the hypothetical bicyclic quinone intermediate as (20) which they suggested could conceivably undergo conversion to citromycetin (19) following C(2)-C(3) cleavage. However, this particular cleavage sequence would not in fact lead to the carbon skeleton of citromycetin.

As well as leading to the essential aromatic amino acids the shikimic acid pathway also provides intermediates for the biosynthesis of secondary metabolites. The biosynthesis of shikimic acid (21) involves a series of reactions starting with the condensation of erythrose-4-phosphate and phosphoenolpyruvic acid (PEP) derived initially from glucose-6-phosphate. Shikimic acid is a key compound in the synthesis of the C₆-C₃ unit.
The shikimic acid pathway appears to play a smaller part in the biosynthesis of the secondary metabolites of fungi and in particular is infrequently used by the fungi imperfecti, which produce a wide range of aromatic compounds by the polyketide route. However, under conditions of excess of carbohydrate with insufficient nitrogen for its conversion to amino acids, the shikimic acid or some other
precursor of amino acids may be used in the synthesis of miscellaneous aromatic metabolites. Crowden studied the metabolism of shikimate-derived aromatic compounds in Polyporus tumulosus, and obtained the most efficient incorporation of radioactivity into the phenol from \(^{14}\text{C}\)-labelled glucose and shikimate. Bassett and Tanenbaum suggested that the biosynthesis of pyrogallol and para-hydroxybenzoic acid in P. patulum could occur either by the shikimic acid pathway or by condensation of acetate units.

Although the carbon atoms of glucose are indirectly incorporated into all fungal metabolites, a few secondary metabolites of fungi are known to incorporate the intact carbon skeleton of glucose, for example kojic acid (22).
Mevalonic acid, which is the precursor of fungal terpenes and steroids, is derived from acetyl-coenzyme A. It is metabolised with loss of the carbonyl group to isopentenyl pyrophosphate which is the branched chain precursor of the polyisoprenoids. This subject has frequently been reviewed\(^5,50,51,52\).

In view of the concern of this thesis with the selective inhibition of penicillic acid formation by derivatives of orsellinic acid, it is of interest to list some naturally occurring orsellinic acid derivatives (fig. 1.1), since some of these could conceivably serve as selective inhibitors, e.g. everninic acid (orsellinic acid 4-methyl ether).

![Chemical structures of orsellinic acid derivatives](image-url)
1.3.2 Biosynthesis of fatty acids and secondary metabolites in cell-free systems

Wakil, Titchener and Gibson\textsuperscript{54,55} found that a biotin-dependent enzyme was involved in the biosynthesis of long-chain fatty acids from acetate. The biotin enzyme, which was first purified by Wakil\textsuperscript{56} from avian liver, turned out to be an acetyl-coenzyme A carboxylase, forming malonyl-coenzyme A as shown in the following equation:
Lynen also suggested that malonyl-coenzyme A might be involved in the synthesis of fatty acids. This process requires NADPH as a reducing agent and a proportionately smaller amount of acetyl-coenzyme A as the primer of the chain-forming sequence. Lynen, Hopper-Kessel and Eggerer suggested the following equation for the biosynthesis of fatty acids:

\[
\text{AcetylCoA} + n \text{ malonyl-CoA} + 2n \text{ NADPH} + 2n \text{ H}^+ \rightarrow \\
\text{CH}_3\left[-\text{CH}_2\text{-CH}_2\right]_n\text{-CO-CoA} + n \text{ CO}_2 + 2n \text{ NADP}^+ + n \text{ H}_2\text{O} + n\text{CoA}
\]

In 1960, Lynen found that the transformation of malonyl-coenzyme A into fatty acids is achieved through intermediates that are covalently bound to thiol groups of the fatty acid synthetase. He classified these into two different types: "central" and "peripheral". Subsequently Lynen suggested that the reactions are catalysed by a "multi-enzyme complex".

No cell-free enzyme preparations capable of synthesising secondary metabolites had been isolated, prior to 1960, when Tanenbaum and Bassett found that treatment of the mycelium of *P. patulum* with dilute ammonia yielded a crude enzymic extract, which catalysed the incorporation of acetyl-coenzyme A into patulin. Light subsequently found that
Scheme 1.12 Hypothetical scheme for the synthesis of 6-methylsalicylic acid via a multi-enzyme complex. \( S_p \) = peripheral thiol site, \( S_c \) = central thiol site.
(1-\(^{14}\)C, 2-\(^{3}\)H) acetate is incorporated into fatty acids and 6-methyl salicylic acid in a culture of \textit{P. griseofulvum}, by the same general condensation process.

Lynen and co-workers\(^{60,61}\) showed that in the absence of NADPH, a \textit{P. patulum} enzyme preparation yields only triacetic acid lactone (23). Consequently in 6-methyl salicylic acid formation, reduction appears to occur at the triacetic acid level, prior to the final condensation with malonyl-coenzyme A.

\[
\text{Triacetic acid lactone (23)}
\]

Subsequently, cell free extracts capable of synthesising orsellinic acid\(^{62}\), and alternariol\(^{26,44}\) were prepared, both systems providing evidence of a multi-enzyme complex, as in fatty acid biosynthesis, thus in the biosynthetic pathway leading to orsellinic acid from acetyl-coenzyme A and malonyl-coenzyme A, the following enzymes and proteins are involved\(^{62}\): transacylases, acyl carrier protein, a condensing enzyme, a cyclization enzyme and an hydrolase.

It has been shown that \textit{P. baarnense} produces the fatty acid palmitic acid and the secondary metabolite orsellinic acid. Mosbach and Bavertaft\(^{63}\) found that the ratio between
palmitic acid and orsellinic acid is not constant, but varies with the concentration of nitrate in the growth medium. A lack of nitrate was accompanied by an increased incorporation of radioactive acetate in palmitic acid. Whereas in the presence of nitrate an increased incorporation into orsellinic acid was observed. Furthermore, it was shown that NADPH has no role in the synthesis of this aromatic polyketide.

A cell-free extract of Alternaria alternata catalysed the formation of alternariol from one mole of acetyl-coenzyme A and six moles of malonyl-coenzyme A. Sjoland and Gatenbeck also found that propionyl-coenzyme A could be utilised instead of acetyl-coenzyme A by this fungus to form the C-ethyl homologue of alternariol.

Radioactive tracer studies by Peterson suggested that A. flavipes cultures are capable of introducing a $C_1$ unit into an aromatic ring, as follows:

\[ 4 \text{CH}_3\text{-COOH} \rightarrow \text{HO} \begin{array}{c} \text{CH}_3 \\ \text{COOH} \\ \text{OH} \end{array} \rightarrow \text{C}_1 \]

\[ \text{HO} \begin{array}{c} \text{CH}_3 \\ \text{COOH} \\ \text{OH} \\ (24) \end{array} \rightarrow \text{HO} \begin{array}{c} \text{CH}_3 \\ \text{CHO} \end{array} \]
Later Gatenbeck and co-workers isolated an enzyme system from *A. flavipes* which is responsible for C-methylation of a metabolite in this culture, and they suggested that C-methylation occurs on a tetraacetic acid structure bound to a protein prior to cyclization of the polyketide, and that C-methylation of an aromatic nucleus is not involved in the formation of compound (24).

1.4 Tetronic acids including penicillic acid

Tetronic acids, which include penicillic acid (2) are those compounds which are structurally related to the lactone (25). It has been usual to regard that 4-methyl tetronic acid (26) as the parent compound of the naturally-occurring fungal tetronic acids which are listed in Fig. 1.2. All of these acids were isolated from culture filtrates of *Penicillium*, or *Aspergillus* species. Clutterbuck et al. found that *P. charlesii* produces 4-methyl tetronic acid (26), carolic acid (27), carolinic acid (29), carlic acid (32) and carolosic acid (31). Bracken and Raistrick isolated dehydrocarolic acid (28) from the metabolism solution of *P. cinerascens*, whereas terrestric acid (30) was isolated from *P. terrestre*. Ceiger and Huber reported that isolation of ascorbic acid (34) from *Aspergillus v. Tieghen*, while Ramakrishnam described it as a metabolite of *A. tamorii kita*. Viridicatic acid (ethyl carlosic acid) (33) was found to be present in culture filtrates of *P. viridicatum*. 
Fig. 1.2 Some tetronic acids and structurally related compounds.
Turner\textsuperscript{75} considered the tetronic acids derivatives as a class of fungal products, which are, at least formally prepared from the condensation of an acetate derived chain with an intermediate of the tricarboxylic acid cycle. In other words derivatives of tetronic acid typically arise by condensation of a carboxyl group of a C\textsubscript{4}-dicarboxylic acid with \(\alpha\)-methylene group of a polyketide.

The tricarboxylic acid cycle (TCA cycle) provides the requisite dicarboxylic acid and subsequently amino acids which can lead to the formation of the corresponding tetramic acids cf. tenuazonic acid, derived from isoleucine and acetoacetate.

![Tenuazonic acid](ex. Alternaria alternata)

Bentley et al.\textsuperscript{77} obtained evidence from radioactive tracer and chemical degradation experiments, which suggested that carolic acid could be derived from the condensation of one molecule of acetyl-coenzyme A, two of malonyl-coenzyme A and a C\textsubscript{4}-carboxylic acid such as succinate. These results were obtained in the course of a study in which they found that labelled acetic acid is incorporated by \textit{P. charlesii} into carolic acid (27) at positions 1, 2, 5, 6, 7 and 8, whereas
labelled malonic acids furnished only carbons 1, 2, 5 and 6. They also found evidence that glucose metabolism in this fungus, may involve the hexose monophosphate pathway in the initial stages, and therefore suggested the following biosynthetic pathway for tetronic acid production, (Scheme 1.13).

**Penicillic acid**

Penicillic acid (2) is a secondary metabolite, which is produced by a variety of fungi, particularly species of the genus *Penicillium*. Penicillic acid was first isolated in 1910 by Alsberg and Black\(^{78}\) from the cultures of *P. puberulum*. This fungus was grown either on a natural medium such as corn meal mash, or on a chemically-defined medium such as Raulin-Thom\(^{79}\).

Penicillic acid is toxic to mammals and has been shown to be carcinogenic in rats\(^{80}\). Alsberg and Black\(^{78}\) found that penicillic was lethal to mice on subcutaneous injection of 200-300mg/kg. The minimal lethal dose for rabbits ranged from 100-200mg/kg\(^{81}\). Its antimicrobial activity was described by Oxford et al.\(^{82,83}\), and Ciegler et al.\(^{84}\) identified the products formed by the reaction between cysteine and glutathione with penicillic acid. Generally, penicillic acid can react with free-SH groups of the above mentioned amino acids (Scheme 1.14). It also inhibits alcohol dehy-
Scheme 1.13 Possible biosynthetic pathways for the formation of tetronic acids from the C$_4$-dicarboxylic acid, malic acid.
drogenase at $1.1 \times 10^{-4} \text{M}$, non-competitively, and lactic-dehydrogenase at $7.2 \times 10^{-5} \text{M}$, competitively. Additional details of the toxicity of penicillic acid are given in a review by Wilson.

![Scheme 1.14 Reaction between penicillic acid and RSH when RSH is cysteine or glutathione.](image)

1.4.1 Chemistry and natural occurrence of penicillic acid

Birkinshaw et al. following chemical degradation studies proposed the structure of penicillic acid as a mixture of 4-keto-3-methoxy-5-methylene-hexa-2-enoic acid (35) and the corresponding $\gamma$-hydroxy lactone (2).

A summary of the chemical reactions of penicillic acid is given in Scheme 1.15.

On the basis of spectrophotometric investigations of penicillic acid and its derivatives, Shaw and Ford showed that penicillic acid exists solely in the lactone form (2)
Scheme 1.15 Chemical degradation of penicillic acid.\(^8^7\)
in neutral aqueous solution. In 0.1N alkali, however, the ionic form corresponds to the ring-open structure (35).

Penicillic acid was first synthesised in 1948 by Raphael\textsuperscript{90} starting from the lactone (36) as shown in Scheme 1.16.

Natural occurrence

Penicillic acid has been found in mouldy tobacco\textsuperscript{91}, corn and beans\textsuperscript{92}. Ciegler and Kurtzman\textsuperscript{93} found that \textit{P. martensii}, \textit{P. palitans}, \textit{P. cyclopium} and \textit{P. puberulum} were able to produce penicillic acid on various agricultural commodities, although commodities with high protein contents did not support toxin synthesis. The extent of toxin production varied with the mould strain, the commodity, and the temperature; it was found that low temperatures (1 to 10°C) favoured toxin accumulation. Lillehoj et al.\textsuperscript{94} showed that an atmosphere enriched with 60% CO\textsubscript{2} (at 5°C and 10°C) reduced penicillic acid production below a detectable level, following inoculation of high moisture corn with \textit{P. martensii}.

Penicillic acid is also produced in appreciable quantities by fungi responsible for the blue-eye disease of corn\textsuperscript{93}. Blue-eye is the name of a common storage disease of this cereal crop involving several species of penicillia, e.g. \textit{P. martensii}\textsuperscript{95}. This mould grows on high-moisture corn even at near-freezing temperatures.
Scheme 1.16 Raphael's synthetic route for the preparation of penicillic acid.

(87% yield)
The isolation of penicillic acid has been reported from the following fungi; *P. lividum*, *P. griseum*, *P. simplicissimum*, *P. cyclopium*, *P. thomii*, *P. roqueforti* (*P. suavolens*), *P. fennelliae*, *P. aurantioirens*, *P. janthinellum*, *P. viridicatum*, *P. palitans*, *P. baarnense*, *P. madriti*, *P. lilacinum*, *P. canescens*, *P. chrysogenum*, *P. olivinoviride*, *Aspergillus ochraceus*, *A. sulphureus*, *A. melleus*, *A. sclerotiorum*, *A. alliaceus*, *A. ostianus*, and *Paecilomyces ehrlichii*.

Penicillic acid is not the only toxic compound produced by *P. cyclopium*. Recently Bond et al. established the structure of two congeneric alkaloids, which were isolated from this fungus when grown on maize meal; these were designated cyclopiamine A (37) and B (38).
1.4.2 Biosynthesis of penicillic acid

Birch, Blance, and Smith\textsuperscript{112} showed that there is no apparent biosynthetic relationship between penicillic acid (2) and the terpenes, based on the negative result obtained on feeding (2-\textsuperscript{14}C) mevalonic acid lactone to \textit{P. cyclopium}. However, they found that CH\textsubscript{3}-\textsuperscript{14}CO\textsubscript{2}H is readily incorporated into penicillic acid by the head to tail linkage of four acetate units, and suggested the following biosynthetic sequence (Scheme 1.17 Path a) involving orsellinic acid (1).

\begin{align*}
\text{(a)} & \\
\text{(b)} & \\
\end{align*}

\textbf{Scheme 1.17} Biosynthesis of penicillic acid via alternative ring cleavages; (a) 1,2 cleavage (Birch\textsuperscript{112}), (b) 4,5 cleavage (Mosbach\textsuperscript{1}).
Mosbach confirmed that orsellinic acid is converted to penicillic acid by *P. baarnense*, but the results of this tracer incorporation experiments indicated that the cleavage of the aromatic ring occurs between positions 4 and 5 (Scheme 1.17, path b).

Bentley and Keil showed that labelled acetate, malonate and glucose are incorporated into penicillic acid in *P. cyclopium* (Scheme 1.7; page 14). From these experiments, it was found that the C (6) and C (7) derive from acetate or glucose, but not from malonate. Malonate was therefore not converted into acetate under their experimental conditions and apparently represents a precursor beyond the acetate level. This chain is consistent with the derivation of penicillic acid from a single acetate-malonate, although no evidence was obtained for a pathway involving cleavage of an aromatic ring.

Cleavage of aromatic rings during the biosynthesis of secondary metabolites are not uncommon. Another example of the field of acetate-malonate derived substances is the fission of the ring of 6-methyl salicylic acid (3) to give patulin (43) which occurs in *P. patulum* (Scheme 1.18). Tanenbaum and Bassett, who isolated various phenolic compounds from this fungus, including gentisaldehyde (42), proposed pathway 'a'. Subsequently, Scott and Yalpani used deuterium-labelled precursors and mass spectroscopic analysis of the products to study the biosynthesis of patulin (43). They found that m-cresol (39) is converted to compound
(43) and that deuterium from m-cresol is also incorporated into toluquinol (40) and gentisaldehyde (42), on the basis of which they proposed pathway 'b' (Scheme 1.18).

Scheme 1.18 Alternative schemes for the biosynthesis of patulin [Tanenbaum and Bassett (path a); Scott and Yalpani (path b)].
Sekiguchi and Gaucher proposed a biosynthetic route to patulin via \( \text{m-cresol, m-hydroxybenzylalcohol, m-hydroxybenzaldehyde, gentisaldehyde and phyllostine} \) (44). More recently however, Sekiguchi isolated an isomer of patulin from \( \text{P. urticae} \) cultures, namely isopatulin (45), and by means of mutant studies showed that this compound is an intermediate in the biosynthesis of patulin. On the basis of these findings, it was suggested that a cleavage between C (4) and C (5) of phyllostine probably accounts for the biosynthesis of patulin.

\[
\text{Isopatulin (45)} \quad \text{Phyllostine (44)} \quad \text{Patulin (43)}
\]

Al-Rawi et al. have confirmed that cleavage of the aromatic ring occurs between C (4) and C (5) of orsellinic acid during the formation of penicillic acid. In this work, they fed \( \text{(}^3\text{H}) \) acetate to a culture of \( \text{P. cyclopium} \), and from \( ^3\text{H-nmr spectra of the product directly determined the resulting} \)
labelling pattern, on the basis of which they proposed the following sequence for penicilllic acid biosynthesis.
However, Axberg and Gatenbeck subsequently suggested the following sequence for the biosynthesis of penicillic acid, which appears to require a reduction in the final stage.

\[
\begin{align*}
\text{(1)} & \quad \text{HO} - \text{Me} - \text{COOH} \\
\text{(46)} & \quad \text{HO} - \text{Me} - \text{COOH} \\
\text{(47)} & \quad \text{HO} - \text{Me} - \text{OH} - \text{OMe} \\
\text{(48)} & \quad \text{HO} - \text{Me} - \text{OH} - \text{OMe} \\
\text{(2)} & \quad \text{Me} - \text{OH} - \text{OMe} \\
\end{align*}
\]

This pathway was proposed on the basis of the incorporation of \((0^{14}\text{CH}_3)\)-orsellinic acid-2-methyl ether (46) and \((0^{14}\text{CH}_3)\)-3-methoxytoluquinone (48) and the corresponding quinol (47) into penicillic acid by \textit{P. cyclopium}. 

In the present study two \textit{P. cyclopium} metabolites were isolated and identified as (47) and (48). It has also been established that cultivation of the fungus in the presence of 5-chloroorsellinic acid stimulates the accumulation of these compounds, while selectively inhibiting the formation of penicillic acid.

The mechanism of penicillic acid biosynthesis is further discussed in Chapter 3 (pages 104-110).
Chapter 2: Synthesis of Potential Inhibitors of Penicillic Acid Biosynthesis

2.1 Introduction ....................... 46
2.2 Results and discussions ............ 47
2.3 Experimental ...................... 67
2.1 Introduction

Orsellinic acid is known to be an intermediate in the biosynthetic pathway leading to penicillic acid in different fungi, such as *P. cyclopium* and *P. madriti*. An attempt was made to examine what effect the addition of simple derivatives of orsellinic acid and structurally related compounds would have on the biosynthesis of penicillic acid.

Since orsellinic acid is not commercially available, it was prepared synthetically by a sequence involving condensation of ethyl acetoacetate and ethyl crotonate to form ethyl dihydroorsellinate, with subsequent oxidative conversion to orsellinic acid via bromination, hydrolysis and debromination.

There is some confusion in the literature concerning the bromination step of this preparative method. Sonn and Santesson suggested that the bromination of ethyl dihydroorsellinate in glacial acetic acid produces ethyl dibromoorsellinate, while Kloss-Clayton and Gaucher-Shepherd claimed that the bromination stage leads to the formation of ethyl dibromodihydroorsellinate. The present study shows that in this synthetic procedure, bromination leads directly to the fully aromatic product, as proposed by Sonn and Santesson. From the analysis of the brominated compound its structure was confirmed as ethyl dibromoorsellinate. Furthermore, it was found that bromination of ethyl dihydroorsellinate (1:1 mole) with bromine in glacial acetic acid and bromine water yielded a mixture of ethyl orsellinate, ethyl monobromoorsellinate, and ethyl di-
bromoorsellinate in addition to some unchanged starting material. It was also found that the aromatisation of ethyl dihydroorsellinate could be carried out with sodium iodate and potassium periodate in acidic media.

Standard procedures were used for the preparation of some derivatives of orsellinic acid (Table 2.11).

In an isotopic synthesis, \((3^{-3}H)-5\text{-chloroorsellinic acid}\) was prepared from 5-chloroorsellinic acid according to the Pt/HTO- general tritium exchange procedure as utilised by Saljoughian\textsuperscript{124} for the synthesis of \((G^{-3}H)\text{-benzene}.

2.2 Results and Discussion

The following synthetic approaches have been reported for the preparation of orsellinic acid\textsuperscript{6};

(i) carboxylation of orcinol\textsuperscript{125}.

(ii) oxidation of protected orcylaldehyde\textsuperscript{126,127}.

(iii) condensation of ethyl acetoacetate (2) and ethyl crotonate (1) to form ethyl dihydroorsellinate\textsuperscript{3} with subsequent conversion to orsellinic acid via bromination, hydrolysis and debromination\textsuperscript{120-123}.

Of the above methods, the last approach provided a satisfactory source of orsellinic acid in reasonable yield. The preparative method described by Gaucher and Shepherd\textsuperscript{123} (in 1971) was used in this work. They proposed the following sequence (Scheme 2.1), although as shown below this is only partially correct.
Scheme 2.1 Synthesis of orsellinic acid (6) (Gaucher and Shepherd).
The condensation of ethyl acetoacetate (2) and ethyl crotonate (1) appears to involve the following stages:

(i) Michael condensation: this reaction is promoted by sodium ethoxide, which generates the carbanion (7) from compound (2) which in turn effects a nucleophilic attack at C(3) of ethyl crotonate (1) with formation of a carbon-carbon bond (Scheme 2.2).

(ii) Claisen condensation: by this process, the base catalyst (OEt) removes a proton from the methyl group at C(6) of compound (8), and the resulting nucleophile then attacks the ester carbonyl at C(1) of compound (9).

The crystalline product of the condensation stage (compound (3)) on thin-layer chromatography (using chloroform — acetic acid) showed only one spot (Rf ≈ 0.34), showing it to be free of starting material.

The u.v. spectrum of this compound in ethanol showed a peak at λ_{max} 282 nm, (ε=32300). This shifted to 255 nm when dilute hydrochloric acid was added, but on adding a slight excess of sodium hydroxide, the 282 nm peak reappeared. This confirmed that ethyl dihydroorsellinate (3) exists in a ketonic form in dilute alcoholic solution (cf. dihydroresorcinol¹²⁸).

The i.r. spectrum of this compound in nujol showed peaks at 1725 cm⁻¹ (ester) and 1625 cm⁻¹ [due to 2,4-diketone (enolic)]. Its melting point behaviour (87-89°C) together with u.v. and i.r. spectral data indicated that it contains a mixture of keto and enol tautomers (Fig. 2.3).
Scheme 2.2 The condensation of ethyl acetoacetate and ethyl crotonate.
The identification of intermediates in the synthesis of orsellinic acid, was carried out as follows:

(1) Chromatography (Table 2.1).
(2) Microanalysis (Table 2.2).
(3) Mass spectroscopy (Table 2.3).
(4) $^1$H n.m.r. spectroscopy (Tables 2.4, 2.5 and 2.6).

Table 2.1 Chromatographic comparison of intermediates in the synthesis of orsellinic acid.

<table>
<thead>
<tr>
<th>Products (Scheme 2.1)</th>
<th>$R_f$ (found)</th>
<th>$R_f$ (lit.)$^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4)*</td>
<td>0.86</td>
<td>0.87</td>
</tr>
<tr>
<td>(5)*</td>
<td>0.57</td>
<td>0.59</td>
</tr>
<tr>
<td>(6) (orsellinic acid)</td>
<td>0.46</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Solvent system: CHCl$_3$:glac. CH$_3$CO$_2$H (9:1v/v)
Silica gel plate; GF. 254,0.25mm.

* Products corresponding to the erroneous structures (4) and (5), (Scheme 2.1).
Table 2.2: Microanalysis of orsellinic acid intermediates.

<table>
<thead>
<tr>
<th>Products</th>
<th>C%</th>
<th>H%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>33.85</td>
<td>2.77</td>
</tr>
<tr>
<td>(4) $C_{10}H_{12}O_4Br_2$</td>
<td>Req.</td>
<td>33.74</td>
</tr>
<tr>
<td>(14) $C_{10}H_{10}O_4Br_2$</td>
<td>Req.</td>
<td>33.92</td>
</tr>
<tr>
<td>Found</td>
<td>29.37</td>
<td>1.81</td>
</tr>
<tr>
<td>(5) $C_8H_8O_4Br_2$</td>
<td>Req.</td>
<td>29.30</td>
</tr>
<tr>
<td>(15) $C_8H_6O_4Br_2$</td>
<td>Req.</td>
<td>29.48</td>
</tr>
<tr>
<td>Found</td>
<td>52.03</td>
<td>5.17</td>
</tr>
<tr>
<td>(6) $C_8H_8O_4\cdot H_2O$ (orsellinic acid)</td>
<td>Req.</td>
<td>51.61</td>
</tr>
</tbody>
</table>
(6) Orsellinic acid $R_1=R_2=H$

(14) Ethyl dibromoorsellinate $R_1=\text{Br}$ and $R_2=\text{Et}$

(15) Dibromoorsellinic acid $R_1=\text{Br}$ and $R_2=\text{H}$

Table 2.3 Mass spectral data of orsellinic acid intermediates.

<table>
<thead>
<tr>
<th>Products</th>
<th>$M^+$ peaks ($m/e$)</th>
<th>Base peaks ($m/e$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(14)</td>
<td>352,354,356</td>
<td>307,309,311</td>
</tr>
<tr>
<td></td>
<td>(1:2:1)</td>
<td>(1:2:1)</td>
</tr>
<tr>
<td>(15)</td>
<td>324,326,328</td>
<td>280,282,284</td>
</tr>
<tr>
<td></td>
<td>(1:2:1)</td>
<td>(1:2:1)</td>
</tr>
</tbody>
</table>

The characteristic triple peaks, $M, M+2$ and $M+4$ (due to $^{79}\text{Br}$ and $^{81}\text{Br}$) and their relative intensities (1:2:1) indicate the presence of two bromine atoms in the products (14) and (15). Base peaks in the above spectra resulted from the loss of $\text{OC}_2\text{H}_5$ from the product (14) and $\text{CO}_2$ from (15).
Table 2.4 $^1$H n.m.r. spectral data (CDCl$_3$) of ethyl dibromoorsellinate (14).

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.37</td>
<td>3(tr,J=7.15Hz)</td>
<td>C(1)-CO$_2$-CH$_2$-CH$_3$</td>
</tr>
<tr>
<td>2.6</td>
<td>3(s)</td>
<td>C(6)-CH$_3$</td>
</tr>
<tr>
<td>4.4</td>
<td>2(q, J=6.95Hz)</td>
<td>C(1)-CO$_2$-CH$_2$-CH$_3$</td>
</tr>
<tr>
<td>6.4$^*$</td>
<td>1(br. s)</td>
<td>-OH (probably C(4)-hydroxy)</td>
</tr>
</tbody>
</table>

*This signal was assigned to the C(4)-OH since C(2)-OH (not observed) would be expected to appear considerably down field due to H-bonding to the ortho carboxy C=O.  

Table 2.5 $^1$H n.m.r. spectrum (CDCl$_3$) of dibromoorsellinic acid (15).

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7</td>
<td>3(s)</td>
<td>C(6)-CH$_3$</td>
</tr>
<tr>
<td>8.1$^*$-8.75</td>
<td>1(br. s)</td>
<td>-OH (probably C(4)-hydroxy)</td>
</tr>
</tbody>
</table>

*This signal was assigned to the C(4)-OH since C(2)-OH (not observed) would be expected to appear considerably down field due to H-bonding to the ortho carboxy C=O.  

129
Table 2.6 $^1$H n.m.r. spectral data (d$_6$-acetone) of orsellinic acid (6).

<table>
<thead>
<tr>
<th>Chemical shift (δ p.p.m.)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.45</td>
<td>3(s)</td>
<td>C(6)-CH$_3$</td>
</tr>
<tr>
<td>6.25</td>
<td>2(br. s)</td>
<td>C(3) and C(5)-H</td>
</tr>
</tbody>
</table>

C(3) and C(5)-H signals appeared in 90MHz spectrum as doublets (at 6.29 and 6.23 p.p.m. respectively; $J \leq 2.5$Hz).

From the microanalytical, mass spectra and n.m.r. spectral data which are listed in Tables 2.1 to 2.5, it becomes clear that the intermediates in the second and third steps of this preparative method (Scheme 2.1) have aromatic structure. These experiments were carried out exactly as described by Gaucher and Shepherd, and the melting points of the products of these two steps were found to be the same as reported by these and other authors.$^{120-122}$ The intermediates in fact appear to correspond to ethyl dibromoorsellinate (14) and dibromoorsellinic acid (15), as previously suggested by Sonn.$^{120}$ and Santesson$^{121}$ although at the time of completing the present study we were unaware of their publications. An alternative interpretation of Sonn's reaction sequence was suggested by Kloss and Clayton
122 (Scheme 2.4, 'b'), however, this does not appear to be correct, since they claimed that ethyl 2,4-dibromo-5-methyl-6-carboxyhexa-1,3-dione (4) was obtained by Sonn's procedure, whereas Sonn correctly suggested the bromination of ethyl dihydroorsellinate in glacial acetic acid led directly to the

Scheme 2.4 Alternative pathways ('a' and 'b') proposed for the synthesis of orsellinic acid. [path 'a' suggested by Sonn (1928) and path 'b' suggested by Kloss and Clayton (1965)].
ethyl dibromoorsellinate. Kloss and Clayton found that in the final step in Sonn's procedure, i.e. the hydrolysis of ethyl orsellinate to orsellinic acid decarboxylation occurs upon acidification.

Since the bromination of ethyl dihydroorsellinate (3) led to the aromatisation of this compound, it was decided to investigate potential intermediates formed during this step. Compound (3) was treated with bromine in glacial acetic acid (1:1 mole). The following compounds were detected on thin-layer chromatography of the reaction mixture using a mixture of chloroform and acetic acid (9:1 v/v): Compounds A (R_f = 0.88), B (R_f = 0.69), C (R_f = 0.57) and D (R_f = 0.37).

Compounds A and D were identified as starting material and ethyl dibromoorsellinate. The purified compound B melted at 139-140°C, its mass spectrum exhibiting two molecular ions at m/e 274 and 276 (1:1) with base peaks appearing at m/e 229 and 231 (1:1) due to ^79Br and ^81Br isotopes. This indicates the presence of one bromine atom both in the molecular ion and the base peak. The molecular weight of this compound is the same as ethyl monobromoorsellinate (17), and the n.m.r. spectral data for compound B is consistent with this structure (Table 2.7).
Table 2.7 $^1$H n.m.r. spectrum of compound B (CDCl$_3$).

<table>
<thead>
<tr>
<th>Chemical shift (δ p.p.m.)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.37</td>
<td>3(tr. J=6.85Hz)</td>
<td>C(1)-CO$_2$-CH$_2$-CH$_3$</td>
</tr>
<tr>
<td>2.45</td>
<td>3(s)</td>
<td>C(6)-CH$_3$</td>
</tr>
<tr>
<td>4.37</td>
<td>2(q. J=6.95Hz)</td>
<td>C(1)-CO$_2$-CH$_2$-CH$_3$</td>
</tr>
<tr>
<td>5.9 (exchangeable with D$_2$O)</td>
<td>1(br. s)</td>
<td>C(4)-OH</td>
</tr>
<tr>
<td>6.4</td>
<td>1(s)</td>
<td>C(3)-H</td>
</tr>
<tr>
<td>12.55 (exchangeable with D$_2$O)</td>
<td>1(br. s)</td>
<td>C(2)-OH</td>
</tr>
</tbody>
</table>

The characteristics of compound C corresponded to those of ethyl orsellinate (16), which structure was confirmed by comparison with an authentic sample (Tables 2.8 and 2.9).
Table 2.8 Some characteristics of compound C and ethyl orsellinate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>m.p. (°)</th>
<th>$M^+$ (m/e)</th>
<th>Base peak (m/e)</th>
<th>$R_f^*$</th>
<th>$\lambda_{max}$ (EtOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>131-132</td>
<td>196</td>
<td>151 (M-OEt)</td>
<td>0.57</td>
<td>265 301</td>
</tr>
<tr>
<td>Ethyl orsellinate</td>
<td>130-131</td>
<td>196</td>
<td>151 (M-OEt)</td>
<td>0.56</td>
<td>265 301</td>
</tr>
</tbody>
</table>

The mixture of CHCl$_3$ and CH$_3$COOH (9:1 v/v) was used as eluent.

By analogy with the mode of bromination of $\beta$-resorcylic acid$^{130}$, bromination of ethyl dihydroorsellinate (which is shown above to involve dehydrogenation) was considered most likely to yield 5-bromoethyl orsellinate (17). The u.v. spectrum of this compound showed two peaks at 296 ($\varepsilon$=11139) and 260 ($\varepsilon$=1879) in ethanol.

![Chemical structure](Image)

(16) Ethyl orsellinate $R = H$
(17) Ethyl 5-bromoorsellinate $R = Br$
Table 2.9 $^1$H n.m.r. spectral data of compound C and ethyl orsellinate (CDCl$_3$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical shift (δ p.p.m.)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C(1)-O-CH$_2$-CH$_3$</td>
</tr>
<tr>
<td>Ethyl orsellinate</td>
<td>1.4</td>
<td>3(tr, $J=6.55$Hz)</td>
<td>C(1)-O-CH$_2$-CH$_3$</td>
</tr>
<tr>
<td></td>
<td>2.45</td>
<td>3(s)</td>
<td>C(6)-CH$_3$</td>
</tr>
<tr>
<td></td>
<td>4.4</td>
<td>2(q., $J=6.75$Hz)</td>
<td>C(1)-OCH$_2$-CH$_3$</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>2(s)</td>
<td>C(3) and C(5)-H</td>
</tr>
<tr>
<td>C</td>
<td>1.37</td>
<td>3(tr, $J=6.95$Hz)</td>
<td>C(1)-O-CH$_2$-CH$_3$</td>
</tr>
<tr>
<td></td>
<td>2.45</td>
<td>3(s)</td>
<td>C(6)-CH$_3$</td>
</tr>
<tr>
<td></td>
<td>4.35</td>
<td>2(q., $J=6.95$Hz)</td>
<td>C(1)-O-CH$_2$-CH$_3$</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>2(s)</td>
<td>C(3) and C(5)-H</td>
</tr>
</tbody>
</table>

Ethyl dihydroorsellinate (3) was treated with bromine water (1:1) and the main product was identified as ethyl orsellinate. Compound (3) was also treated with iodine monobromide when the reaction yielded a mixture of ethyl orsellinate and ethylmonobromoorsellinate. The identification of these products was carried out by comparing their $R_f$ values,
melting points and u.v. spectral data with those of authentic samples.

Further, the effect of the following reagents was investigated on the solution of ethyl dihydroorsellinate in acetic acid media; (i) KClO₃, (ii) KClO₄, (iii) NaIO₃, (iv) KIO₄. The ethereal extracts of the above mixtures on chromatography, indicated that only NaIO₃ and KIO₄ yielded ethyl orsellinate. When these reactions were carried out at different pH values (i.e. 2, 6 and 10), it was found that sodium iodate and potassium periodate only react with ethyl dihydroorsellinate at pH 2.

The addition of bromine to the solution of ethyl dihydroorsellinate in glacial acetic acid firstly produces ethyl orsellinate, the substitution reactions on the aromatic ring taking place on further reaction with bromine. The following scheme illustrates why the aromatisation does not seem to occur in the debromination stage as was suggested by Gaucher¹²³ and Kloss.¹²²
3- or 5- bromo ethyl
orsellinate (R₁ or R₂ = Br).

Scheme 2.5 Probable bromination sequence for ethyl dihydro-orsellinate (in preparation of orsellinic acid).

On refluxing a number of hydroaromatic compounds with D.D.Q (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) in benzene aromatisation¹³²,¹³⁶ has frequently been observed. An attempt was therefore made to aromatise ethyl dihydroorsellinate by this method, but under the following conditions, only unchanged starting materials were recovered.
(I) 3:1 moles D.D.Q., 10hrs. reflux in benzene.

(II) 2:1 moles D.D.Q., 10hrs. reflux in benzene.

(III) 1:1 moles D.D.Q., 10hrs. reflux in benzene.

No brominated compounds were observed on thin-layer chromatography, when ethyl dihydroorsellinate was treated with NBS (N-bromosuccinamide) in the presence of a catalytic amount of dibenzoyl peroxide under conditions which have been successfully utilised for the bromination of 5-pentyl-3,4-dihydropyran-2-one.\textsuperscript{133}

For the preparation of ethyl-5-bromo-6-methyl-1-carboxyl-cyclohexa-2,4-dione (2,4) an attempt was made to obtain the latter compound by condensation of ethyl acetoacetate and ethyl-\(\alpha\)-bromo crotonate. However, under similar conditions to those used for the preparation of ethyl dihydroorsellinate a mixture of products resulted. Ethyl-\(\alpha\)-bromo crotonate was prepared according to the Moureu method,\textsuperscript{134} involving two stages starting from ethyl crotonate. Its n.m.r. spectrum corresponded to the required structure.

\underline{Table 2.10 N.m.r. spectral data for ethyl \(\alpha\)-bromo-crotonate.}

<table>
<thead>
<tr>
<th>Chemical shift ((\delta) p.p.m.)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>3(tr., (J=7.45)Hz)</td>
<td>-CO-CH(_2)-CH(_3)</td>
</tr>
<tr>
<td>2.03</td>
<td>3(d., (J=7.15)Hz)</td>
<td>CH(_3)-CH=CH-</td>
</tr>
<tr>
<td>4.17</td>
<td>2(q., (J=7.15)Hz)</td>
<td>-CO-CH(_2)-CH(_3)</td>
</tr>
<tr>
<td>6.7</td>
<td>1(q., (J=7.75)Hz)</td>
<td>CH(_3)-CH=CH-</td>
</tr>
</tbody>
</table>
In the present work an attempt was made to prepare certain chloro derivatives of orsellinic acid (6) and structurally related compounds. 5-Chloroorsellinic acid (19) and 3-chloroorsellinic acid (20) were prepared according to methods given by Santesson. 2-Chloroorcinol (23) and 5-chloro-β-resorcylic acid (26) were prepared by chlorination of orcinol (27) and β-resorcylic acid, in a similar manner to the preparation of 5-chloroorsellinic acid, using sulphuryl chloride. The structure of (23) was confirmed on comparing its melting point with the decarboxylation product of 5-chloroorsellinic acid, while the structure of compound (26) was established through comparison of the melting point of its decarboxylated product with an authentic sample of 4-chlororesorcinol (28). The selectivity of the monochlorination of the above mentioned compounds results from the gradual release of free chlorine by sulphuryl chloride in benzene, especially in the absence of a catalyst at room temperature. The methylation of orsellinic acid and orcinol were also carried out by standard procedures.

\[
\left[\text{3-}^3\text{H}\right]-\text{5-chloroorsellinic acid (29) was prepared according to the method utilised for the synthesis of} \\
\left[{\text{G-}}^3\text{H}\right]-\text{benzene.}^{136}
\]

The specific activity of the product (31.8 μCi/mg), recovered by preparative-tlc was sufficient to permit the localisation of the aromatic tritium atom by \textsuperscript{3}H-n.m.r. (Fig. 2.6). The tritium and corresponding proton resonances were very similar (δ 6.35 and 6.45 p.p.m. respectively).
Fig. 2.6 N.m.r. spectra (d$_6$-acetone) of [3-$^3$H]-5-chloro-orsellinic acid (a) $^1$H n.m.r. spectrum 
(b) $^3$H n.m.r. spectrum showing exclusive triton-labelling at C-3 (6.45 p.p.m.).
### Table 2.11 Synthesised potential inhibitors of penicillic acid.

<table>
<thead>
<tr>
<th>Number</th>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
<th>R&lt;sub&gt;4&lt;/sub&gt;</th>
<th>R&lt;sub&gt;5&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6)</td>
<td>Orsellinic acid</td>
<td>Me</td>
<td>CO&lt;sub&gt;2&lt;/sub&gt;H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>(19)</td>
<td>5-Chloroorsellinic acid</td>
<td>Me</td>
<td>CO&lt;sub&gt;2&lt;/sub&gt;H</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
</tr>
<tr>
<td>(20)</td>
<td>3-Chloroorsellinic acid</td>
<td>Me</td>
<td>CO&lt;sub&gt;2&lt;/sub&gt;H</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>(21)</td>
<td>3,5-Dichloroorsellinic acid</td>
<td>Me</td>
<td>CO&lt;sub&gt;2&lt;/sub&gt;H</td>
<td>Cl</td>
<td>Cl</td>
<td>H</td>
</tr>
<tr>
<td>(22)</td>
<td>Orsellinic acid-4-methyl ether</td>
<td>Me</td>
<td>CO&lt;sub&gt;2&lt;/sub&gt;H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>(23)</td>
<td>2-Chloroorcinol</td>
<td>Me</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
</tr>
<tr>
<td>(24)</td>
<td>4-Chloroorcinol</td>
<td>Me</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>(25)</td>
<td>Orcinol-mono-methyl ether</td>
<td>Me</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>(26)</td>
<td>5-Chloro-β-resorcylic acid</td>
<td>H</td>
<td>CO&lt;sub&gt;2&lt;/sub&gt;H</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
</tr>
<tr>
<td>(27)</td>
<td>Orcinol</td>
<td>Me</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>(28)</td>
<td>4-Chloro-resocinol</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
</tr>
<tr>
<td>(29)</td>
<td>[3-&lt;sup&gt;2&lt;/sup&gt;H]-5-Chloroorsellinic acid</td>
<td>Me</td>
<td>H</td>
<td>&lt;sup&gt;3&lt;/sup&gt;H</td>
<td>Cl</td>
<td>H</td>
</tr>
</tbody>
</table>
2.3 Experimental

All the spectra obtained from the following instruments, except those mentioned in text;

(1) Perkin-Elmer n.m.r. spectrometer R-24A.
(2) Mass spectrometer M.S.12.
(3) Unicam SP8000 U.V. Spectrophotometer.
(4) Perkin-Elmer IR-577.

* All the $^1$H n.m.r. spectra obtained in CDCl$_3$, using T M S as internal reference, except those which are mentioned in text.

2.3.1 Preparation of anhydrous ethyl alcohol (c.f. Vogel$^{137}$).

Magnesium turnings (1.25g) and iodine (0.25g) were placed in a 500ml round bottomed flask. After adding absolute ethanol (18ml), the flask was fitted with a double surface condenser, and a drying tube. The reaction mixture was then warmed until the iodine had disappeared, at which stage there was no sign of any hydrogen evolution. A further 250ml of absolute ethanol was added to the ethylated magnesium, and the mixture refluxed for 30 minutes. Then 150ml of the alcohol was distilled off directly into a three-necked, round bottomed flask, which was used for subsequent experiments.
2.3.2 Preparation of ethyl dihydroorsellinate

To the flask containing super-dry ethanol (150ml.), equipped with a mechanical stirrer and a condenser, was added 11.5g of sodium. The mixture was then warmed with stirring until the sodium has disappeared. Ethyl acetoacetate (65.1g) was gradually added, followed by ethyl crotonate (57.1g) and the reaction mixture refluxed with stirring until a thick cream-like slurry formed. At this stage, heating was stopped and the reaction mixture stirred for 3 hrs. It was then neutralised with 1M H$_2$SO$_4$ (the pH was adjusted to 7). The Na$_2$SO$_4$ was removed by vacuum filtration. The filtrate was diluted with distilled water (100ml) and acidified (pH=4) with 6M HCl, prior to extraction with three 400ml. portions of chloroform. The combined chloroform extracts were again dried over anhydrous Na$_2$SO$_4$, and chloroform was then evaporated under vacuum. From this 94.5g crude material was obtained, 2.0g of this compound was recrystallised from benzene and petroleum ether (40-60°C); recovery 80%, m.p. 87-89°C (lit. 123 88-90°C).

2.3.3 Preparation of ethyl dibromoorsellinate

To a 1l, 3-necked, flask containing 300ml of glacial acetic acid, 92.0g of the above mentioned crude material was added. The flask was then fitted with a mechanical stirrer and a condenser. When the compound had completely dissolved, 55 ml of bromine was gradually added to the
reaction mixture, and stirring was continued over night. A white precipitate was then collected, washed with water, and dried (95.0g). 1.0g of this product was recrystallised from ethanol (recovery 85.1%; m.p. 143-4°, lit.123 143-4°).

2.3.4 Preparation of dibromoorsellinic acid

The crude product of the above (94.0g) was dissolved in concentrated H₂SO₄ (300ml) and was stirred for 1 hr. at room temperature. The solution was then transferred into a 2l. beaker half-filled with crushed ice, and a white precipitate was obtained. The reaction mixture was subsequently extracted with two 800ml portions of diethyl ether, and the combined ethereal extracts were in turn extracted with three 400ml portions of 0.5M NaHCO₃. The combined sodium bicarbonate extracts were then immediately acidified with 6M HCl to pH=3, and the white solid formed was filtered, and dried (69.2g; yield 80.6%). The crystallisation of this compound in methyl alcohol was not successful, unlike the result reported by Kloss and Clayton.122 It was however, recrystallised (1.0g) from glacial acetic acid-water; recovery 72.6%, m.p. 213-214° (lit.123 212-213°).

2.3.5 Preparation of orsellinic acid

Debromination of the last product was carried out by the following methods.
(i) Using Pd on charcoal (5%) as catalyst;

Dibromoorsellinic acid (16.0 g) was dissolved in glacial acetic acid (250 ml) and transferred to the hydrogenation instrument. To this solution, were added sodium acetate (8.0 g) and Pd/C (5%; 16.0 g), and the mixture was stirred under 3 atm. of hydrogen for 4 hrs. The reaction mixture was then filtered, and 500 ml of distilled water was added to the filtrate. The solution was extracted with two 500 ml portions of ether. After washing the combined ethereal extracts with two 250 ml of water, the ether phase was dried over anhydrous magnesium sulphate and then evaporated to dryness (4.1 g; Yield 48.5%).


In a 500 ml conical flask, dibromoorsellinic acid (8.3 g) was dissolved in 2M sodium hydroxide (200 ml) at 0°C. Then Raney's nickel (8.0 g) was added, with stirring in small portions (in 1 hr. intervals), to the reaction mixture at below 4°C. After adding 0.5 ml ethanol to the mixture, it was filtered into a flask containing cool conc. hydrochloric acid (70 ml). The filtrate was extracted with three portions of 500 ml ether, and the combined extracts dried over anhydrous magnesium sulphate. The ether was then removed (3.7 g; yield 86%).
Debromination of dibromoorsellinic acid under 1 atm. H₂ (A) using Pd on C (5%), (B) using Pd on CaCO₃; this catalyst was prepared according to Busch and Stoves' method. ¹³⁹

In both instances, after 6 hrs. of shaking the reaction mixture at room temperature, the yields obtained were less than 5%.

The crude products, in each method were recrystallised from a mixture of glacial acetic acid and distilled water. The orsellinic acid was collected as white needle form crystals, which were dried in a vacuum oven for 24 hrs., (recovery 75% and m.p. 175-176°C, lit. ¹²¹ 174-175°C).

2.3.6 Bromination of ethyl dihydroorsellinate (1:1mole)

Ethyl dihydroorsellinate (5.94g) was dissolved in glacial acetic acid (20ml) inside a 100ml, 3-necked, flask equipped with a magnetic stirrer and a condenser. To this solution, bromine (4.8g) in acetic acid (20ml) was gradually added within 3 hrs. with stirring at 40°C. The reaction mixture was then poured into a 500ml beaker which already contained 200ml of 10% Na₂CO₃. This solution was then extracted with two 100ml portions of ether, and the combined ethereal extracts washed with four 100ml of water. After drying the ethereal extract over anhydrous magnesium sulphate, the solution was concentrated. Thin-layer chromatography, using CHCl₃;CH₃COOH (9:1 v/v) as solvent system, showed that
it was a mixture of ethyl dibromoorsellinate, ethyl 5-bromo-
orsellinate, ethyl orsellinate and the starting material.
The aromatic compounds were separated by column chromatography
 technique, using chloroform and ethyl acetate mixture (19:1
v/v) as eluent. The column was made up from silica gel
120 mesh (height=150cm). Ethyl dibromoorsellinate was recrystal-
tallised from a mixture of water-ethanol (0.91g, m.p. 143-4°C;
yield 8.5%). Ethyl monobromoorsellinate was recrystallised
from water-acetic acid mixture and the crystals obtained
were dried in a vacuum oven at 30°C for 2 days (2.79g,
m.p. 139-140°C; yield 33.2%). Ethyl orsellinate was recrystal-
tallised by the same method as described for ethyl mono-
bromoorsellinate (2.10g, m.p. 131-2°C; yield 35.1%).

2.3.7 Bromination of ethyl dihydroorsellinate with bromine water

To 10ml solution of ethyl dihydrobromoorsellinate (2.0g)
in glacial acetic acid, 10ml of 10% bromine water was
gradually added (in 1 hr.) with continuous stirring
at room temperature. At this stage, the solvents were evapo-
rated and the residue obtained was washed with two 50ml
portions of water. The residue (1.35g) was then recrystal-
tallised from acetic acid-water mixture (0.98g, m.p. 131-
132°C, Rf=0.56 silica tlc- CHCl3:CH3COOH = 9:1, its u.v.
spectrum showed two maxima in ethanol at 265nm and 301nm).
2.3.8 Bromination of ethyl dihydroorssellinate with iodine monobromide

(i) Preparation of iodine monobromide. Finely divided iodine (2.6g) in glacial acetic acid (6ml) was treated with bromine (0.5ml) at 50°C.

(ii) Iodine monobromide which was already prepared was gradually added to ethyl dihydroorssellinate (4.0g) solution in glacial acetic acid (10ml) with stirring at 40°C. After 4 hrs. of stirring, the reaction mixture was filtered, the filtrate was then treated with 100ml aqueous solution of sodium bisulphite (1.6g) and sodium bicarbonate (16g). After 12 hrs., the reaction mixture was filtered and the solid washed with two 20ml portions of water, it was then dried in a vacuum oven (30°C) for a period of 2 days (yield 2.51g). Thin-layer chromatography using a CHCl₃:CH₃COOH mixture (9:1v/v) as solvent system, showed this to be a mixture of ethyl orsellinate and ethyl-5-bromoorsellinate. Pure compounds were obtained from preparative chromatography (30mg of mixture was used) using the above mentioned solvent system on silica gel (GF 254, 0.5mm). [Ethylorsellinate; m.p. 131-132°C, Rf ≈ 0.56, λ<sub>max</sub><sup>EtOH</sup> 265 and 301.; Ethyl-5-bromoorsellinate, m.p. 139-140°C, Rf ≈ 0.68, λ<sub>max</sub><sup>EtOH</sup>(ε =11139) and λ<sub>260</sub> (ε =1879)].
2.3.9 Effect of some oxidizing reagents on ethyl dihydroorsellinate

The following reagents were added to the five test tubes containing ethyl dihydroorsellinate (0.2g.) in acetic acid (1ml);

(a) 1ml of saturated solution of KClO$_3$ in water.
(b) 1ml of saturated solution of KClO$_4$ in water.
(c) 1ml of saturated solution of NaIO$_3$ in water.
(d) 1ml of saturated solution of KIO$_4$ in water.
(e) 1ml of concentrated HClO$_4$.

These tubes were then placed in a water bath at 40°C. Thin-layer chromatography of ethereal extracts of the samples (at varying times) indicated that only those which contained IO$_3^-$ and IO$_4^-$ yielded ethyl orsellinate (Rf~0.56) in 30 mins. In the case of IO$_3^-$ and IO$_4^-$ respectively 50 and 70 per cent of the starting material had changed to ethyl orsellinate after 24 hours.

In another experiment 1ml of a saturated solution of KIO$_4$ in water was added to three test tubes containing 2ml of alcoholic solution of ethyl dihydroorsellinate (0.2g.). After adjusting the pH in the following order, pH 2, pH 6 and pH 10, the test tubes were then placed in a water bath and the temperature was kept at 40°C. After 4 hrs. they were extracted separately with two 5ml portions of ether in acidic medium (pH 2). The combined ethereal extract of each sample was evaporated under reduced pressure. Among these
residues only pH=2 showed a spot at ethyl orsellinate zone on thin-layer chromatogram. From preparative chromatography of this residue ethyl orsellinate was recovered (81.5mg , m.p. 130-131°C, yield 41%).

2.3.10 Condensation of ethyl acetoacetate with α-bromocrotonate

Ethyl crotonate (27.0g ) was slowly treated by a solution of bromine (37.8g.), in glacial acetic acid (20ml ), with stirring at room temperature for 1.5 hrs. After adding formic acid (a few drops), the mixture was treated with a solution of 1M NaHCO\textsubscript{3}, and extracted with two 80ml. portions of ether. The combined ethereal extracts were evaporated under vacuum, and the remainder was refluxed with sodium acetate (9.0g ) in ethanol (150ml ) (in a dark place) for 4 hrs. After removing NaBr and ethanol, the solution was mixed with 40ml sodium bicarbonate (5%) and extracted with two 50ml. portions of ether. The combined extracts were dried over Na\textsubscript{2}SO\textsubscript{4}, and ether was evaporated under vacuum (27.1g ).

Ethyl α-bromocrotonate was treated with ethyl acetate under the same conditions as described for condensation of ethyl acetate and ethyl crotonate (p. 67 ). Under these conditions no solid was separated, and the obtained solution on thin-layer chromatogram (using CHCl\textsubscript{3}:CH\textsubscript{2}COOH (9:1v/v)) showed seven spots.
2.3.11 Bromination of ethyl dihydroorsellinate with NBS

Ethyl dihydroorsellinate (0.5g) was dissolved in CCl₄ (50ml) and refluxed with N-bromo-succinimide (0.45g) in the presence of dibenzoyl peroxide (0.15g) under a mercury lamp (400W). The mixture on chromatography plate showed only starting materials.

This procedure was repeated in benzene, and no product was obtained.

2.3.12 Preparation of 5-chloroorsellinic acid.¹⁴⁰,¹²¹

Dry orsellinic acid (4.2g, recrystallised from benzene) was dissolved in dry ether (100ml) in a 250ml two necked, round bottomed flask, equipped with stirrer and a drying tube. This solution was treated at room temperature with sulphuryl chloride (3.375g) in dry ether (20ml). After 4 hrs. the reaction mixture was washed with two 30ml portions of water, the ether was then evaporated under reduced pressure, and the residue obtained was recrystallised from water. The crystals were dried in a vacuum oven at 30°C for 24 hrs. [3.09g, yield 61.33%, m.p. 196-197°C; lit.¹²¹ 182.5-184°C (recrystallised from benzene), Rf=0.70] microanalytical and n.m.r spectral data are as follows:
Microanalytical data:

<table>
<thead>
<tr>
<th></th>
<th>C%</th>
<th>H%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>47.15</td>
<td>3.34</td>
</tr>
<tr>
<td>Calculated</td>
<td>47.42</td>
<td>3.47</td>
</tr>
</tbody>
</table>

N.m.r. spectral data:

<table>
<thead>
<tr>
<th>Signal position (S;PPm)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.45</td>
<td>1(s)</td>
<td>Ar-H</td>
</tr>
<tr>
<td>2.67</td>
<td>3(s)</td>
<td>Ar-CH₃</td>
</tr>
</tbody>
</table>

Mass spectral data:

- M⁺ peaks at m/e 204 and 202 (3:1)
- Base peaks at m/e 160 and 158 (3:1)

2.3.13 Preparation of 3-chloroorsellinic acid

This compound was prepared in three stages from orsellinic acid.

(A) 5-iodoorsellinic acid - A mixture of dry orsellinic acid (1.68g) and iodine (2.5g) in ether (50ml) was treated with yellow mercuric oxide (2.1g) in small portions. After 30 mins. refluxing, the reaction mixture was kept at room temperature for 2 hrs. The mixture was then filtered and the filtrate washed with 50ml of aqueous KI (10%) and water (50ml). Ether was then removed and the residue obtained (2.48g, yield 84%) was recrystallised from benzene (recovery 88%, m.p. 172-173°C).
(B) 5-Iodo-3-chloroorsellinic acid: 5-Iodoorsellinic acid (2g ) in dry ether (60ml ) was treated with 3ml of sulphuryl chloride in ether (30ml ) and the reaction mixture was then stirred at room temperature. After 24 hrs. it was washed with five 100ml portions of water, and the ether was then removed. The residue obtained (1.42g ) was recrystallised from benzene (1.21g , 54%, m.p. 199-200°C).

(C) 3-Chloroorsellinic acid: The above product (1.10g ) was dissolved in 35ml of 2M NaOH, and it was then treated with 25-50mg. portions of Raney nickel alloy when the evolution of gas became rapid. At this stage, the solution was filtered into 7.0ml of conc. HCl, and the filtrate diluted with water (70ml ). This solution was extracted with three 100ml portions of water. After removing ether, the residue was recrystallised from benzene (0.57g , 83%, m.p. 173-174°C; lit. 172-174°C, Rf = 0.70) microanalytical data:

<table>
<thead>
<tr>
<th></th>
<th>C%</th>
<th>H%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>47.30</td>
<td>3.37</td>
</tr>
<tr>
<td>Calculated</td>
<td>47.42</td>
<td>3.48</td>
</tr>
</tbody>
</table>

Table 2.12 ¹H n.m.r. spectral data of 3-chloroorsellinic acid (d₆-acetone).

<table>
<thead>
<tr>
<th>Signal position</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.46</td>
<td>1(s)</td>
<td>Ar-H</td>
</tr>
<tr>
<td>2.66</td>
<td>3(s)</td>
<td>Ar-CH₃</td>
</tr>
</tbody>
</table>
2.3.14 Preparation of \([3^{-3}H] -5\)-chloroorsellinic acid

The preparation was achieved in a similar way to the method used for \([G^{-3}H] -benzene\), as reported by Saljoughian.\(^{124}\) Since tritium is a \(\beta\) -emitter a trial experiment was firstly carried out for preparation of \([3-D]\) -5-chloroorsellinic acid.

Preparation of catalyst - Pt was prepared according to Calf's method.\(^{141}\) In a 100ml beaker, 100mg of sodium borohydride was slowly added to a suspension of platinum oxide (40mg) in 50ml of water. Hydrolysis of the excess sodium borohydride was accomplished by warming the reaction mixture at 70\(^0\)C. The catalyst was collected, washed with water to remove salts, and the water finally decanted.

5-Chloroorsellinic acid (8.2mg), in a small test tube, was dissolved in dioxane (0.2ml); Pt\(^0\) and HTO (10\(\mu\)l, 50mCi/ml) was then added to this solution. The reaction mixture was frozen, the tube evacuated from the air and sealed over a flame. The test tube was then heated in an oil bath at 110\(^0\)C. After 18 hrs. the reaction mixture was filtered and the solid washed with six 2ml portions of acetone. The solution was then freeze-dried, and the residue obtained dissolved in methanol (2.5ml) and the solution again freeze-dried (the process was repeated three times). Pure \([3^{-3}H] -5\) -chloroorsellinic acid (5.495mg) was then obtained from preparative chromatography using CHCl\(_3\); CH\(_2\)COOH (9:1v/v).
2.3.15 Preparation of 3,5-dichloroorsellinic acid

Dry orsellinic acid (0.42g) in dry ether (40ml) was treated at room temperature with sulphuryl chloride (1ml, 1.485g) in ether (4ml). After 18 hrs., the reaction mixture was washed with two 15ml portions of water, the ethereal solution was then evaporated under vacuum, and the residue obtained recrystallised from benzene (0.513g, yield 87%, m.p. 187-188°C, Rf ≈ 0.78). Its mass spectrum showed the M+ peaks at m/e 240, 238 and 236 (8:5:1), the base peaks appeared at m/e 196, 194 and 192 (8:5:1).

2.3.16 Mono-chlorination of orcinol

(A) Dry orcinol (1.24g) in dry ether (100ml) was treated at room temperature with sulphuryl chloride (1.35g, 0.9ml) in dry ether (10ml). The reaction mixture was stirred for 4 hrs., and washed with two 50ml portions of water. The ethereal solution was then evaporated under reduced pressure and the residue recrystallised from benzene (0.92g, 58%, m.p. 137-8°C, Rf ≈ 0.6). Microanalytical data:

<table>
<thead>
<tr>
<th></th>
<th>C%</th>
<th>H%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>53.36</td>
<td>4.24</td>
</tr>
<tr>
<td>Calculated</td>
<td>53.02</td>
<td>4.41</td>
</tr>
</tbody>
</table>
Table 2.13 $^1$H n.m.r. spectral data of 2-chloroorcinol (D$_2$O; using DSS as reference).

<table>
<thead>
<tr>
<th>Signal position ($\delta$ ppm)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4</td>
<td>2 (br. s)</td>
<td>Ar-H</td>
</tr>
<tr>
<td>2.4</td>
<td>3 (s)</td>
<td>Ar-CH$_3$</td>
</tr>
</tbody>
</table>

The mass spectral data:

$M^+$ peaks at m/e 160 and 158 (3:1)

Base peaks at m/e 160 and 158 (3:1)

(B) To a 10ml aqueous solution of 5-chloroorsellinic acid (18.0mg) a solution of 2M HCl (2 drops) was added, and the reaction mixture refluxed for 2 days. The solution was evaporated to dryness (12.0mg) and thin-layer chromatography using CHCl$_3$;CH$_2$COOH showed a spot with the same Rf as the above mentioned mono-chlorinated compound. This compound recrystallised from benzene (10.2mg, m.p. 137-138°C; lit. m.p. 137°C).

The melting point (137-138°C) of the products of procedure A and B was undepressed in the mixture, therefore the mono-chloro compound (A) was identified as 2-chloroorcinol.

Preparation of 4-chloroorcinol

An acidic aqueous solution (10ml, pH 3) of 3-chloroorsellinic acid (18.0mg) was refluxed for 2 days. The solvent was then evaporated under vacuum (10.8mg), and the residue recrystallised from benzene (9.1mg, m.p. 132-133°C).
2.3.17 Monochlorination of β-resorcylic acid

Dry β-resorcylic acid (8.0g) in dry ether (200ml) was treated at room temperature with sulphuryl chloride (8.9g, 6.0ml) in dry ether (50ml). After 4 hrs. stirring, the reaction mixture was washed with two 50ml portions of water, and the ethereal solution was then evaporated under vacuum. The residue obtained was recrystallised from water (8.07g, m.p. 216-217°C, Rf ≈ 0.71).

Microanalytical data:

<table>
<thead>
<tr>
<th></th>
<th>C%</th>
<th>H%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found</td>
<td>40.62</td>
<td>3.20</td>
</tr>
<tr>
<td>Required (C₇H₅ClO₄)</td>
<td>44.58</td>
<td>2.67</td>
</tr>
<tr>
<td>Required (C₇H₅ClO₄·H₂O)</td>
<td>40.70</td>
<td>3.41</td>
</tr>
</tbody>
</table>

An aqueous solution of 5-chloro-β-resorcylic acid (0.2g in 20ml, pH=2) was refluxed for 2 days, and then evaporated to dryness. The solid obtained was recrystallised from benzene (0.95g, m.p. 106-107°C, m.p. of 4-chlororesorcinol 106.5-107.5°C).

2.3.18 Preparation of orsellinic acid-4-methyl ether

(i) Diazomethane was prepared from diazald according to the method given by Vogel. In a 250ml conical flask, diazald (6.42g) was dissolved in ether (90ml). After cooling
the solution, potassium hydroxide (1.2g) in ethanol (30ml) was added, and left at room temperature for 5 mins. The reaction mixture was then distilled off, and ethereal solution collected in a flask which had been cooled previously to -70°C in a mixture of dry ice and acetone.

In a 250ml conical flask, equipped with a magnetic stirrer, orsellinic acid (1.68g) in methanol (40ml) was gradually treated with pre-produced diazomethane in ether, at room temperature. After 1 hr. stirring, the reaction products were dissolved in 2M NaOH (20ml). The solution was then refluxed (2 hr), cooled, acidified with 3M HCl (pH 2), and extracted with two 50ml portions of ether. The combined ethereal extract was in turn extracted with two 50ml portions of 0.1M NaHCO₃ and the combined sodium bicarbonate extracts then acidified to pH 2. After the extraction of this solution with two 50ml of ether, the combined ethereal extract was evaporated under vacuum, and the residue obtained then recrystallised from ethanol-water. The crystals were collected and dried in a vacuum oven (30°C) within 10hrs., (1.41g, m.p. 174, (157°C).

Table 2.14 ¹H n.m.r. spectral data of orsellinic acid-4-methyl ether (d₆-acetone).

<table>
<thead>
<tr>
<th>Chemical shift (δ p.p.m.)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.34</td>
<td>1(s)</td>
<td>Ar-H(between OH and OMe)</td>
</tr>
<tr>
<td>6.32</td>
<td>1(s)</td>
<td>Ar-H(between OMe and Me)</td>
</tr>
<tr>
<td>3.82</td>
<td>3(s)</td>
<td>Ar-OCH₃</td>
</tr>
<tr>
<td>2.54</td>
<td>3(s)</td>
<td>Ar-CH₃</td>
</tr>
</tbody>
</table>
2.3.19 Preparation of O-methyl orcinol

A mixture of dry orcinol (1.24g) and potassium carbonate (2.1g) in dry acetone (40ml) was refluxed with methyl iodide (0.95ml) for 8 hrs. The reaction mixture was then acidified with 0.5M H₂SO₄ (pH 2) and extracted with three 50 ml portions of ether. The combined ethereal extract was evaporated under vacuum, and orcinol monomethyl ether was then separated by column chromatography using CHCl₃:CH₃COOH (95:5 v/v) as eluent (0.516g, m.p. 133-4°C, R_f ≈ 0.72).

Table 2.15. ¹H n.m.r. spectral data of O-methyl orcinol (d₆-acetone).

<table>
<thead>
<tr>
<th>Chemical shift ( δ p.p.m.)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4</td>
<td>3(br.s)</td>
<td>Ar-H</td>
</tr>
<tr>
<td>3.9</td>
<td>3(s)</td>
<td>Ar-O-CH₃</td>
</tr>
<tr>
<td>2.45</td>
<td>3(s)</td>
<td>Ar-CH₃</td>
</tr>
</tbody>
</table>

2.3.20 Sulphonation of orcinol

The following methods were utilised, which parallel those given for the synthesis of 1,3-dihydroxybenzene-4-sulphonic acid.

(i) Using H₂SO₄

The mixture of orcinol (0.71g) and conc. H₂SO₄ (0.5ml) was heated for 3 hrs. It was then dissolved in water (100ml) and extracted with two 50ml portions of ether. The combined ethereal extracts were concentrated (20ml).
This solution on thin-layer chromatography using CHCl₃ and CH₃COOH (9:1v/v) showed 4 spots and the main component was found to be unchanged starting material.

(ii) Using ClSO₃H.¹⁴⁴

Orcinol (1.42g) was dissolved in nitrobenzene (5ml) and slowly treated with the solution of ClSO₃H (0.67ml) in nitrobenzene (1.7ml) with stirring for 3 hr. at 40°C. The solid was filtered under vacuum, washed with five 20ml portions of benzene, and it was then dried in a vacuum oven (0.356g). This solid was observed to liquefy when exposed to air, and to undergo a rapid degradation on filter paper to a black residue. From its n.m.r spectrum (in D₂O, using DSS as internal standard), it was identified as orcinol disulphonic acid.

<table>
<thead>
<tr>
<th>Chemical shift (δ p.p.m.)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3</td>
<td>3(s)</td>
<td>Ar-CH₃</td>
</tr>
<tr>
<td>6.35</td>
<td>1(s)</td>
<td>Ar-H</td>
</tr>
</tbody>
</table>

(iii) Using H₂NSO₃H.

The mixture of orcinol (1.42g) and NH₂SO₃H (0.44g) was heated (50°C) and stirred for 2 hr. The reaction mixture was then dissolved in water (20ml) and extracted with ether. Ethanol (20ml) was added to residue, the mixture filtered and the filtrate was concentrated. This was kept
at 4°C for 20 days, the crystals obtained were collected and recrystallised from ethanol (0.160g, m.p. 226-228°C). Its n.m.r. spectrum in D₂O, using DSS as internal standard, showed two peaks at 2.12ppm (3s), and 6.2ppm (1s).
Chapter 3: Effects of Inhibitors on the Biosynthesis of Penicillic Acid.

3.1 Biosynthesis of penicillic acid ........ 88
   3.1.1 Introduction ......................... 89
   3.1.2 Results and discussion ............... 90
   3.1.2.1 Growth of P.cyclopium in submerged and surface cultures ..................... 91
   3.1.2.2 Course of fermentation ............... 92
   3.1.2.3 Isolation of the P.cyclopium metabolites 95

3.2 Inhibition studies ....................... 111
   3.2.1 Introduction .......................... 112
   3.2.2 Results and discussion ............... 115
   3.2.3 Appendix ............................. 134
   3.2.4 Experimental .......................... 151
3.1 Biosynthesis of Penicillic Acid.
3.1.1 Introduction

The growth and replication of *P. cyclopium*, like any other fungus depends upon environmental conditions such as; temperature, aeration and nutritional factors. Detailed knowledge of various aspects of the course of metabolism is essential before attempting the exploration of individual metabolic pathways. An attempt was therefore made to examine the course of fermentation of *P. cyclopium*, on Raullin-Thom medium, and its relationship to the biosynthesis of penicillic acid.

The detection, isolation and identification of penicillic acid (4) and biosynthetically related metabolites was carried out using thin-layer chromatography and u.v. spectroscopic analysis of the chloroform extracts of acidified metabolism solutions. In addition to penicillic acid (4) and orsellinic acid (1) two other metabolites, 3-methoxy-2,5-toluquinone (3) and 3-methoxy-2,5-toluquinol (2) were also isolated from culture filtrates of submerged cultures after 3 days' growth.

The rate of growth of this fungus is at its maximum between 2 and 5 days in the submerged culture, and from 5 to 10 days in surface culture. The highest rate of penicillic acid biosynthesis in submerged cultures occurs between 3 and 4 days after inoculation. This was confirmed by the result of the incorporation of $^{14}$C-labelled acetate into penicillic acid. Under the same conditions, the incorporation of
$^{14}$C-labelled formate was found to be similar as that of $^{14}$C-labelled acetate.

*P. cyclopium* did not grow well on the Czapek-Dox medium and no penicillic acid was detected in the metabolism solution.

3.1.2 Results and discussion

A high yielding culture of *P. cyclopium* $^{79,146}$ (IMI, 89372) was used in the present investigations. It grows well on potato dextrose agar (P.D.A.) slopes and Raulin-Thom medium which was consequently used for the present biosynthetic and inhibition studies. Czapek-Dox medium was however used for some trial experiments with this fungus.
3.1.2.1 Growth of *P. cyclopium*.

(A) Slopes grown on P.D.A. at 24°C, formed white mycelium after only 24 hours, changing to dark green following sporulation after a further 2 days.

(B) Surface cultures grown on Raulin-Thom medium at 24°C, covered the surface with a thin white mycelial pad after 2 days. It grew with deep folding from the edges and thickened from the third day until the fifth or sixth day. At this stage the mycelium became grey on the surface and creamy yellow on the underside, while the colour of the metabolism solution changed from pale yellow to brown. After this period, an increase in the amount of mycelium and a change in the colour of the metabolism solution to deep brown were the only subsequent differences to be observed.

(C) Submerged cultures grown on Raulin-Thom medium at 24°C, formed small white spheres of mycelium after 24 hours. These grew rapidly between one and three days. At this stage the mycelium started to develop a brown colouration becoming completely brown after 4 days' growth; the metabolism solution was coloured pale brown.

A large variation in mycelial growth was observed in submerged cultures, which were inoculated directly from suspended spores. More uniform growth was obtained on using an inoculum of vegetative mycelium after initial incubation of the spore-inoculated medium for 30 hours. This procedure gave
a good growth response with minimum variations in mycelial yield.

_P.cyclopium_ did not grow well on Czapek-Dox medium either in surface or submerged cultures.

### 3.1.2.2 Course of fermentation.

A number of submerged cultures were harvested at varying times ranging from 1 to 8 days after inoculation. The same experiment was carried out for surface cultures (5 to 15 days of growth). Data observed included mycelial weight, residual glucose, pH of culture filtrate and the yield of its chloroform extract; these were plotted against the age of the culture (Figs. 3.1 and 3.2).

As shown, rapid changes during growth in submerged cultures appear between 2 and 4 days, the corresponding change in surface culture occurs between 5 and 9 days. In both instances, the pH variations showed that the minimum pH occurs at the stage of maximum yield. The decrease of pH during the course of metabolism could be related to the consumption of nitrogen which is present in the medium as ammonium salts, the _NH_4^+ ions being replaced by H+. Rapid growth of the fungus in submerged cultures rather than in surface cultures is due to more efficient aeration under these conditions. The rate of glucose consumption is consistent with the high metabolic activity of this fungus during the growth phase. The subsequent decrease in mycelial weight
Fig. 3: Course of fermentation of *P. cyclopium* in submerged culture (60ml) at 24°C.
Day of Growth

Day of Growth

Course of fermentation of P. cyclopium in surface culture (300 ml.) at 24°C.

Mycelial wt.

Growth wt.

Glucose (g/ftask)

Penicillic acid (mg/ftask)

Penicillic acid

HCl ext.

Glucose (%)
is not accompanied by a significant increase in penicillic acid concentration and probably represents the stage when autolysis starts.

3.1.2.3 Isolation of *P. cyclopium* metabolites.

Penicillic acid and its related intermediates were found to exist in the metabolism solution. Since the complete solvent extraction of intermediates which have phenolic characteristics was not possible above pH 3, the filtrate was adjusted to pH 2 before extraction. Chloroform was a satisfactory solvent for extraction of the metabolites from culture filtrates. The detection, isolation and identification of penicillic acid and its biosynthetically related metabolites was carried out using thin-layer chromatography and spectrophotometric analyses of the chloroform extracts of the metabolism solutions.

Results of thin-layer chromatography showing the course of the fermentation in submerged cultures are listed in Table 3.1. Among the components existing in the chloroform extracts the metabolites C, D, E and H were found to be associated with the pathway of penicillic acid formation, however the relationship of the other components to this pathway is still unknown. Components F and G appeared as bright blue fluorescing zones on irradiating at 350nm.
Table 3.1 Results of thin-layer chromatography of chloroform extracted materials obtained from media of *P. cyclopium* in submerged cultures t.l.c., silica GF 254, 0.25mm, with CHCl₃:CH₃OH:H₂O:HO₂H = 250:25:24:1 (organic phase).

<table>
<thead>
<tr>
<th>Component</th>
<th>Rf</th>
<th>Chromatographically equivalent substances</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>~ 0.1</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>~ 0.15</td>
<td>Orcinol</td>
</tr>
<tr>
<td>C</td>
<td>~ 0.2</td>
<td>Orsellinic acid</td>
</tr>
<tr>
<td>D</td>
<td>~ 0.35</td>
<td>Penicilllic acid</td>
</tr>
<tr>
<td>E</td>
<td>~ 0.45</td>
<td>3-Methoxy-2,5-toluquinol</td>
</tr>
<tr>
<td>F</td>
<td>~ 0.6</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>~ 0.8</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>~ 0.85</td>
<td>3-Methoxy-2,5-toluquinone</td>
</tr>
<tr>
<td>I</td>
<td>~ 1</td>
<td></td>
</tr>
</tbody>
</table>
Their mass spectra showed the following molecular ions: 
\[ M^+_F = 254 \] and \[ M^+_G = 444 \]. Since A, F, G and I were observed at all stages of growth (Table 3.1) they are possibly unrelated to the biosynthetic pathway leading to penicillic acid.

As shown in Table 3.1, on the basis of thin-layer chromatography, orsellinic acid was detected in chloroform extracts of 2 day old submerged cultures. This was confirmed from the u.v. spectra of the above residue in ethanol (\( \lambda = 254 \)) and the original filtrate (\( \lambda = 257 \)) (ca.= 0.5 mg/flask). The normal levels of orsellinic acid in the surface culture were examined by Bentley and Keil\(^79\), who found that the filtrates of surface cultures on the 3rd and 7th days of growth respectively contained 0.002 mg and 0.015 mg per ml of orsellinic acid. The chemical structure and spectral characteristics of this compound have been extensively discussed in chapter 2.

Orcinol in trace amounts was found on thin-layer chromatograms of the chloroform extracted filtrate after the third day of growth. It is possible that this compound resulted from a non-enzymatic decarboxylation of orsellinic acid.

Axberg and Gatenbeck\(^119\) found, through radioisotopic tracer experiments, that 3-methoxy-2,5-toluquinol (2) and its related quinone (3) were incorporated into penicillic acid. Subsequently Better and Gatenbeck\(^147\) isolated
compound (2) from the metabolism solution of *P. baarnense* growing on Czapek-Dox medium. In addition to barnol this fungus produces orsellinic acid and penicillic acid.  

\[
\begin{align*}
\text{HO} & \quad \text{CH}_3 \\
\text{HO} & \quad \text{C}_2\text{H}_5 \\
\text{OH} & \quad \text{CH}_3
\end{align*}
\]

**Barnol**

It was found that the relative yields of these substances are dependent on the growth medium, i.e., when Czapek-Dox medium was used orsellinic acid and penicillic acid formed in substantial amounts, whereas only a trace amount of barnol was detected, but on Raulin-Thom medium the fungus produces barnol as the main metabolite.

An attempt was made to establish whether or not 3-methoxy-2,5-toluquinol can be biosynthesised from *P. cylcopium*. Chloroform extracted residues of submerged R-T cultures on thin-layer chromatography indicated (after 3 days of growth) the presence of compounds (2) and (3) which was confirmed by comparison with authentic samples. Under the above conditions, typical yields were 1.6mg and 2.1mg per 50 ml of submerged culture. These compounds were crystallised
from carbon tetrachloride, when 3-methoxy-2,5-toluquinol was obtained as white needles, m.p. 131-132°C, $\lambda_{\text{max}} = 290\text{nm}$ ($\varepsilon = 3830$). 3-Methoxy-2,5-toluquinone was obtained as yellow crystals (ex CCl$_4$), m.p. 155-156°C, $\lambda_{\text{max}} = 264\text{nm}$ ($\varepsilon = 4178$).

Using a two dimensional silica gel (GF254) thin-layer chromatogram, it was found that 3-methoxy-2,5-toluquinol is readily oxidised in air to the related quinone. Both compounds were observed as dark spots when viewed at 350nm. The mass spectrum of compound (3) showed a molecular ion peak at $M^+ = 152$ (100%) and further abundant fragments were seen at m/e 137 (32%), m/e 122 (100%) and m/e 109 (60%). The n.m.r. spectral data for this compound are listed in Table 3.2.

Table 3.2 N.m.r. spectral data (in CDCl$_3$) of 3-methoxy-2,5-toluquinone (3).

<table>
<thead>
<tr>
<th>Chemical shift ($\delta$ p.p.m.)</th>
<th>No. of protons</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.04</td>
<td>3</td>
<td>$-\text{CH}_3$</td>
</tr>
<tr>
<td>3.79</td>
<td>3</td>
<td>$-\text{OCH}_3$</td>
</tr>
<tr>
<td>5.98</td>
<td>1</td>
<td>$H_b$</td>
</tr>
<tr>
<td>6.6</td>
<td>1</td>
<td>$H_a$</td>
</tr>
</tbody>
</table>
Penicillic acid differs from other tetronic acids in being optically inactive and does not give the usual colour test with aqueous ferric chloride. Bentley and Keil described a colorimetric method for the estimation of penicillic acid which is based on the development of a red colour with concentrated ammonia. They initially utilised optical density measurements at 545nm after 10 minutes at room temperature. Later it was found that the optical density did not stabilise even after 1 hour. It was, however, useful as a sensitive qualitative test.

Since the u.v. spectrum of penicillic acid in ethanol gives a characteristic peak at 225nm ($\varepsilon = 12830$, lit. $\varepsilon = 12750$), it was utilised as the basis of an assay procedure in the present study. It was found that penicillic acid appeared as the principal culture filtrate metabolite after the third day of growth in submerged cultures (Table 3.1). As shown in Fig. 3.3, the fungus has the highest capability of penicillic acid biosynthesis between 3 and 4 days' growth.
This was confirmed by the rate of incorporation of (1-\(^{14}\text{C}\))-labelled acetate into penicillic acid using mycelia of varying age.

As shown in Fig. 3.2, the biosynthesis of penicillic acid in surface cultures starts around the fifth day with the maximum production occurring about 11 days after inoculation.

As previously mentioned (p. 14) the carbon skeleton of penicillic acid is derived from 4 acetate units, the O-methyl carbon corresponding to a C\(_1\) unit (ex-methionine or formate).\(^{79}\) (1-\(^{14}\text{C}\))-acetate and \(^{14}\text{C}\)-formate were incorporated with similar efficiencies (3.97% and 3.85% respectively).

The efficiency of incorporation of \(^{14}\text{C}\)-labelled acetate was observed to parallel approximately the rate of formation of penicillic acid (Fig. 3.3).
Fig. 3.3 Rate of penicillic acid formation during growth in submerged culture.

- Penicillic acid (mg/12 hrs.).
- Incorporation of [1-14C] labelled acetate into penicillic acid by fresh mycelia of submerged cultures at different ages.
Filtrate metabolites of P. cyclopium
acetate and \(^{14}C\)-formate into CHO-J-extracted culture

Fig. 3.4 Autoradiogram showing the incorporation of \(^{14}C\)-acetate.
The biosynthesis of penicillic acid via a 1,2-cleavage of orsellinic acid was suggested by Birch, following an investigation of the incorporation of \( (1-^{14}\text{C})-\text{acetate} \) (Scheme 3.1, path 'a'). However, chemical degradation of penicillic acid derived from \( [^{14}\text{C}-\text{carboxy and } 2-^{14}\text{C}] \)-orsellinic acid, led Mosbach to conclude that cleavage of the ring occurs between C(4) and C(5) (Scheme 3.1, path 'b').

\[ \text{Scheme 3.1 Alternative hypothetical cleavages of the aromatic ring of orsellinic acid(1).} \]
Further support for the Mosbach mechanism was provided by Al-Rawi et al.\(^2\) from the interpretation of \(^{3}\text{H}\)-n.m.r. spectra of penicillic acid obtained from tritiated acetate, which led to the suggested epoxide scheme (3.4, path b) for the biosynthesis of penicillic acid from orsellinic acid. The operation of the C(4) and C(5) cleavage of the ring followed from the presence of tritium on C(5). The observed selective partial exchange of tritium at C(5) of the hypothetical chiral intermediate (8), could result from the rapid enzyme-catalysed stereospecific isomerisation of (7) to (8), coupled together with a slow simultaneous non-enzymic reversible interconversion of these intermediates. This would effect some loss of tritium and in the reverse direction would yield a racemate of C(8). Seto et al.\(^{150}\) have also obtained evidence for a C(4)-C(5) cleavage of the aromatic ring based on \(^{13}\text{C}\) n.m.r spectral data of penicillic acid derived from (1,2-\(^{13}\text{C}\))-acetate. They observed spin-spin coupling between C(2) and C(3) as well as C(6) and C(7), which contrasts with the coupling required for a pathway involving C(1)-C(2) cleavage.
Scheme 3.2 Possible mode of incorporation of $^{13}$C-$^{13}$C doubly labelled acetate into penicillic acid (4).
Axberg and Gatenbeck\textsuperscript{119} on the basis of labelling studies suggested Scheme (3.3) for the biosynthesis of penicillic acid. This involves a Baeyer-Villiger type of oxidative ring opening reaction. The final step in this scheme requires a reduction of the product of Baeyer-Villiger reaction (5)\textsuperscript{151}.

\begin{center}
\begin{tikzpicture}
  \node (A) at (0,0) {\begin{tabular}{c}
    \textbf{Baeyer-Villiger} \\
    \textbf{oxidation}
  \end{tabular}};
  \node (B) at (2,0) {\begin{tabular}{c}
    \textbf{Reduction}
  \end{tabular}};
  \node (C) at (0,-2) {\begin{tabular}{c}
    \textbf{MeO} \\
    \textbf{Me}
  \end{tabular}};
  \node (D) at (2,-2) {\begin{tabular}{c}
    \textbf{MeO} \\
    \textbf{Me}
  \end{tabular}};
  \node (E) at (0.5,-1) {\begin{tabular}{c}
    \textbf{C(5)}
  \end{tabular}};
  \draw[->] (A) -- (B);
  \draw[->] (B) -- (C);
  \draw[->] (A) -- (E);
  \draw[->] (D) -- (E);
\end{tikzpicture}
\end{center}

Scheme 3.3 Baeyer-Villiger type of oxidative ring opening reaction (Axberg and Gatenbeck.\textsuperscript{119})

As shown, the partial loss of tritium on C(5) cannot be directly explained by a Baeyer-Villiger type of reaction. This together with the higher oxidation state of (5), necessitating a final reductive step, allows at least two possible
pathways based on the following epoxide Schemes (3.4; A and B) for the biosynthesis of penicillic acid. As indicated (page 99), the ease of oxidation of the quinol (2) to the related quinone (3), suggests that compound (3) may not be a true intermediate in the biosynthetic pathway of penicillic acid, since it is readily formed on aerial oxidation of 3-methoxy-2,5-toluquinol in media and on tlc-chromatography.

It has recently been demonstrated that phyllostine\textsuperscript{117} (10) is an intermediate in the formation of patulin (11). Its structural similarity to epoxydon (9), which has been isolated from \textit{Phyllosticta} species\textsuperscript{153}, would be consistent with the mediation of epoxydon in this biosynthetic pathway, as shown in Scheme (3.5). The aromatic ring cleavage involved in penicillic acid (4) formation and also that leading to patulin (11) are both unusual, in that they require a lower degree of oxidation than the majority of known oxidative ring scissions.\textsuperscript{113} This could support a mechanism such as that obtained in Scheme (3.4, A), involving cleavage through the hypothetical epoxide (6), which exhibits a close structural similarity to patulin-precursor epoxydon (9).
Scheme 3.4 Alternative epoxide schemes (A and B) for penicillic acid biosynthesis.\textsuperscript{152}

* Chiral carbon atoms
Scheme 3.5 Alternative epoxide schemes (A and B) for the biosynthesis of patulin.\textsuperscript{152}

* Chiral carbon atoms.
3.2 Inhibition studies
3.2.1 Introduction

Current knowledge of advanced stages involved in the biosynthesis of a wide range of secondary metabolites, suggests the feasibility of a rational approach to the strategic design of selective antimetabolites. In principal, this parallels the classical application of competitive inhibitors to the interception of primary metabolic pathways, such as the specific inhibition by fluorocitrate (12) of the citric acid cycle enzyme aconitase,\textsuperscript{154,155} the inhibition by sulfanilamide (14) of the biosynthesis of folic acid\textsuperscript{156} (16), and that of succinic dehydrogenase by malonate\textsuperscript{157} (17). This competitive inhibitory effect arises from the structural similarity of the inhibitor to a normal metabolite in the specific pathway under consideration (Fig. 3.5).

In a sequence involving cleavage of a carbon-carbon bond, cultivation of the parent organism, in the presence of suitably modified intermediates carrying additional substituents at either of the two key carbon atoms, could conceivably selectively inhibit the relevant enzyme responsible for this cleavage reaction. This is a predictable consequence of the competition between a normal intermediate and its analogue for an active site on the enzyme catalysing that specific step. When applied to secondary metabolic pathways, such antimetabolites need not necessarily inhibit any of the essential primary metabolic processes involved in growth and cell reproduction.
Inhibitor

\[
\begin{align*}
&\text{CH}_2 - \text{CO}_2\text{H} \\
&\text{CH(\text{OH})-CO}_2\text{H} \\
&\text{CHF - CO}_2\text{H}
\end{align*}
\]

(normal metabolites)

\[
\begin{align*}
&\text{CH}_2 - \text{CO}_2\text{H} \\
&\text{CH(\text{OH})-CO}_2\text{H} \\
&\text{CH}_2 - \text{CO}_2\text{H}
\end{align*}
\]

\[(12)\]

\[(13)\]

\[(14)\]

\[(15)\]

\[(16)\]

\[(17)\]

\[(18)\]
Some inhibitor candidate phenols.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>R_1</th>
<th>R_2</th>
<th>R_3</th>
<th>R_4</th>
<th>R_5</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Orsellinic</td>
<td>CO_2H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Me</td>
</tr>
<tr>
<td>(20) 5-Chloroorsellinic acid</td>
<td>CO_2H</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>Me</td>
</tr>
<tr>
<td>(21) 3-Chloroorsellinic acid</td>
<td>CO_2H</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>Me</td>
</tr>
<tr>
<td>(22) 2,5-Dichloroorsellinic acid</td>
<td>CO_2H</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>Me</td>
</tr>
<tr>
<td>(23) 3,5-Dibromoorsellinic acid</td>
<td>CO_2H</td>
<td>Br</td>
<td>H</td>
<td>Br</td>
<td>Me</td>
</tr>
<tr>
<td>(24) Orsellinic acid-4-methyl ether</td>
<td>CO_2H</td>
<td>H</td>
<td>Me</td>
<td>H</td>
<td>Me</td>
</tr>
<tr>
<td>(25) β-Resorcylic acid</td>
<td>CO_2H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>(26) 5-Chloro-β-resorcylic acid</td>
<td>CO_2H</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
</tr>
<tr>
<td>(27) Orcinol</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Me</td>
</tr>
<tr>
<td>(28) 2-Chloroorcinol</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>Me</td>
</tr>
<tr>
<td>(29) Orcinol-monomethyl ether</td>
<td>H</td>
<td>H</td>
<td>Me</td>
<td>H</td>
<td>Me</td>
</tr>
<tr>
<td>(30) Resorcinol</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>
In the present study, it has been demonstrated that the biosynthesis of penicillic acid is selectively inhibited by low concentrations of 5-chloroorsellinic acid (20), (Fig. 3.6).

Cultivation of *P. cyclopium* with 5-chloroorsellinic acid was found to stimulate the accumulation of 3-methoxy-2,5-toluquinone and the corresponding quinol, both of which have been reported as intermediates formed prior to cleavage of the aromatic ring. The inhibitory effect of the candidate inhibitors was also examined using an autoradiographic procedure previously applied to a study of the biosynthesis of penicillins from 14C- and 35S-labelled precursors. The selective nature of the inhibitory effect was clearly apparent on inspection of autoradiograms, which not only confirmed the inhibition of penicillic acid formation by 5-chloroorsellinic acid, but also detected the accumulation of other probable intermediates, including orsellinic acid, 3-methoxy-2,5-toluquinol and its related quinone.

3.2.2 Results and Discussion.

Cultivation of *P. cyclopium* was carried out in the presence of a variety of concentrations of 5-chloroorsellinic acid (20). The yield of penicillic acid, mycelial growth and also subsequent growth (i.e.; growth during to the period of cultivation in the presence of the inhibitor relative to the control), are illustrated graphically in Fig. 3.7(a). In this
case, the inhibitor was added after 30 hours growth, and the cultures were harvested after a total of 6 days incubation.

These results demonstrate the selective inhibition of penicillic acid biosynthesis under conditions which only partially restrict growth relative to that of the control. Thin-layer chromatograms obtained from chloroform extracts of acidified culture filtrates showed the inhibition of penicillic acid formation by 5-chloroorsellinic acid and also detected the concomitant accumulation of other metabolites; such as 3-methoxy-2,5-toluquinol and 3-methoxy-2,5-toluquinone. The maximum accumulation of these intermediates was observed at a concentration of $8.6 \times 10^{-4} \text{M}$ of 5-chloroorsellinic acid. Under these conditions no penicillic acid was obtained and the yield of the quinone was approximately 0.07 mg/ml of culture filtrate.

An autoradiographic approach was used to examine the metabolic activity in the presence of inhibitors. This technique was based on that previously applied to a study of the biosynthesis of penicillins from $^{14}\text{C}$- and $^{35}\text{S}$-labelled precursors. $^{158} 14\text{C}$-labelled acetate was utilised to demonstrate the formation of penicillic acid, since its carbon skeleton is known to be derived from four molecules of this precursor.

Fresh mycelium was grown (72 hours) in submerged culture and resuspended in water in the presence of 5-chloroorsellinic acid ($4.9 \times 10^{-4} \text{M}$), and (1-$^{14}\text{C}$)-acetate ($5 \mu\text{Ci}$/2ml). Autoradio-
Front

3-Methoxy-2,5-toluquinone

Penicillic acid

Orsellinic acid

$R_f = \text{Orcinol}$

Origin

Fig. 3.8  Autoradiogram showing the effect of orsellinic acid (OA) analogues on penicillic acid formation.

<table>
<thead>
<tr>
<th>No.</th>
<th>OA derivatives $^a$</th>
<th>Conc., Mx10$^{-4}$</th>
<th>Activity of CHCl$_3$ ext. $^b$ c.p.m. x10$^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OA</td>
<td>14.8</td>
<td>5.3</td>
</tr>
<tr>
<td>2</td>
<td>5-Chloro-OA</td>
<td>4.9</td>
<td>12.8</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>0</td>
<td>45.7</td>
</tr>
<tr>
<td>4</td>
<td>OA-4-methyl ether</td>
<td>1.37</td>
<td>23.2</td>
</tr>
</tbody>
</table>

a) Addition to resuspended *P. cyclopium* mycelium (72hr old submerged culture) plus (1-$^{14}$C)-acetate (6.66x10$^6$ cpm/2ml).

b) CHCl$_3$ extract of acidified filtrate (2ml) after 16hr. incubation.
Fig. 3.9 

Autoradiogram showing the effect of different concentrations of 5-chloroorsellinic acid on penicillic acid formation.

<table>
<thead>
<tr>
<th>No.</th>
<th>Concentration (Mx10^-4)</th>
<th>Activity of CHCl_3 ext. (c.p.m.x10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.08</td>
<td>9.3</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
<td>13.9</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>8.3</td>
</tr>
<tr>
<td>4</td>
<td>0.185</td>
<td>23.4</td>
</tr>
<tr>
<td>5</td>
<td>0.12</td>
<td>22.3</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>33.2</td>
</tr>
</tbody>
</table>
graphic comparison (Fig. 3.8) of chloroform extracts obtained from this culture and that of a control, clearly confirmed that 5-chloroorsellinic acid not only inhibits penicillic acid formation but also stimulates the accumulation of other known intermediates, namely orsellinic acid (1) and 3-methoxy-2,5-toluquinol (2) and the related quinone form. Continuation of the above experiment with lower levels of 5-chloroorsellinic acid concentration showed that the inhibitory effect is still apparent at 1.2x10^{-5}M (Fig. 3.9). As shown, the rate of accumulation of the precursors of penicillic acid [e.g. compounds (1), (2) and (3)] depends on the concentration of the inhibitor. The maximum yield of these metabolites occurs at the level of 3.08x10^{-4}M concentration when penicillic acid is no longer produced. This indicates the selective nature of the inhibitory effect of 5-chloroorsellinic acid on penicillic acid biosynthesis. The role of other components existing in chloroform extracts (e.g. compounds A, B and C) is still unknown. Since these compounds are present in all chloroform extracts they may well be unrelated to the biosynthesis of penicillic acid.

To determine whether 5-chloroorsellinic acid is converted to the chloro derivative of the intermediates of penicillic acid, the fungus was cultivated in the presence of (3-^{3}H)-5-chloroorsellinic acid. Thin-layer scanning of the chloroform extract of the culture medium only detected the presence of the corresponding chloroorcinol formed by loss of CO_{2}, in addition to unchanged starting material (40.7%). This decar-
Boxylation occurs spontaneously on incubating the chloroorsellinate with sterile medium adjusted to pH 3 for 5 days at 24°C, consequently it is unlikely that this reaction is catalysed by any of the enzymes involved in the normal biosynthetic pathway leading to penicillic acid. Similar results were obtained on incubating freshly grown mycelium with a low concentration of [3-\(^{3}\)H]-5-chloroorsellinic acid for 18 hours.

In a further experiment, the fungus was grown in the presence of 5-chloroorsellinic acid for periods of 60 hours to 4 days, when penicillic acid biosynthesis was completely inhibited. The resulting mycelium was subsequently used to prepare an autoradiogram which demonstrated its ability to form penicillic acid and also 3-methoxy-2,5-toluquinone (Fig. 3.10). This finding indicates the selective role of the inhibitor in blocking the active site of a corresponding orsellinic acid metabolising enzyme. This contrasts with the accumulation of intermediates by auxotrophic mutants, which is a consequence of the absence of genes responsible for the biosynthesis of individual enzymes in essential metabolic sequences. It was also observed that mycelium, grown in the presence of inhibitor concentrations which completely block penicillic acid formation, is able to resume production of this acid on resuspending in water in the absence of inhibitor.
Fig. 3.10 Autoradiogram showing the incorporation of (1-$^{14}$C)-acetate into penicillic acid with a culture previously grown in the presence of 5-chloroorsellinic acid.

The mycelium was cultivated in the presence of the inhibitor (8.6x10^-4 M) over the growth period 60-96 hr. prior to resuspending in aqueous $^{14}$C-acetate, but without further addition of 5-chloroorsellinate.
On the basis of the results of autoradiographic studies to determine the optimum concentration of inhibitor, an experiment was carried out to measure the percentage of acetate incorporation into 3-methoxy-2,5-toluquinone. In this experiment, the mycelium of a 50ml submerged culture (72 hours) was resuspended in a modified Raulin-Thom medium, and incubated with \([2-^{14}C]\)-acetate (200\(\mu\)Ci) in the presence of 5-chloro-OA; the resulting quinol was then oxidised to the corresponding quinone. The percentage of incorporation of acetate and the specific activity of the quinone were found to be 7.14% and 6.48\(\mu\)Ci/mg respectively.

Since a secondary metabolite is not normally considered to play a direct role in the growth of the parent organism, this parameter is not likely to be directly influenced by the presence of a specific inhibitor of such a secondary metabolite. However, in these studies, some inhibition of growth in addition to secondary metabolite formation was frequently observed at higher concentrations, which is probably a reflection of a type of general or non-specific inhibition common to many phenols. As shown in Fig. (3.7a) 5-chloroorsellinic acid at the level of \(2.05 \times 10^{-4}\)M which reduces penicillic acid formation to 64%, shows only a negligible effect on fungal growth. The inhibitory effect of 5-chloroorsellinic acid was examined at an early stage of growth by incorporating the acid in the culture media prior to inoculation with \(P\. cyclopium\). Both mycelial weight and penicillic acid production were partially reduced (Fig. 3.11a) relative to the experiments with established cultures (Fig. 3.7a).
Fig. 3.11(a) Influence of 5-chloroorsellinic acid on mycelial growth and penicillic acid production. (Inhibitor was added at the time of inoculation and mycelia harvested after 7 days' growth).

Fig. 3.11(b) Influence of orsellinic acid on mycelial growth and penicillic acid production cf. (a).
Fig. 3.12 Comparison of the production of penicilllic acid in the presence of 5-chloroorsellinic acid and orsellinic acid. (cf. Fig. 3.11).
This indicated a more pronounced general inhibition type of behaviour, although the relatively greater effect on penicillic acid yields demonstrated some selective inhibition of the biosynthetic pathway (Fig. 3.12).

A corresponding series of experiments designed to examine the effect of orsellinic acid addition, both at the time of inoculation and to established cultures (Fig. 3.7b 3.11b), showed that this phenolic acid also behaves as a general inhibitor at relatively high concentrations. At first sight this may appear surprising since orsellinic acid is an apparent obligatory precursor of penicillic acid, however in view of the previous observations, this is probably simply another instance of the general inhibitory effect of phenolic substances. It is conceivable that feed-back control mechanisms may also contribute to the inactivation of earlier enzymes involved in this biosynthetic sequence. Another potentially relevant inhibitory effect has been demonstrated for a variety of enzymes which have been shown to be inhibited by their own substrate at higher concentrations, for example the influence of pyruvate on lactic dehydrogenase.\textsuperscript{159}

Cultivation of the fungus with orsellinic acid-4-methyl ether showed this simple derivative to be a relatively potent inhibitor. The result of feeding experiments over a range of concentrations between $0.91 \times 10^{-4}$M to $4.5 \times 10^{-4}$M are presented in Fig. 3.7d. At a concentration of only
1.8 \times 10^{-4} \text{M} \text{ the methyl ether can reduce penicillic acid formation by 50\%, however in contrast to 5-chloroorsellinic acid this inhibitor exerted an equally pronounced inhibition of mycelial growth. This result is consequently more characteristic of general rather than selective inhibition, the reduced production of penicillic acid reflecting the lower rate of growth and enzyme synthesis. A corresponding autoradiogram showed that the inhibition of penicillic acid formation occurred without any apparent accumulation of the previously - observed advanced intermediates (Fig. 3.8). An interesting aspect of this finding, is that orsellinic acid 4-methyl ether occurs naturally in an esterified form in lichens as the depside evernic acid.}^{160}

\[
\begin{align*}
\text{MeO} & \quad \text{Me} \\
\text{OH} & \quad \text{Me} \\
\text{COO} & \quad \text{COOH}
\end{align*}
\]

Evernic acid
3-Chloroorsellinic acid was also fed to the growing culture at various concentrations. The results of this experiment (Fig. 3.7c) shows that this compound only appears to exert a general inhibitory effect on fungal growth at higher concentrations. At the level which with 5-chloroorsellinic acid reduces penicillic acid formation by 50%, 3-chloroorsellinic acid exerts a comparatively negligible effect, reducing the yield by only 5%. An autoradiogram showing the effect of this inhibitor on the formation of penicillic acid is shown in Fig. 3.16 (appendix).

The results shown in Tables 3.4, 3.5 and 3.10 (appendix) indicated that 5-chloroorsellinic acid and the 3-chloro isomer exhibit slightly higher inhibitory effects than some other phenolic compounds, including β-resorcylic acid, resorcinol, and orcinol, possibly reflecting a specific effect of the chlorine atom. Autoradiograms obtained on incubation of the mycelium with β-resorcylic acid, 5-chloro-β-resorcylic acid and 3-chloroorsellinic acid are shown in Fig. 3.16 (p.137). The results showed that 5-chloro-β-resorcylic acid effected a greater inhibition of penicillic acid formation than β-resorcylic acid, but the effect was less pronounced than that of 5-chloroorsellinic acid which under these conditions resulted in ~90% inhibition. As also shown in these autoradiograms, 5-chloroorsellinic acid alone stimulated the accumulation of 3-methoxy-2,5-toluquinone.
Further investigation of the effect of halogen substituents on fungal growth was carried out using 3,5-dibromoorsellinic acid. As shown in Table 3.10 (appendix) pronounced inhibition of growth was observed; thus the fungus was not able to grow when cultivated at a concentration of $2.56 \times 10^{-4}$M of the dibromo compound. Dibromoorscinol was recovered from chloroform extracts of the acidified media used in these experiments, which shows the ease of decarboxylation of dibromoorsellinic acid similar to the effect previously observed with $[3^{-3}H]$-5-chloroorsellinic acid (page 121).

A general inhibitory effect of 3,5-dichloroorsellinic acid was also observed under standard autoradiographic conditions (Fig. 3.18, p.139). At a concentration of $1.05 \times 10^{-5}$M, penicillic acid biosynthesis was reduced to 56% as estimated by uptake of $^{14}$C-acetate; with this inhibitor no accumulation of the biosynthetic intermediates was observed. A comparable effect was also observed with 2-chloroorcinol at a level which with orcinol showed no significant effect, indicating that the chlorine substituent is responsible for the inhibitory effect activity (Fig. 3.17; appendix).

Orcinol monomethyl ether and orcinol were also fed to growing cultures of *P. cyclopium*. The results (Table 3.10, p.145) show that where orcinol exerts slight, non-specific inhibitory activity (i.e. growth and penicillic acid production) at $1.34 \times 10^{-3}$M, orcinol monomethyl ether profoundly inhibits the biosynthesis of penicillic acid, the effect on growth being
less pronounced. These results were also confirmed by autoradiographic studies (Fig. 3.17; appendix).

Typical results of the relative inhibitory effects of low concentrations of selected orsellinic acid derivatives on the metabolism of \textit{P. cyclopium} are demonstrated in Table 3.3. While 5-chloroorsellinic acid effectively blocks penicillic acid formation at levels which only partially reduce mycelial growth, orsellinic acid 4-methyl ether inhibits both growth and penicillic acid formation at appreciably lower concentrations. In contrast, 3-chloroorsellinic acid exhibits only slight inhibitory activity. This is consistent with the idea that interference with the normal orsellinic acid substituents at carbon atoms 4 and 5 (i.e. OH and H respectively) may selectively inhibit the C(4)-C(5) cleavage involved in the pathway leading from orsellinic acid to penicillic acid.

The selective nature of the inhibitory effect of low concentrations of 5-chloroorsellinic acid is clearly apparent from the results shown in the composite autoradiogram (Fig. 3.13), which also demonstrated the concomitant accumulation of other metabolites (i.e., 3-methoxy-2,5-toluquinol, the corresponding quinone, and orsellinic acid), the yields of which were dependent upon the concentration of the inhibitor, (cf. A\textsubscript{1} and B\textsubscript{1}).
<table>
<thead>
<tr>
<th>Orsellinic acid derivative</th>
<th>Concn. $^{\text{b}}$</th>
<th>Mycelial wt. $^{\text{c}}$</th>
<th>Penicillic acid yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mx10$^{-4}$</td>
<td>mg/flask</td>
<td>% growth</td>
</tr>
<tr>
<td>Orsellinic acid (OA)</td>
<td>0</td>
<td>542</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>563</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>560</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>9.9</td>
<td>561</td>
<td>104</td>
</tr>
<tr>
<td>5-Chloro-OA</td>
<td>0</td>
<td>475</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td>462</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>4.1</td>
<td>396</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
<td>320</td>
<td>67</td>
</tr>
<tr>
<td>3-Chloro-OA</td>
<td>0</td>
<td>470</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td>438</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>4.1</td>
<td>465</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
<td>370</td>
<td>79</td>
</tr>
<tr>
<td>OA-4-methyl ether</td>
<td>0</td>
<td>369</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>339</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>287</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td>191</td>
<td>52</td>
</tr>
</tbody>
</table>

Table 3.3 Influence of orsellinic acid analogues on mycelial growth and penicillic acid production of *P. cyclophium*.

(a) Addition to culture after 30 hr. growth at 24°C.
(b) Initial concentration of orsellinic acid and its analogues.
(c) Mycelial weight 6 days after inoculation (average of duplicate flasks).
Fig. 3.13 Autoradiograms showing the influence of 5-chloroorsellinic acid on penicillic acid formation. The effect of incubating \textit{P. cyclopium} mycelium with (\textsuperscript{1-\textsuperscript{14}}C)-acetate in the presence of 5-chloroorsellinic acid at 2.47\times10^{-4}M (A\textsubscript{1}) and 4.90\times10^{-4}M (B\textsubscript{1}) is shown relative to controls obtained with H\textsubscript{2}O (A\textsubscript{2} and B\textsubscript{2}).
Conclusion

The finding that penicillic acid accumulation is inhibited at sub-lethal concentrations of 5-chloroorsellinic acid, supports the concept that this derivative of orsellinic acid selectively combines with an active site on the enzyme which catalyses cleavage of the aromatic ring. It also supports the view that penicillic acid is a secondary metabolite which is not directly essential to cell growth and reproduction. In addition, it demonstrates the feasibility of this strategy for designing selective inhibitors of secondary metabolism based on a knowledge of the advanced stages in a biosynthetic sequence.

In respect of the enhanced formation of advanced intermediates which occurs in the presence of the 5-chloroorsellinic acid, this corresponding characteristic has been frequently observed in studies with auxotrophic mutants. Here the accumulation of an intermediate in a particular pathway is typically a consequence of the deletion of genes responsible for the synthesis of specific enzymes in the normal metabolic sequence. In the present context, the primary effect of the inhibitor would not be expected to influence the biosynthesis of the cleavage enzyme, but rather serve as an antimetabolite which competes for access with the normal substrate for its active site.
3.2.3 Appendix
Fig. 3.14  Autoradiogram of culture filtrate extracts of resuspended *P. cylopium* mycelium (72hr growth).

(1) Czapek-Dox medium.

(2) Raulin-Thom medium.
Fig. 3.15  Autoradiogram showing the uptake of acetate by mycelium of *P. cyclopium*, at different times of incubation in the presence of 5-chloroorsellinic acid (2.47x10^-4 M).
Fig. 3.16 Influence of some phenolic compounds on resuspended (cf. p.128) mycelium (72hrs.) of P. cyclopium.

<table>
<thead>
<tr>
<th>No.</th>
<th>Phenolic compound</th>
<th>Concentration (M x 10^-4)</th>
<th>CHCl3 ext. (c.p.m. x 10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-Chloro-OA</td>
<td>2.47</td>
<td>19.6</td>
</tr>
<tr>
<td>2</td>
<td>3-Chloro-OA</td>
<td>6.17</td>
<td>3.2</td>
</tr>
<tr>
<td>3</td>
<td>Control (1)</td>
<td>0</td>
<td>30.3</td>
</tr>
<tr>
<td>4</td>
<td>Control (2)</td>
<td>0</td>
<td>32.3</td>
</tr>
<tr>
<td>5</td>
<td>5-Chloro-OA</td>
<td>2.47</td>
<td>12.0</td>
</tr>
<tr>
<td>6</td>
<td>Orcinol</td>
<td>4.0</td>
<td>34.6</td>
</tr>
<tr>
<td>7</td>
<td>β-Resorcylic acid</td>
<td>3.2</td>
<td>31.8</td>
</tr>
<tr>
<td>8</td>
<td>5-Chloro-β-resorcylic acid</td>
<td>2.6</td>
<td>24.0</td>
</tr>
</tbody>
</table>
Fig. 3.17 Autoradiogram showing the effect of orcinol and (cf. p. 129) its derivatives on penicillic acid production.

<table>
<thead>
<tr>
<th>No.</th>
<th>Derivatives</th>
<th>Concentration (M x 10^{-4})</th>
<th>Activity of CHCl₃ ext. (c.p.m.) x 10^{-4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Orcinol monomethyl ether</td>
<td>0.72</td>
<td>3.6</td>
</tr>
<tr>
<td>2</td>
<td>Orcinol</td>
<td>1.61</td>
<td>45.3</td>
</tr>
<tr>
<td>3</td>
<td>2-Chloro orcinol</td>
<td>1.89</td>
<td>12.6</td>
</tr>
<tr>
<td>4</td>
<td>2-Chloroorcinol</td>
<td>1.26</td>
<td>16.0</td>
</tr>
<tr>
<td>5</td>
<td>2-Chloroorcinol</td>
<td>0.63</td>
<td>29.1</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>0</td>
<td>34.2</td>
</tr>
</tbody>
</table>
Fig. 3.18 Autoradiogram showing the influence of 3,5-dichloroorsellinic acid (DCOA) on resuspended mycelium of \textit{P. cyclopium}.

<table>
<thead>
<tr>
<th>No.</th>
<th>Conc. of DCOA (Mx10^{-4})</th>
<th>CHCl$_3$ ext. (c.p.m.)x10^{-4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.05</td>
<td>20.9</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>37.4</td>
</tr>
<tr>
<td>Concentration (mg/l)</td>
<td>% Control</td>
<td>% Growth</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------</td>
<td>----------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.2</td>
<td>0</td>
</tr>
<tr>
<td>2.1</td>
<td>6.2</td>
<td>0</td>
</tr>
<tr>
<td>4.1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>4.2</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>6.6</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>10.4</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td>% mycelial wt.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2.4 Influence of 5-phosphoroseltic acid on mycelial growth and penicilllic acid production by P. cepacia (a) % growth = % change in mycelial weight 6 days after inoculation. (b) % Growth E. coli = % growth on E. coli lawn. (c) % Growth P. aeruginosa = % growth on P. aeruginosa lawn. (d) % Penicilllic acid = % of duplicate flasks. (e) Δ Growth = % change in mycelial weight 3 days after inoculation.
### Table 3.5: Influence of 3-chloroorsellinic acid on mycelial growth and penicillin production by *P. chrysogenum* (addition to culture after 30 h, 100 µg/ml)

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% Growth</th>
<th>Δ Growth</th>
<th>Penicillin acid (mg/flask)</th>
<th>% Growth</th>
<th>Penicillin acid (mg/flask)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.4</td>
<td>70</td>
<td>37</td>
<td>19</td>
<td>370</td>
<td>79</td>
</tr>
<tr>
<td>46.9</td>
<td>44</td>
<td>31.5</td>
<td>315</td>
<td>465</td>
<td>65</td>
</tr>
<tr>
<td>100</td>
<td>88</td>
<td>31.5</td>
<td>99</td>
<td>288</td>
<td>12.5</td>
</tr>
<tr>
<td>3.0</td>
<td>100</td>
<td>30.5</td>
<td>10</td>
<td>436</td>
<td>6</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>30.5</td>
<td>0</td>
<td>436</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>436</td>
<td>0</td>
</tr>
</tbody>
</table>

*Notes:* 
- Δ = subsequent growth.
- % Growth = weight 6 days after inoculation.
- Mycelial weight 6 days after inoculation.
- Initial concentration of 3-chloroorsellinic acid.
\[ \text{percentage of subsequent growth relative to control} = \nabla \% \text{ (d) } \]
\[ \nabla = \text{ subsequent growth of duplicate flasks (averages) } \]

Myxetall wet weight 6 days after inoculation 

(a) Initial concentration of ascertainment acid 

(b) Myxetall after 30 hrs. growth (addition of p.126 penticillin acid production by P. cyclophorum) 

<table>
<thead>
<tr>
<th>% control</th>
<th>84</th>
<th>28.5</th>
<th>105</th>
<th>391</th>
<th>104</th>
<th>561</th>
<th>9.9</th>
<th>5.0</th>
<th>2.5</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>102</td>
<td>39.0</td>
<td>105</td>
<td>390</td>
<td>103</td>
<td>560</td>
<td>5.0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>104</td>
<td>39.5</td>
<td>106</td>
<td>395</td>
<td>104</td>
<td>563</td>
<td>5.5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>34.0</td>
<td>100</td>
<td>372</td>
<td>100</td>
<td>542</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.6: Influence of ascertainment acid on myxetall growth and P. cyclophorum growth (mg/laker)
### Table 3.7

<table>
<thead>
<tr>
<th>% Control</th>
<th>% Growth</th>
<th>% Growth</th>
<th>% Growth</th>
<th>% Growth</th>
<th>% Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>11</td>
<td>21</td>
<td>52</td>
<td>191</td>
</tr>
<tr>
<td>69</td>
<td>12.5</td>
<td>59</td>
<td>117</td>
<td>78</td>
<td>287</td>
</tr>
<tr>
<td>75</td>
<td>0.0</td>
<td>65</td>
<td>85</td>
<td>92</td>
<td>379</td>
</tr>
<tr>
<td>100</td>
<td>25.5</td>
<td>100</td>
<td>100</td>
<td>369</td>
<td>0</td>
</tr>
</tbody>
</table>

\( \text{Initial concentration of orsellinic acid-4-methyl ether} \)
\( \text{Mycelial weight 6 days after inoculation (average of duplicate flasks).} \)
\( \text{Subsequent growth.} \)
\( \% \text{ k.} = \text{percentage of subsequent growth relative to control.} \)

\[ \text{Table 3.7 Influence of orsellinic acid-4-methyl ether on mycelial growth and pentitollic acid production by P. nivosum (addition to culture after 30 hrs. growth).} \]
Table 3.8 Influence of 5-chloroorsellinic acid on mycelial growth and penicillic acid production (addition to cultures at the time of inoculation). cf. p.124

<table>
<thead>
<tr>
<th>Concn.(^a) Mx10(^{-4})</th>
<th>Mycelial wt.(^b) mg/flask</th>
<th>% growth</th>
<th>Penicillic acid % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1039</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2.1</td>
<td>880</td>
<td>85</td>
<td>60</td>
</tr>
<tr>
<td>4.1</td>
<td>825</td>
<td>79</td>
<td>20</td>
</tr>
<tr>
<td>8.2</td>
<td>316</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.9 Influence of orsellinic acid on mycelial growth and penicillic acid production (addition to culture at the time of inoculation). cf. p.124

For both tables  
\(a\) = initial concentration of orsellinic acid or its 5-chloro derivative.

\(b\) = Mycelia harvested after 7 days' growth.
<table>
<thead>
<tr>
<th>phenolic compound&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Conc.&lt;sup&gt;b&lt;/sup&gt; (M&lt;sub&gt;x&lt;/sub&gt;10&lt;sup&gt;-4&lt;/sup&gt;)</th>
<th>Mycelial wt.&lt;sup&gt;b&lt;/sup&gt; (mg/flask)</th>
<th>% growth</th>
<th>CHCl&lt;sub&gt;3&lt;/sub&gt; extract&lt;sup&gt;b&lt;/sup&gt; (mg/flask)</th>
<th>Penicillic acid&lt;sup&gt;c&lt;/sup&gt; (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resorcylic acid</td>
<td>0</td>
<td>960</td>
<td>100</td>
<td>79.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>910</td>
<td>94</td>
<td>71.2</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>10.8</td>
<td>806</td>
<td>84</td>
<td>67.1</td>
<td>80</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>0</td>
<td>985</td>
<td>100</td>
<td>81.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>945</td>
<td>96</td>
<td>81.8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>827</td>
<td>84</td>
<td>61.7</td>
<td>80</td>
</tr>
<tr>
<td>Resorcinol-onononononono-ethyl ether</td>
<td>0</td>
<td>864</td>
<td>100</td>
<td>90.7</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>13.4</td>
<td>823</td>
<td>95</td>
<td>86.5</td>
<td>90</td>
</tr>
<tr>
<td>3,5-dibromoorsellinic acid</td>
<td>0</td>
<td>1097</td>
<td>100</td>
<td>71.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>790</td>
<td>72</td>
<td>69.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2.6</td>
<td>641</td>
<td>58</td>
<td>16.3</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>481</td>
<td>44</td>
<td>7.6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5.1</td>
<td>30</td>
<td>3</td>
<td>6.5</td>
<td>0</td>
</tr>
</tbody>
</table>

Influence of some phenolic compounds on mycelial growth and penicillic acid production.

(a) Addition to spore-inoculated submerged culture after 24 hr. growth.

(b) Cultures were harvested 7 days after inoculation.

(c) % Penicillic acid on the basis of t.l.c.
Table 3.1: Influence of Penicillopeptone on mycelial growth, yield of penicillic acid, and pH.

<table>
<thead>
<tr>
<th>pH (av.)</th>
<th>Penicillopeptone (mg/kg)</th>
<th>Medium growth</th>
<th>Mycelial wt. (mg/flask)</th>
<th>Penicillic acid yield (mg/flask)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: All data points are based on average values from 3 replicates.

Control conditions provided in parentheses (C).
**Fig. 3.19** Visible spectrum of a mixture of glucose and glucose oxidase reagent after incubation (15 min./37°C).

**Fig. 3.20** Plot of optical density at 435nm against concentration of glucose.
Fig. 3.21 Plot of optical density at 254nm against (cf. p.158) concentration of orsellinic acid.
Fig. 3.22 Plot of optical density at 264nm against (cf. p.158) concentration of 3-methoxy-2,5-toluquinone.
Fig. 3.23 Plot of optical density at 225nm against (cf. p.157) concentration of penicilliac acid.
3.2.4 Experimental
3.2.4 Experimental

3.2.4.1 Preparation and Inoculation of the Solid Medium (PDA)

The solid medium contained potato extract (prepared by boiling potato slices (200g) in water (1.01) for 45 mins.), 2% glucose, and 2% agar. Portions of hot medium (10ml) were poured into the culture tubes and autoclaved (at 15 lbs/sq.in. pressure for 15 mins.). The culture tubes were then allowed to cool on a sloped surface.

Previously prepared slopes were inoculated from a sporing slope of *P. cyclopium* (IMI, 89372, obtained from the Commonwealth Mycological Institute, Kew, Surrey) by the transfer of spores on a wire in sterile surroundings; slopes were then incubated in a stationary incubator for seven days at 24°C.

3.2.4.2 Preparation and Inoculation of the Liquid Media

Composition of the media are as follows:

I) Raulin Thom medium; D(+) glucose (anhydrous, 50g), tartaric acid (2.67g), ammonium tartrate (2.67g), magnesium carbonate (0.27g), zinc sulphate (hepta-hydrate, 0.047g), ferrous sulphate (hepta-hydrate, 0.047g), ammonium sulphate (0.17g), potassium carbonate (0.4g), and diammonium hydrogen phosphate (0.4g) are dissolved in distilled water (1l). The initial pH of the medium is 4.0.
II) Czapek-Dox Medium; D(+) -glucose (anhydrous, 50g), sodium nitrate (2.0g), potassium chloride (0.5g), magnesium sulphate (hepta-dydrate, 0.5g), ferrous sulphate (hepta-hydrate, 0.01g) and potassium dihydrogen phosphate (1.0g) are dissolved in distilled water (1.0l). The initial pH of the medium is 6.

In either case, 350ml and 60ml of media were placed into 1l, and 250ml conical flasks respectively. The flasks were then plugged with non-absorbent cotton and sterilised by autoclaving at 15 lbs/sq.in. pressure for 20 minutes. The big flasks were provided for surface, and small flasks for submerged cultures.

Sterile solution of Tween 80 (0.01%, 10ml) was poured into a heavily sporing slope and the surface of the slope was scratched with a wire. An aliquot (3ml) of the spore suspension was then poured into the 1l flasks under sterile conditions and kept in a stationary incubator at 24°C. An aliquot (1ml) of the spore suspension was used for submerged cultures and kept in an orbital incubator (240 r.p.m.) at 24°C.

Large variations in mycelial growth were observed in the submerged cultures. The following method was therefore used for inoculation; a submerged culture was prepared in the same way as the above cultures. After 30 hrs. of growth, 2ml of the mixed young mycelium and media was used for inoculation of a new flask.
3.2.4.3 Course of Fermentation of P. cyclopium

Submerged cultures were harvested in varying time of incubation between 1 and 8 days, and the following measurements were carried out (the same procedure was followed for surface cultures harvested in the range of 3 to 15 days growth);

I) Mycelial weight

Mycelium was pressed dry on a Buchner funnel and then dried in a vacuum desiccator over fresh phosphorus pentoxide for 2 days.

II) Measuring of pH

The pH of the culture filtrates was determined using an E.I.L.-23A pH meter, against standard buffers of pH 4 at 20°C.

III) Measuring of glucose

Glucose concentration of the filtrates was determined by the following methods:

a) Spectrophotometric method - the technique is based on enzymatic oxidation of glucose to gluconate. In this reaction, the hydrogen peroxide obtained is treated with diammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) to give a coloured complex.

In practice, a standard solution (0.1ml) of glucose (0.455mg /100ml) was placed in a test tube, and a mixture of phosphate buffer, enzyme and chromogen (5ml, pH = 7) was then added. The reaction mixture was kept in a water bath.
(37°C) for 15 minutes, and an aqueous solution of H₂SO₄ (20%, 2ml) was then added with shaking the mixture. The optical density of this solution was measured in varying wavelengths ranging from 370-600nm. Each time the absorbency of the blank solution was adjusted at zero (this solution contained the standard contents except glucose; and the volume was made up with adding water (0.1ml). The typical spectrum is shown in Fig. 3.19 (p.147). According to this experiment λ_max of the reaction mixture was found to stand at 435nm. This wavelength was used for measuring a series of standards and samples of glucose. The calibration curve is shown in Fig. 3.20 (p.147).

b) In this method rotation (α) of polarised sodium light in diluted metabolism solutions (5ml of 5 times diluted filtrates) was measured by a polarimeter. A related concentration of glucose was obtained from a calibration curve of the glucose standards ranging from 0.2 to 1.0g/100ml. Since some of the metabolism solutions were heavily coloured as well as having a low glucose content, this method was not considered successful for these studies.

(IV) Chloroform extracts of the filtrate - the efficiency of extraction of the fungal metabolites from Raulin-Thom medium was determined as follows:
Amount of the fungal metabolites added to 60ml of Raulin Thom medium 61.2mg.

pH of the medium 2

Volume of chloroform used for first extraction 40ml.

Volume of chloroform used for second extraction 40ml

Weight of the residual of the first extraction 51.6mg

Weight of the residual of the second extraction 4.7mg

Total of the fungal metabolites extracted 56.3mg

The efficiency of extraction \[
\frac{56.3 \times 100}{61.2} = 92\%
\]

3.2.4.4 Detection of the Metabolites

Standard technique of thin-layer chromatography on silica gel GF 254 (0.25mm thick) was used for a preliminary detection of the metabolites in chloroform extracts of culture filtrates (at pH 2) and acetone extract of mycelium. This was carried out by using different solvent systems such as:

A) organic phase of the mixture of \( \text{CHCl}_3; \text{CH}_2\text{OH}; \text{H}_2\text{O}; \text{HCOOH} \) \((250:24:25:1 \text{ v/v})\) \(^{119}\)

B) mixture of \( \text{CHCl}_3; \text{CH}_3\text{COOEt}; \text{HCOOH} \) \((60:40:1 \text{ v/v})\) \(^{162}\)

C) mixture of \( \text{CHCl}_3; \text{C}_2\text{H}_5\text{OH} \) \((19:1 \text{ v/v})\) \(^{163}\)
The following instruments were used for analysis of the metabolites:

Uv - spectrophotometer, Unicam Sp-8000.
Ir - spectrophotometer, Perkin-Elmer 157G.
Nmr - spectrometer, Perkin-Elmer R-24A.
Mass spectrometer MS-12.

3.2.4.5 Isolation of the Metabolites

This was carried out by preparative chromatography using silica gel plate (1mm thickened). The zones which appeared on preparative chromatogram were eluated as follows;

The zone scraped was placed in a small column (1 x 20 cm) and the metabolite eluated with a flow of methanol (10ml). The collected solution was then evaporated under a reduced pressure to dryness residue, and it used for further investigations.

Penicillic acid:

Since uv-spectrum of penicillic acid in ethanol gives a distinguishable peak at 225nm, the following method was used for estimation of this acid;

Chloroform extracted residue of the filtrate was dissolved in ethanol and its absorbency measured against ethanol (as blank) at 225nm. The corresponding concentration was then obtained from calibration curve (Fig. 3.23 appendix).
Isolation of penicillic acid was carried out from 11 days old surface and 7 days old submerged cultures of P. cyclopium on Raulin-Thom. In these cases the combined chloroform extracts of the metabolism solution was evaporated under reduced pressure, and the residue recrystallised from chloroform-cyclohexane mixture (1:3). Penicillic acid obtained as a white crystalline solid m.p. 83-84°C.

Orsellinic acid:

The orsellinic acid zone on preparative t.l.c (chloroform extracted residue of 3 days old submerged culture) was scraped and eluated with methanol. Optical density of this solution was then measured at 254 nm, and the corresponding concentration was deduced from calibration curve (Fig. 3.21, p.148) of orsellinic acid.

3-methoxy-2,5-toluquinol and 3-methoxy-2,5-toluquinone:

These compounds were also detected in the chloroform extracted residue of submerged cultures (3 days old) on t.l.c. plate using solvent A. The metabolites were isolated by preparative thin-layer chromatography similar to the above method. 3-methoxy-2,5-toluquinol was crystallised from cold carbon tetrachloride (white crystals, m.p. 131-132°C lit.147 130-131°C). The crystallization of 3-methoxy-2,5-toluquinone in the same solvent yielded yellow needle form crystals (m.p. 155-156°C, lit.119 155-156°C).
3.2.4.6 Course of fermentation of P.cyclopium in the presence of potential inhibitors.

In these experiments the inhibitor was added after 30 hrs. to $F_2$ (i.e. second stage) submerged cultures. The $F_2$ cultures were obtained following inoculation with an initial $F_1$ culture previously grown for 30 hrs. The submerged cultures were harvested after a total growth period of 7 days. For trial experiments the feeding compounds were added to $F_1$ cultures at the time of inoculation and also 24 hrs. old cultures.

3.2.4.7 Autoradiographic studies

a) Control culture; the fresh mycelium (1g) was incubated with $[1^{14}\text{C}]-$sodium acetate (5$\mu$Ci) in sterile water (2ml) for 16 hrs. The culture was then harvested and the filtrate extracted with two 4ml portions of chloroform (at pH 2). The combined chloroform extract was evaporated under reduced pressure, and the residue obtained used for providing a thin-layer chromatogram. An X-ray film (Kodak; cat. No. 3056207) was exposed with a thin-layer chromatogram for 48 hrs., and then developed.

b) In the presence of potential inhibitors; the above procedure was repeated when the mycelium was incubated in different concentrations of potential inhibitors.

The X-ray films were processed by a standard procedure using recommended solutions for developing and fixing.
List of Schemes, Figures and Tables

Scheme 1.1 C(4)-C(5) cleavage of orsellinic acid leading to the biosynthesis of penicillic acid ............ 3

Table 1.1 Biosynthetic classification of secondary metabolites ............................................. 5

Scheme 1.2 Alternative synthetic routes to orcinol .......... 8

Scheme 1.3 Robinson's hypothesis for the biosynthesis of orsellinic acid .......................... 8

Scheme 1.4 Biosynthesis of modified phenolic polyketides.... 11

Scheme 1.5 Head-to-tail linkage of seven acetate units leading to the formation of alternariol and griseofulvin ................................................ 12

Scheme 1.6 Biosynthesis of orsellinic acid from $^{18}O$-labelled acetate .......................... 13

Scheme 1.7 Pathway of penicillic acid formation from one acetate and three malonate units .......... 14

Scheme 1.8 Possible stereospecificity of polyenolate formation ........................................... 16

Scheme 1.9 Alternative pathways for the cyclisation of polyketides ....................................... 17

Scheme 1.10 Cyclisation of the phenalenone precursor.......... 17

Scheme 1.11 Alternative biosynthetic pathways of citromycetin via (a) linear polyketide chain (b) branched chain intermediate.......... 18

Fig. 1.1 Naturally occurring or analogues of orsellinic acid ........................................... 23
Scheme 1.12 Hypothetical scheme for the synthesis of 6-methyl salicylic acid via a multi-enzyme complex .................................................................. 25

Fig. 1.2 Some tetronic acids and structurally related compounds ................................................. 29

Scheme 1.13 Possible biosynthetic pathways for the formation of tetronic acids from the $\text{C}_4$-dicarboxylic acid, malic acid ................................................................. 32

Scheme 1.14 Reaction between penicilllic acid and RSH, when RSH is cysteine or glutathione .......... 33

Scheme 1.15 Chemical degradation of penicilllic acid ....... 34

Scheme 1.16 Raphael's synthetic route for the preparation of penicilllic acid .................................. 36

Scheme 1.17 Biosynthesis of penicilllic acid via alternative ring cleavages; 1,2-cleavage and 4,5-cleavage ................................................................. 38

Scheme 1.18 Alternative schemes for the biosynthesis of patulin .................................................. 40

Scheme 2.1 Synthesis of orsellinonic acid (Gaucher and Shepherd) .............................................. 48

Scheme 2.2 The condensation of ethyl acetoacetate and ethyl crotonate ........................................ 50

Fig. 2.3 Some possible keto-enol tautomers of ethyl dihydroorsellinate ....................................... 51
Table 2.1 Chromatographic comparison of intermediates in the synthesis of orsellinic acid .......... 51
Table 2.2 Microanalysis of orsellinic acid intermediates .............................................. 52
Table 2.3 Mass spectral data of orsellinic acid intermediates ........................................ 53
Table 2.4 $^1$H nmr spectral data (CDCl$_3$) of ethyl dibromo-orsellinate ............................ 54
Table 2.5 $^1$H nmr spectrum (CDCl$_3$) of dibromoorsellinic acid ...................................... 54
Table 2.6 $^1$Hnmr spectral data ($d_6$-acetone) of orsellinic acid ........................................ 55
Fig. 2.4 Alternative pathways proposed for the synthesis of orsellinic acid ......................... 56
Table 2.7 $^1$H nmr spectrum of compound B ................................................................. 58
Table 2.8 Some characteristic of compound C and ethyl orsellinate .................................... 59
Table 2.9 $^1$H nmr spectral data of compound C and ethyl orsellinate .................................. 60
Fig. 2.5 Probable bromination sequence for ethyl dihydroorsellinate (in preparation of orsellinic acid) .............................................................. 62
Table 2.10 $^1$H nmr spectral data for $\alpha$-bromocrotonate ............................................. 63
Fig. 2.6 N.m.r. spectra of [3-$^3$H]-5-chloorsellinic acid .................................................. 65
Table 2.11 Synthesised potential inhibitors of penicillic acid ........................................ 66
Table 2.12 $^1$H nmr spectral data of 3-chloroorsellinic acid ........................................ 78
Table 2.13 $^1$H nmr spectral data of 2-chloroorsinol ........ 81
Table 2.14 $^1$H nmr spectral data of orsellinic acid-4-methyl ether ........................................ 83
Table 2.15 $^1$H nmr spectral data of 0-methyl orcinol ...... 84
Fig. 3.1 Course of fermentation of *P. cyclopium* in submerged culture (60ml) at 24°C ........ 93
Fig. 3.2 Course of fermentation of *P. cyclopium* in surface culture (300ml) at 24°C ............... 94
Table 3.1 Results of thin-layer chromatography of chloroform extracted materials obtained from media of *P. cyclopium* in submerged cultures .......... 96
Table 3.2 $^1$H nmr spectral data of 3-methoxy-2,5-toloqui-none ........................................ 99
Fig. 3.3 Rate of penicillic acid formation during growth in submerged culture ..................... 102
Fig. 3.4 Autoradiogram showing the incorporation of $(1^{-14}C)$-acetate and $^{14}C$-formate into CHCl$_3$-extracted culture filtrate metabolites of *P. cyclopium* ........................................ 103
Scheme 3.1 Alternative hypothetical cleavages of the aromatic ring of orsellinic acid ............. 104
Scheme 3.2  Possible mode of incorporation of $^{13}$C-$^{13}$C doubly labelled acetate into penicillic acid ........................................ 106

Scheme 3.3  Bayer-Villiger type of oxidative ring opening reaction .................................. 107

Scheme 3.4  Alternative epoxide schemes for penicillic acid biosynthesis .................. 109

Scheme 3.5  Alternative epoxide schemes for the biosynthesis of patulin .......................... 110

Fig. 3.6  Some inhibitor candidate phenols ................................................................. 114

Fig. 3.7  Influence of orsellinic acid analogues on mycelial growth and penicillic acid production by *P. cyclopium* ...................................................... 116

Fig. 3.8  Autoradiogram showing the effect of orsellinic acid analogues on penicillic acid formation ................................................................. 118

Fig. 3.9  Autoradiogram showing the effect of different concentrations of 5-chloroorsellinic acid on penicillic acid formation ........................................ 119

Fig. 3.10  Autoradiogram showing the incorporation of $(1-^{14}$C$)$-acetate into penicillic acid with a culture previously grown in the presence of 5-chloroorsellinic acid .............................. 122

Fig. 3.11  Influence of 5-chloroorsellinic acid and orsellinic acid on mycelial growth and penicillic acid production ................................................. 124
Fig. 3.12  Comparison of the production of penicillic acid in the presence of 5-chloroorsellinic acid and orsellinic acid .......................... 125

Table 3.3  Influence of orsellinic acid analogues on mycelial growth and penicillic acid production of *P. cyclopium* .................................................. 131

Fig. 3.13  Autoradiograms showing the influence of 5-chloroorsellinic acid on penicillic acid formation ................................................................. 132

Fig. 3.14  Autoradiogram of culture filtrate extracts of resuspended *P. cyclopium* mycelium ............................ 135

Fig. 3.15  Autoradiogram showing the uptake of acetate by mycelium of *P. cyclopium*, at different time of incubation in the presence of 5-chloroorsellinic acid ............................................. 136

Fig. 3.16  Influence of some phenolic compounds on resuspended mycelium of *P. cyclopium* .................... 137

Fig. 3.17  Autoradiogram showing the effect of orcinol and its derivatives on penicillic acid production .......................... 138

Fig. 3.18  Autoradiogram showing the influence of 3,5-dichloroorsellinic acid on resuspended mycelium of *P. cyclopium* ............................................. 139

Table 3.4  Influence of 5-chloroorsellinic acid on mycelial growth and penicillic acid production by *P. cyclopium* .................................................. 140
Table 3.5  Influence of 3-chloroorsellinic acid on mycelial growth and penicillic acid production by *P. cyclopium* .......................... 141

Table 3.6  Influence of orsellinic acid on mycelial growth and penicillic acid production by *P. cyclopium* ........................................ 142

Table 3.7  Influence of orsellinic acid 4-methyl ether on mycelial growth and penicillic acid production by *P. cyclopium* ..................... 143

Table 3.8  Influence of 5-chloroorsellinic acid on mycelial growth and penicillic acid production ................................................. 144

Table 3.9  Influence of orsellinic acid on mycelial growth and penicillic acid production .......... 144

Table 3.10 Influence of some phenolic compounds on mycelial growth and penicillic acid production ........... 145

Table 3.11 Influence of 5-chloroorsellinic acid on mycelial growth, pH and penicillic acid production by *P. cyclopium* ......................... 146

Fig. 3.19  Visible spectrum of a mixture of glucose and glucose oxidase reagent .......................... 147

Fig. 3.20  Plot of optical density at 435nm against concentration of glucose ......................... 147

Fig. 3.21  Plot of optical density at 254nm against concentration of orsellinic acid ................. 148
Fig. 3.22  Plot of optical density at 264nm against concentration of 3-methoxy-2,5-toluquinone .... 149

Fig. 3.23  Plot of optical density at 225nm against concentration of penicillic acid ............... 150
References


120. Sonn, A., Ber., 61, 926, (1928).
126. Hoesch, K., Ber., 46, 886, (1913).
152. Thomas, R., personal communication.


