SECONDARY METABOLITES OF PHYTOPATHOGENIC FUNGI

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

IAN WILLIAM EASTON

March 1984

To my parents

SECONDARY METABOLITES OF PHYTOPATHOGENIC FUNGI

by Ian W. Easton

ABSTRACT

Little commercial use has been made of natural products as herbicides, although such compounds could offer significant improvements over currently available synthetic herbicides. The present study of certain plant disease inducing fungi has been carried out with the aim of isolating known or previously unknown metabolites and examining them for any phytopathogenic action.

One microorganism, Alternaria citri, a pathogen of several citrus fruit species, yielded several known compounds including 2-acetylquinazolinone, dihydrocanadensolide and parasiticolide A, which are not, however, host-specific toxins.

The fungus Alternaria mali was found to produce several polyketides, one of which has not previously been isolated. This compound was characterized by spectroscopy and X-ray crystallography. A biosynthetic pathway to this new compound has been postulated.

Further investigation of the X-ray crystal structures of 2-acetylquinazolinone and parasiticolide A have also been made.

ACKNOWLEDGEMENTS

The project was funded by an S.E.R.C. award for which I express my gratitude. There are a number of people I also wish to thank who have been involved with the project.

I am grateful to Professor I.O.Sutherland of the University of Liverpool, Department of Organic Chemistry, for the use of his laboratories and thank all the technical staff for their help throughout the project. Many thanks to Mrs. A. Simpson for typing this thesis and making life easy for me.

I am indebted to Dr. D.J.Chadwick for his assistance in matters pertaining to crystallography, Dr. J.S.E. Holker for his help and discussions about biosynthesis, and especially to Dr. R.A.W. Johnstone for his guidance and encouragement through the past three years.

Finally, thanks to all my friends, especially Julia, Larry, Richard, Cherie, Louise, Brian, Pete, Iwan, Jim and Guy for preserving my sanity.

CONTENTS PAGE

INTRODUCTION

Mould metabolites have been a source of interest to chemists for over a century, although early work on fungal products was limited due to their complexity. Many of these investigations were concerned with systematic cataloguing of the metabolites in the hope that these products would assist their classification of microorganisms into groups and species (chemical taxonomy).

Man's use of biologically active natural products in crude drug preparations has formed the basis of modern medicinal chemistry. Most early extracts of natural products were from plant sources, notably morphine from the opium poppy, and quinine from cinchona bark.

The discovery of penicillin, followed by the cephalosporins and their use in treating bacterial infection radically altered this situation and, since the late 1940's, a great deal of work has been done on fungal metabolites. Isolation and screening of mould metabolites with potential biological activity has yielded many other compounds with interesting properties. However, more recently, crude extracts from fungi and other microorganisms have been used as fungicides and insecticides.[1,2]

Generally, little use has been made of natural products as herbicides, although there have been many reports concerning the isolation of phytotoxic material from cultures of pathogenic fungi.[3]

The potential importance of having a naturally occurring herbicide can be understood when the compounds

presently available for herbicidal use are considered critically.

Until the middle 1940's, the only herbicides available were inorganic compounds such as iron (III) sulphate, ammonium sulphate, arsenic (III) oxide and sodium chlorate. The disadvantages of these compounds are that they are non-specific, a high concentration is required for any degree of control, and they also possess high mammalian toxicity, especially in the case of arsenic salts. Sodium chlorate is still used for total weed eradication in gardens, but not on an agricultural scale.

The first synthetic organic compounds to be used as herbicides were certain chlorinated phenoxy acids such as 2,4-dichlorophenoxyacetic acid (2,4-D), which were found to exhibit plant hormone activity comparable with naturally occurring indoleacetic acid. Use of these compounds enabled control of dicotyledonous plants such as willow herb and fat hen in cereal crops. Some of these herbicides are still among the most important. Various esters and salts of 2,4-D, for instance, are in widespread use as systemic herbicides in cereals and other crops.

Another important group of synthetic organic herbicides are the bipyridyls, diquat and paraquat, which are used for total weed eradication. The problem with many of these chemicals is their high mammalian toxicity and, in some cases, ther persistence in the environment. 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), for instance, although not itself particularly hazardous, may contain

through faulty manufacturing processes, small amounts of 2,3,7,8-tetrachlorodibenzo-p-dioxin which is extremely harmful at levels of less than 0.01 mg/kg body weight.[4] Although different conditions for the manufacture of 2,4,5-T have been devised, the possible presence of dioxin is still a problem. Because of unpleasant toxic side-effects from many of these herbicides, more research is being directed towards the screening of natural products as potential herbicides. A natural product used as a herbicide may well be more biodegradable, more selective, and less toxic to mammals than the currently used synthetic compounds. Possibly, in the future, synthetic materials could be replaced by biologically derived compounds. These would be "third generation" herbicides, similar to the insect hormones and plant extracts currently used as "third generation" insecticides. Many natural products are also active at low concentrations as, for example, the plant growth regulator, gibberellic acid, which is active at a concentration of 0.1 p.p.m.

It was one of the aims of the work described in this thesis to obtain known and previously unknown metabolites of phytopathogenic fungi, and to determine the level and type of their activity using an established screening method.

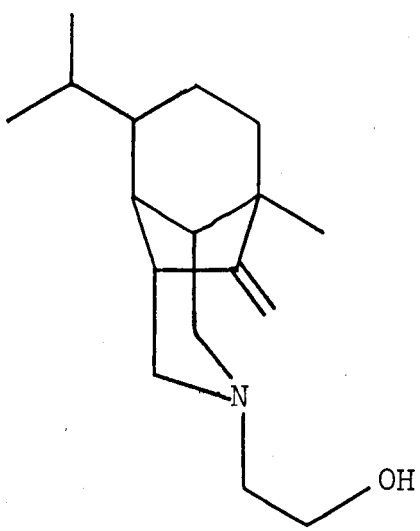
A survey of the literature concerned with fungal metabolites reveals that, although many fungi produce phytotoxic compounds, few of these are selective in action, and usually it is the foliage of plants which are affected, resulting in scorching or wilting. In most cases, this is

not the mode of action of the disease induced by the pathogen, and there is little evidence to connect the toxin with the disease.

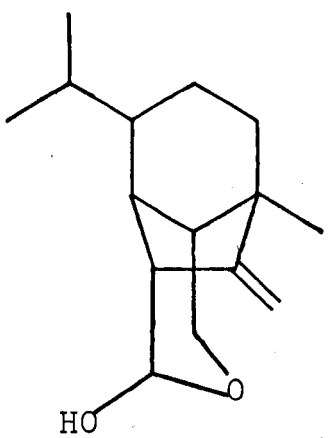
In fact, although the concept that plant pathogens act by producing toxins originated in 1886,[5] proof that plant diseases could be induced by toxigenic action was not obtained until much later.[6] Early studies on production of phytotoxins by microorganisms were carried out by growing the pathogens in artificial culture media. Attempts to extract toxic material from infected plants were not very successful.[7] Eventually, it was appreciated that, to obtain proof of the causal role of a toxin in plant disease, the toxin must be present in the infected host and extracted from it.[8] Also, certain toxins (phytoalexins) may be produced by the host in response to infections.[9] While the failure of an isolated toxin to reproduce the disease may simply mean that the toxin has to act in conjunction with others in vivo, reproduction of the symptoms does not in itself implicate the toxin. This is because plants are strictly limited in their variety of responses. For instance, chlorosis, necrosis and wilting are each produced by a wide variety of irritants. A number of terms have been used in an attempt to classify toxins, such as "vivotoxin", "pathotoxin", "mycotoxin", and "host-specific toxin". However, all these toxins can suitably be described as 'Phytotoxins".

One toxin which has been shown to play a major part in the host-pathogen relationship is victorin, first isolated

from the fungus Helminthosporium victoriae in 1947.[10] Victorin is highly specific and is harmful only to those plants which bear the gene for susceptibility to the pathogen. It causes severe leaf blight in addition to basal stem and root necrosis. Isolation and characterization were complicated by its instability but victorin has been reported to be a pentapeptide linked to a tricyclic amine moiety.[11] The amino acids were identified as aspartic acid, glutamic acid, glycine, valine and one of the leucines.[12] Victoxinine, the amine moiety, has been characterised as (I)[13] and synthesised from naturally occurring prehelminthosporal (II).



(I)



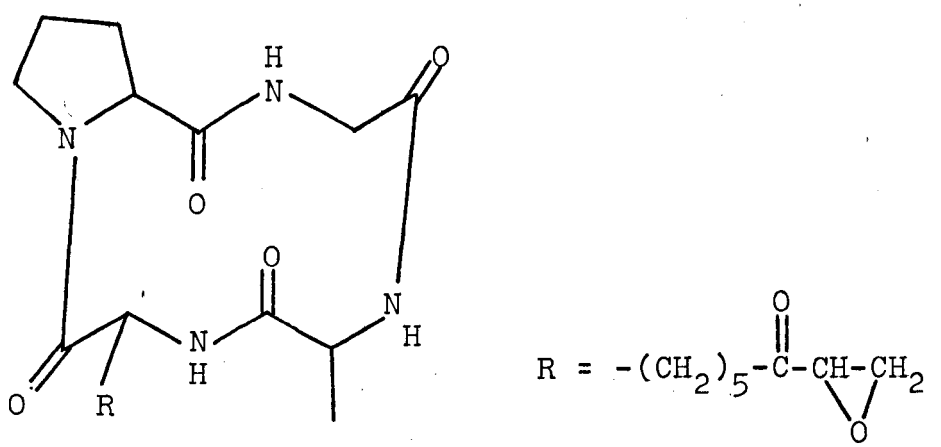
(II)

The mode of action of victorin is not fully understood but it is though to involve changes in cell permeability

resulting in respiratory changes; chlorosis appears to be a secondary effect caused by attack on the leaf protoplast.

Periconia circinata, the pathogen in milo disease of grain sorghum, was found to produce a toxin which was more stable and easier to isolate from cultures.[14] It too only inhibits growth in some strains of the plant. The toxin would appear to be peptidic as acid hydrolysis gives aspartic acid, glutamic acid, alanine and serine. Since then, two more host-specific toxins have been isolated from P.circinata.[15] These are related, and contain multiple residues of aspartic acid, and one or more moles of an uncharacterised polyamine moiety.

Several host-specific toxins have been isolated from certain Helminthosporium species. One of these from H.Carbonum,[16] which attacks corn, has been found to be a cyclic peptide containing, amongst others, proline, alanine, and α-amino-2,3-dehydro-3-methylpentanoic acid.[17] Another cyclic peptide from H.carbonum has recently been characterised as structure (III), which duplicates the

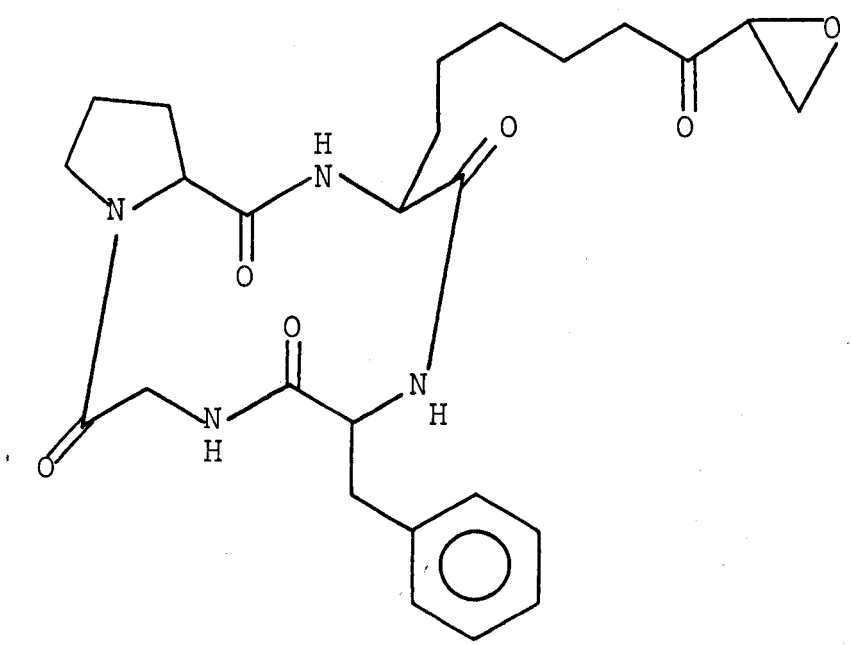


(III)

effect of the fungus on certain species of corn.[18] Besides proline and two alanine residues, it contains the novel amino acid, 2-amino-9,10-epoxy-8-oxodecanoic acid.

These compounds are labile, but have found use in agriculture. By using fungal extracts, breeding programmes may be carried out to produce disease resistant strains. An alternative sequence for H.carbonum toxins has been suggested by Walton,[19] by digesting the peptide in D- and L-amino acid oxidase.

Other cyclic tetrapeptides containing 2-amino-9,10-epoxy-8-oxodecanoic acid are known to occur. Three biologically active compounds, cyl-1,-2, and -3 have been isolated from Cylindrocladium scoparium,[20] causative of many diseases in higher plants. Cyl-2 has been characterised as compound (IV).[21]

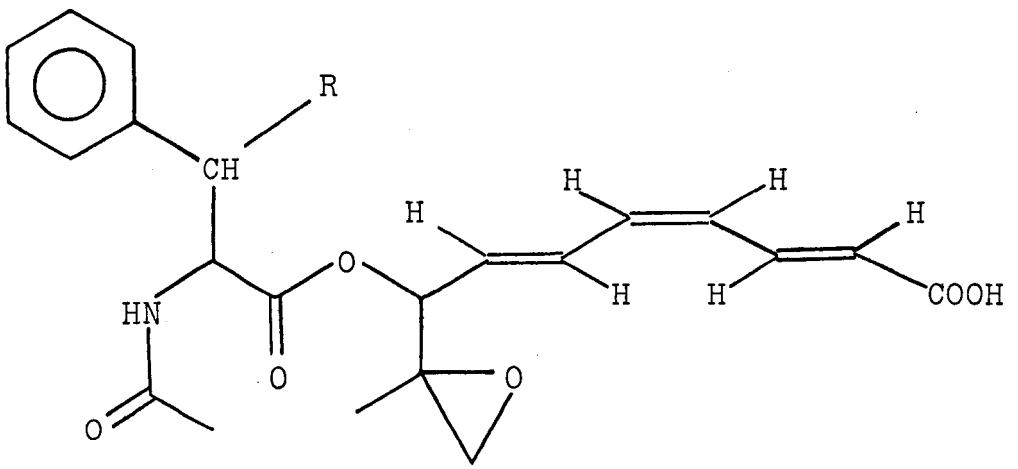


(IV)

Another cyclic tetrapeptide, chlamydocin, from Diheterospora chlamydospora[22] has been characterised using D- and L-amino acid oxidase.

During the past fifteen years, host specific toxins have become the centre of much interest and research. It had generally been believed that phytotoxic metabolites of plant pathogens lead to visible injury on host plants, resulting in the reproduction of some disease symptoms, but are not an initial inciting agent of disease. This traditional view has been challenged by host-specific toxin (HST) researchers who are emphasising that such toxins are a primary determinant for pathogenicity.[23] The present knowledge of HST's has come almost entirely from saprophytic pathogens, such as Alternaria, and those listed above. Strangely, many of these appear to be "man-made diseases", which occur only on newly bred or introduced cultivars of crop plants. For example, black spot disease affecting Japanese pears suddenly appeared after widespread planting of a new natural mutant, "Nijisseiki", about seventy years ago. Most of the older cultivans apparently are immune to the disease. Alternaria kikuchiana, the causative fungus, was found to contain a host-specific factor,[24] and this seems to be the first report of an HST problem. More recent investigations have shown that virulent strains of this fungus produce three HST's (A.K. toxins) in cultures, two of which have been isolated and shown to be pure.[25] These products produced veinal necrosis on susceptible leaves in amounts as small as 0.01 and 0.1 $\mu g\ cm^{-3}$

respectively; there was little or no toxicity to resistant pears or to other non-host plants. Two of the A.K. toxins have been characterised by derivitisation and (particularly) n.m.r. studies.[26]

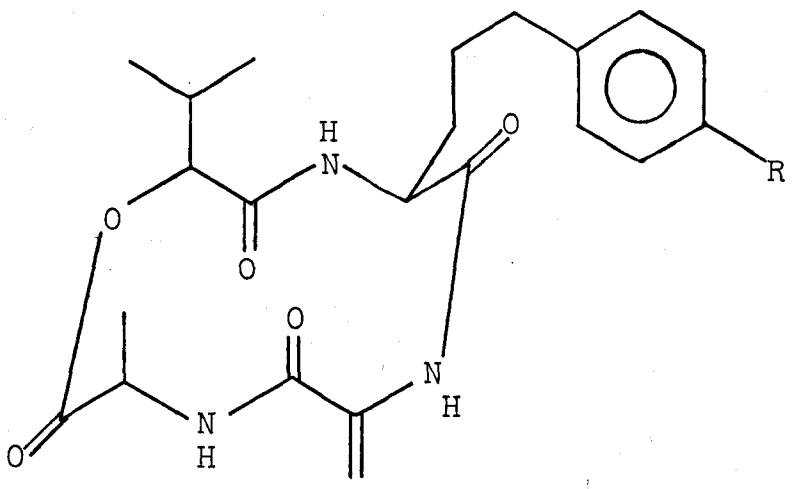


(V) A.K. Toxin I R = CH_3

(VI) A.K. Toxin II R = H

Highly virulent isolates of Alternaria mali, the fungus responsible for leaf spot disease of apple, produce

several toxins (A.M. toxins) in cultures, each with a high degree of host-specificity.[27] Of these toxins, the major three have been isolated in crystalline form, A.M.-toxin (I),[28] A.M. toxin (II) and A.M. toxin (III). A.M. toxin (I) has also been isolated independently and named alternariolide.[29] The structures of A.M. toxin (I) and A.M. toxin (III) have been confirmed by chemical synthesis.[30]



(VII) A.M. toxin I R = OCH_3

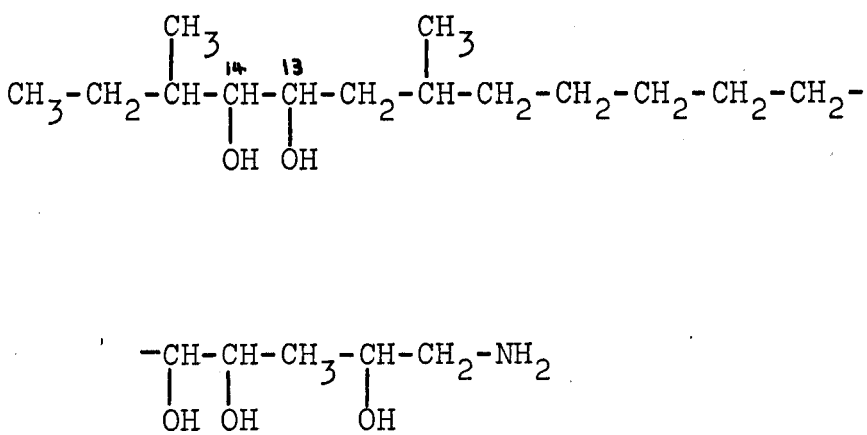
(VIII) A.M. toxin II R = H

(IX) A.M. toxin III R = OH

A.M. toxin I induced veinal necrosis within twenty-four hours and caused an instantaneous increase of electrolyte loss at approximately 10^{-9}M in the highly susceptible

cultivan "Indo" and at a concentration of over 10^{-5}M in the considerably resistant cultivan "Jonathon", approximately 10,000 times higher concentration being required. In contrast, A.K. toxin from A.kikuchiana did not cause symptoms in resistant pears, even at very high concentrations. The reason for the difference between both combinations is due to their genetic background; the susceptibility of apple to A.mali is controlled by multiple dominant genes, and the susceptibility of Japanese pear to A.kikuchiana is by a single dominant gene.[25]

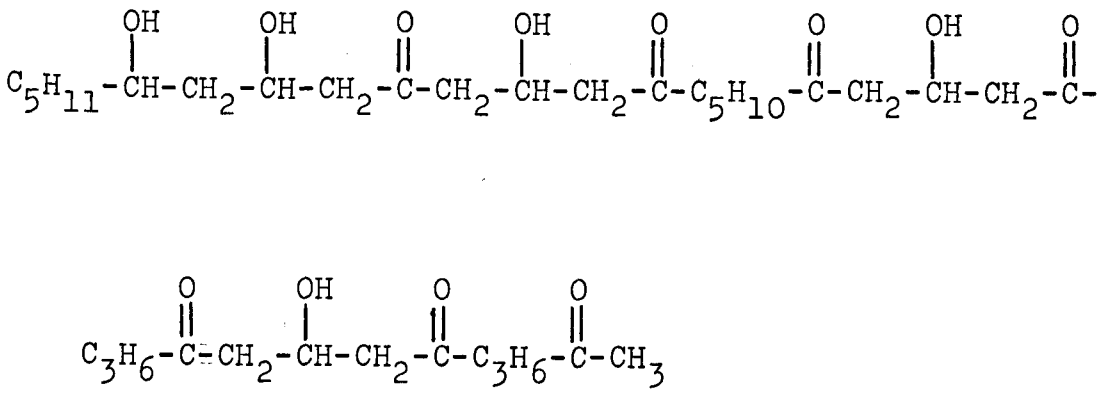
Stem canker of fresh market tomatoes, caused by Alternaria alternata f. sp. lycopersici, has also excited interest.[31] Bottini and Gilchrist found that the disease was caused by two similar host-specific, phytotoxic compounds of novel structure.[32] The compounds are both esters of 1,2,3-propanetricarboxylic acid, and 1-amino-11,15-dimethylheptadeca-2,4,5,13,14-pentol (X). The sites of esterification are a terminal carboxyl of the acid and C_{13} (major component) and C_{14} of compound (X).



(X)

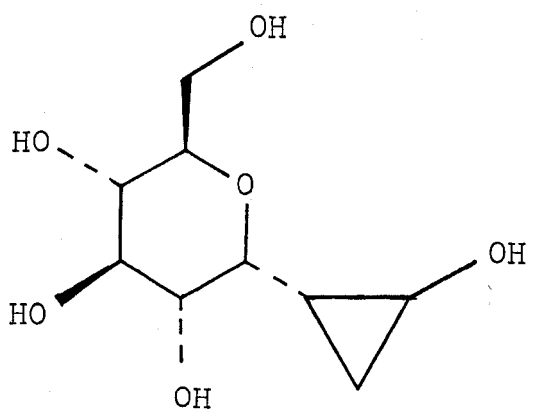
These toxins exhibit biological activity at a concentration of less than 10 ng ml^{-1} on the susceptible host. Another example of HST's from Alternaria species is shown with citrus brown spot, caused by a distinct strain of A.citri. It was first recorded on the Emperor mandarin of Australia in 1966, and since 1974 it has become prevalent on the Dancy tangerine of Florida. The toxin causing these effects has been isolated, but not yet characterised.[33]

Another class of HST consists of long-chain polyketols. One such compound was isolated by Kono and Daly[34] from Helminthosporium maydis race T, the causal agent of corn blight. This chemical is active at a level of 4 - 8 ng per leaf. The structure has been tentatively assigned as shown (XI).



(XI)

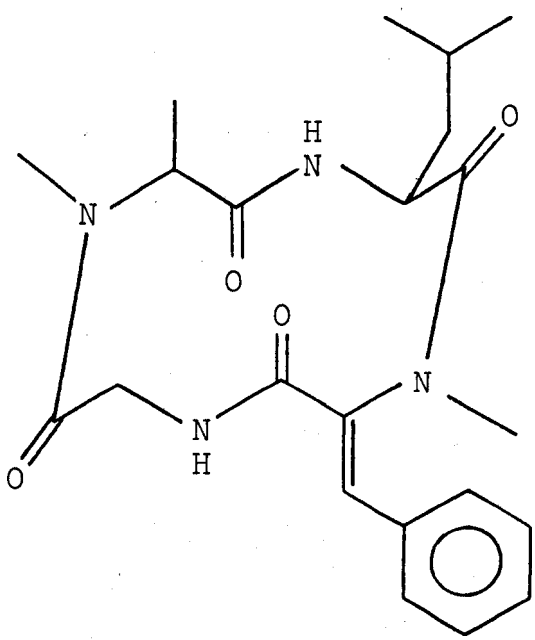
Helminthosporium sacchari has been shown to exhibit host-specific characteristics and an HST was isolated and shown to be 2-hydroxycyclopropyl-α-D-galactopyranoside,[35] trivially named helminthosporoside (XII), a structure unlike any other HST.



(XII)

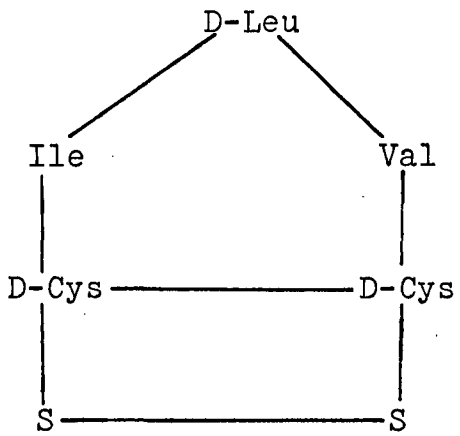
Helminthosporoside causes elongated reddish-brown necrotic streaks, or "runner lesions" on susceptible cultivars of sugar cane.

Several cyclic peptides are known to be phytotoxic as well as those mentioned above, but are not host-specific. Alternaria tenuis Nees, responsible for chlorosis in the cotyledons of cotton seedlings, produces the cyclic tetrapeptide tentoxin [36] (XIII).



(XIII)

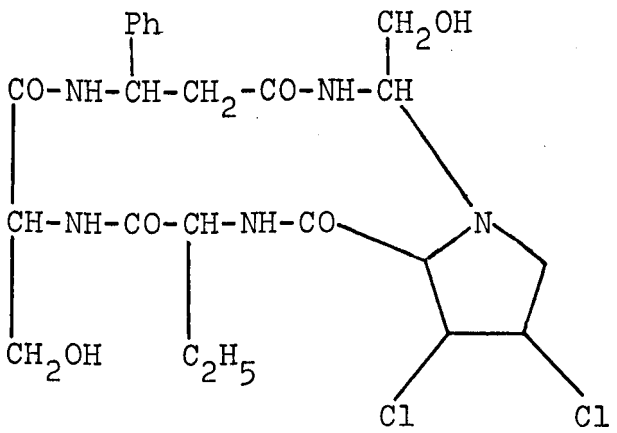
Other cyclic peptide toxins should be mentioned. Five malformins, which occur as metabolites of Aspergillus niger have been reported.[37,38] The malformins compose a family of closely related cyclic pentapeptides, characterised by a disulphide bridge between two cysteine residues. They are of biological interest because of their antibiotic activity, cytotoxicity, inhibition of adventitous root formation, and unique ability to cause malformations of the stems and petioles of bean plants, and curvature of roots of corn. Malformin A[38] (XIV), B_2, and C[39] have been completely characterised.



(XIV)

Two cyclic chlorine-containing pentapeptides, islanditoxin[40] and cyclochlorotine[41] (XV), have been isolated and identified from the fungus Penicillium islandicum. These compounds are of interest because they cause acute and chronic liver toxicoses in mammals.

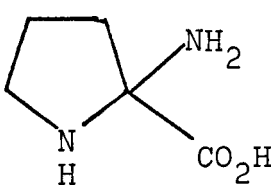
Another non-specific toxin is the cyclic heptapeptide, rhizonin A,[42] from Rhizopus microsporus.



(XV)

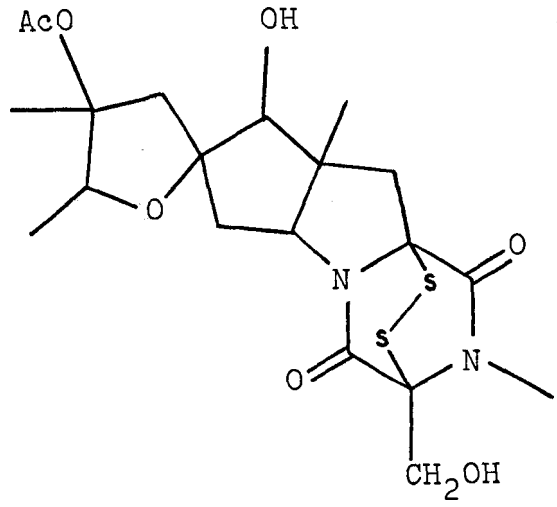
This molecule contains two residues of the novel amino acid N-methyl-3-(2-furyl)alanine.

There are also some less complex phytotoxic metabolites; Turner et al.,[43] found that culture broths of Aspergillus wentii caused yellowing and scorching of leaves in higher plants. The active constituent was isolated and found to be an unusual amino acid (XVI).



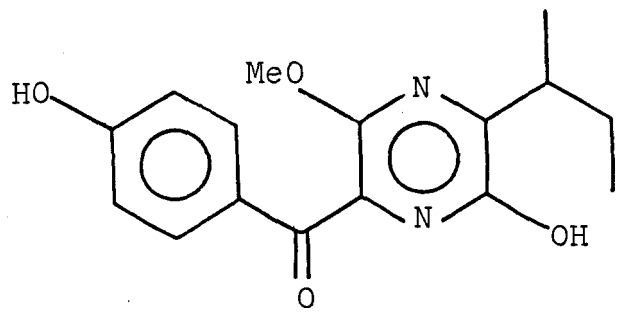
(XVI)

Phoma lingams, a parasite of Crucifera, produces a compound, sirodesmin (XVII),[44] which is phytotoxic and mycotoxic.



(XVII)

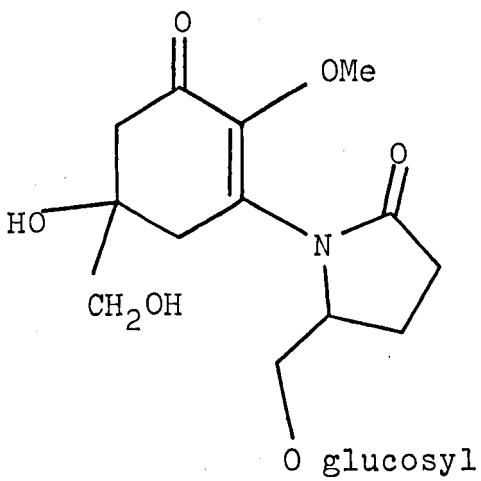
Another phytotoxin which is derived from amino acids is septorine (XVIII).[45] This compound is produced by Septoria nodorum, a common parasite of wheat.



(XVIII)

Peptide phytotoxins are unlikely ever to be used as herbicides due to problems associated with their large-scale synthesis which would make them economically unattractive, but some of the HST's have been used in research programmes to develop resistant varieties of crop.

As well as helminthosporoside, several other glycosidic toxins are known, though none have so far been found to be host-specific. Two phytotoxins containing the glucoside group were isolated from Ascochyta pisi,[46] and found to be the open-chain and ring forms (XIX) of mycosporine-2-glucoside.



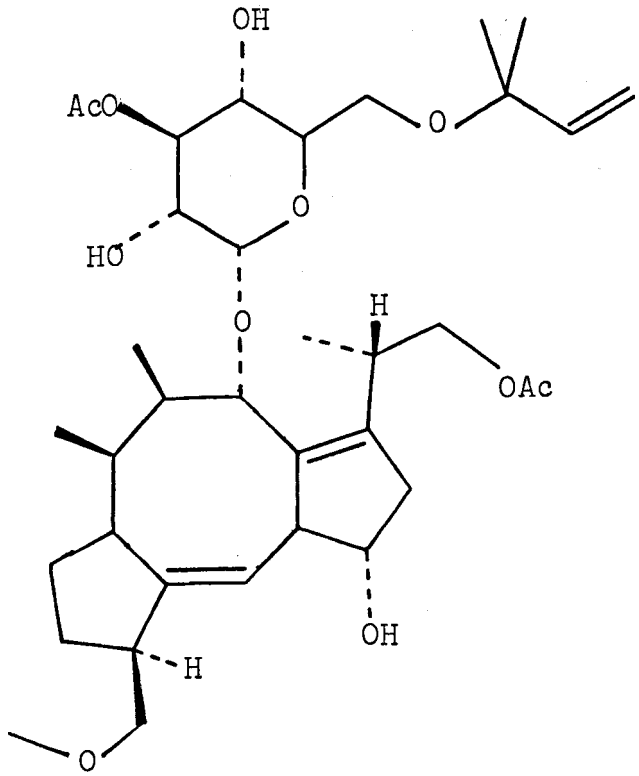
(XIX)

A phytotoxic polysaccharide is produced by Colletotrichum trifolii,[47] which causes paling, desiccation , wilting and death of alfalfa. The toxin also contains 1 - 2% of protein. A similar compound has been isolated from Fusarium oxysporum

lycopersici and cubense,[48] which elicits browning and phytoalexin production in green bean cotyledons. In liquid cultures, Ceratocystis ulmi, the Dutch Elm pathogen, accumulates various large heteropolymers containing polysaccharide and protein. Several different glycoproteins have been distinguished on the basis of molecular weight, and it has been found that the composition of glycoprotein is strikingly similar to that of the outer part of the cell wall.[49] This suggests that many glycoproteins are structurally incomplete cell wall material that is secreted. Glycoproteins have been strongly implicated in wilt symptoms of C.ulmi. In 1979, a phytotoxic protein, cerato-ulmin (molecular weight 13,000) containing almost no polysaccharide, was purified[50] but the nature of phytotoxins produced by C.ulmi remains unclear.

Cultures of Verticillium dahliae secrete large amounts of complex polymeric substances containing proteins, lipids, and polysaccharides[51,52] (PLP). These PLP substances induce classic wilt symptoms in cotton plants. The carbohydrate and protein composition of PLP is very similar to that of the alkali-soluble fraction of cell walls. PLP may, therefore, be a by-product of cell wall biosynthesis.

A somewhat smaller molecule is the highly phytotoxic terpenoid, fusicoccin (XX), isolated from Fusicoccum amygdali Del., and characterised by Barton et al.[53] F.amygdali is responsible for wilting in peach and almond trees and is a serious pest in Italy. The mode of action of fusicoccin has received much attention, and is thought to involve widening of stomata resulting in increased loss of water.



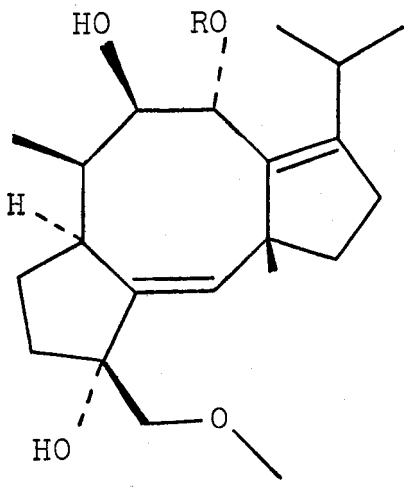
(XX)

Fusicoccin is the most phytotoxic of an increasing group of related metabolites.[54]

Other metabolites with a similar terpenoid skeleton are known. Sassa[55] has isolated several materials from an unknown fungus, 501-7W, which were termed cotylenins. The aglycone part of the molecule (named cotylenol) was determined and found to be as shown (XXI). Later, the structures of the side-groups were characterised.[56]

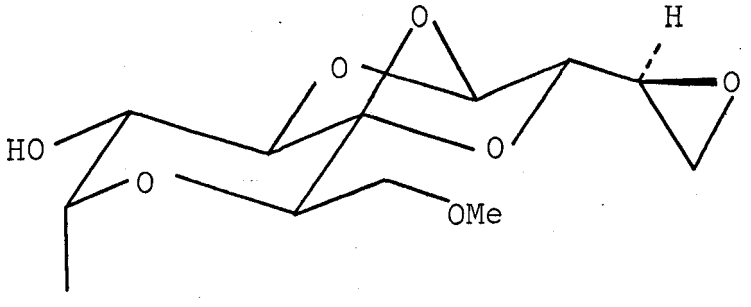
The ophiobolins are an interesting group of sesterterpenes with a similar skeleton. Ophiobolin A was first isolated in 1958 from Cochliobolus miyabeanus, and its structure (XXV) elucidated by X-ray crystallography.[57]

Ophiobolin B (XXVI) was also isolated from the same source,[58] and ophiobolin C (XXVII) (initially called zizanin A) was isolated from Ophiobolus heterostropus.[59]



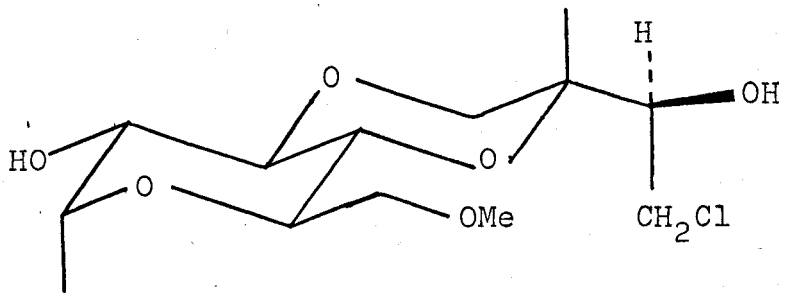
(XXI)

Cotylenol A, R =



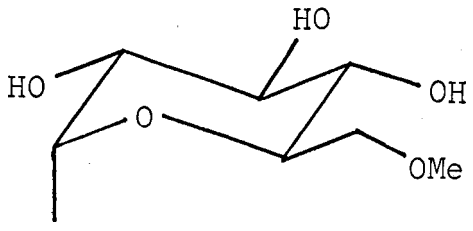
(XXII)

Cotylenol C, R =

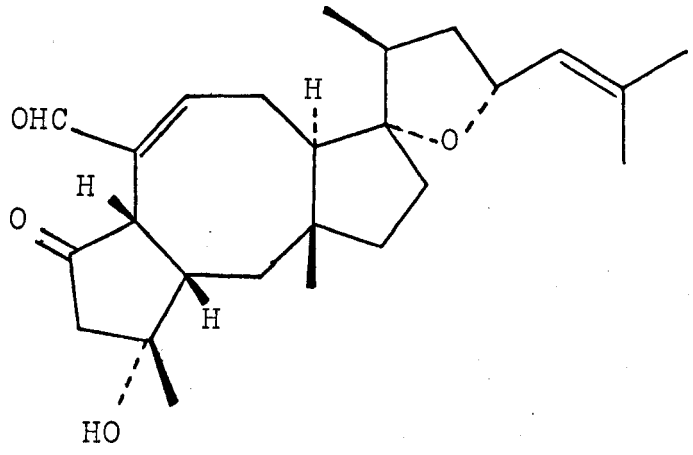


(XXIII)

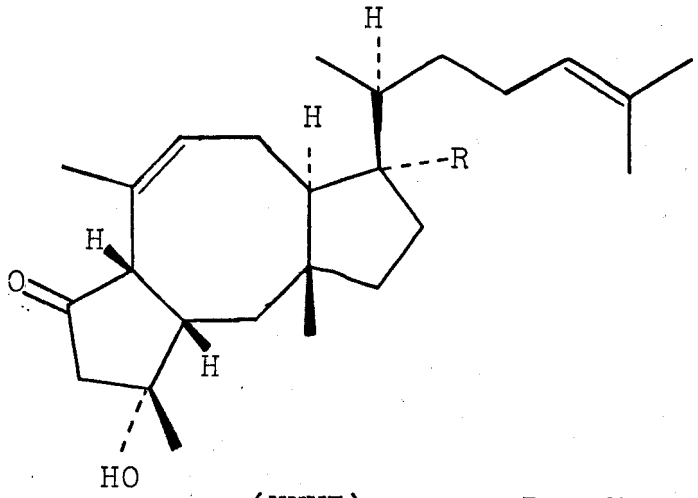
Cotylenol E, R =



(XXIV)



(XXV)

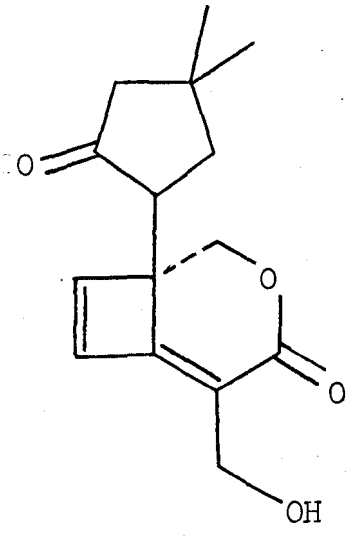


(XXVI) R = OH

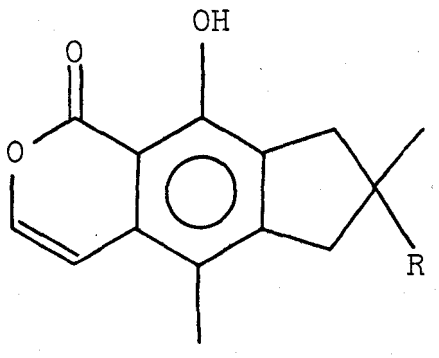
(XXVII) R = H

Five ophiobolins have been isolated from Helminthosporium maydis race T.[60]

Many other terpenoids are known to be phytotoxic. Fomes Annosus (Fr), a wood-rotting fungus, produces the well-known sesquiterpene, fomannosin (XXVIII), which has been shown to be toxic to pine needles.[61] Three more phytotoxic substances, fomannoxin (XXIX),[62] fomajorin S (XXX)[63] and fomajorin D(XXXI) have been isolated from the same source.

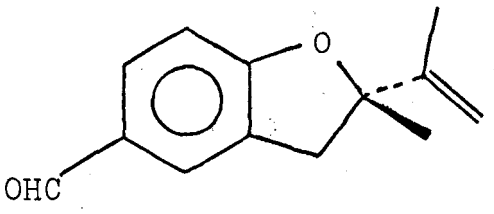


(XXVIII)



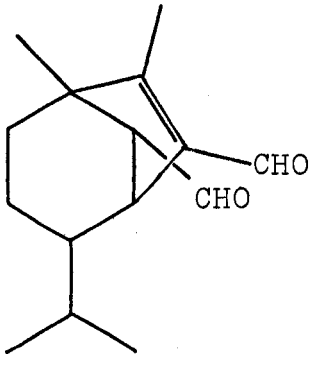
(XXX) R = CO_2H

(XXXI) R = CH_3

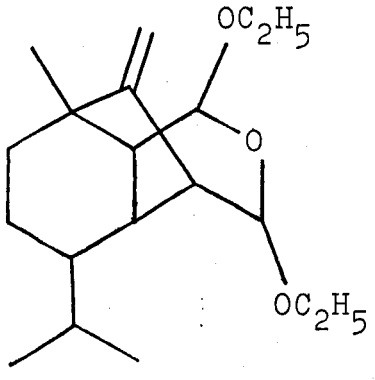


(XXIX)

One of the earliest reports of a herbicidal sesquiterpene concerned a metabolite from Helminthosporium sativum, which is responsible for leaf and root rot on many cereal crops in North America.[64] A compound was isolated and named helminthosporal (XXXII), but this turned out to be an artefact formed on heating the acetal (XXXIII).[65]



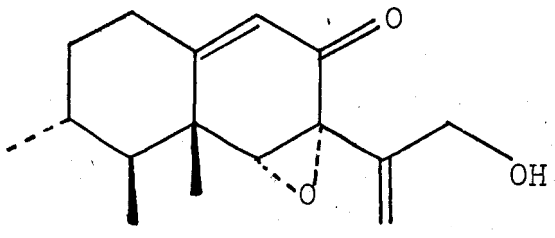
(XXXII)



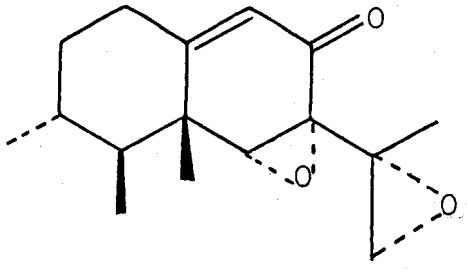
(XXXIII)

This acetal is probably also an artefact formed during the isolation procedure from the action of ethanol on an unstable dimeric acetal.

A phytotoxin, phomenone (XXXIV), has been isolated from Phoma exigua.[66] The related compound, phaseolinone (XXXV), has been found in cultures of Macrophomina phaseolina,[67]



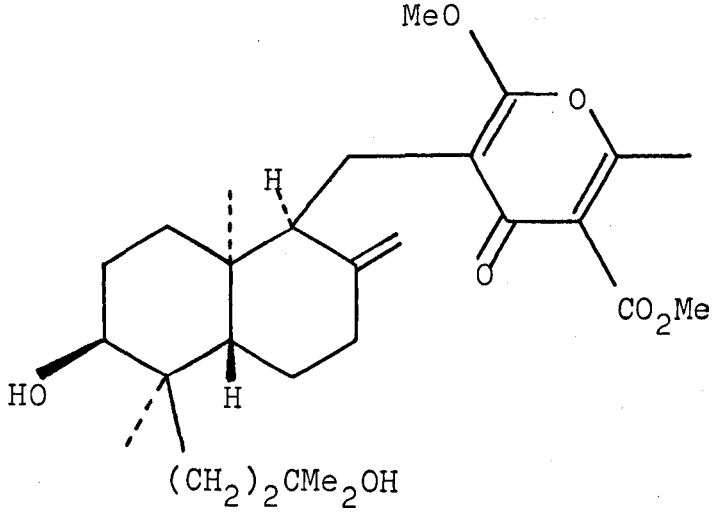
(XXXIV)



(XXXV)

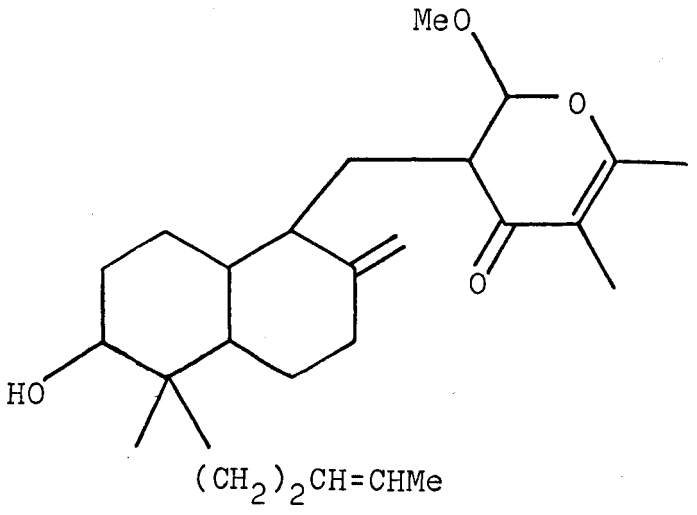
a widely distributed fungus which attacks soya bean, ground nut, beans and tobacco by wilting, root rot and inhibiting seed germination.

The toxic terpenoid acetylcolletotrichin has been isolated from Colletotrichum capsici, which attacks many plant species and can be a serious pest on the chilli pepper in the Indian sub-continent.[68] The molecular structure of acetylcolletotrichin has been determined by X-ray diffraction methods.[69] It was found that the compound does not actually contain an acetyl group, so it has been suggested that the name be changed to colletotrichin (XXXVI).



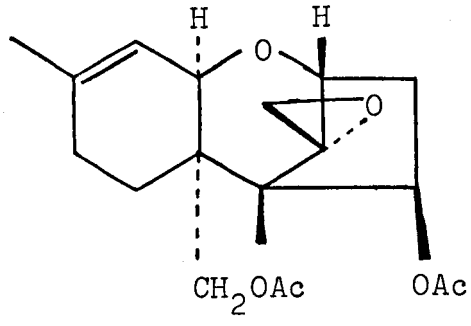
(XXXVI)

Another compound, colletochin (XXXVII), with a similar skeletal structure to colletotrichin, has been obtained from Colletotrichum nicotianae,[70] and has been found to exhibit phytotoxic properties.

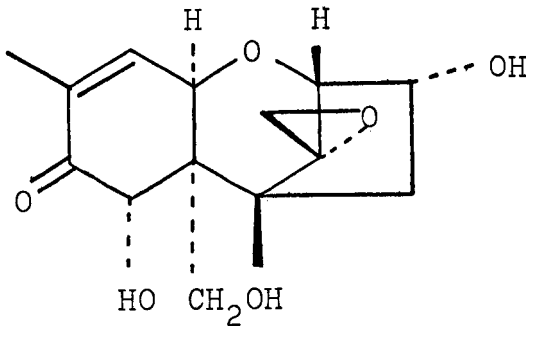


(XXXVII)

One of the largest groups of fungal terpenoids, and potentially one of the most important groups mycotoxic to animals and man, are the tricothecenes. The tricothecenes as a group have been extensively studied and show a wide range of biological effects such as antibacterial, antiviral, antifungal, and cytostatic activity; some are phytotoxic and all show some degree of animal toxicity, including insecticidal activity. The tricothecenes comprise a group of sesquiterpenes produced by various species of fungi imperfecti, and characterised by the 12,13-epoxy-tricothec-9-ene ring system. The group can be subdivided into four subgroups based on chemical differences. The first two groups differ mainly by the presence or absence of a carbonyl function at C-8. An example of a tricothecene that does not contain a carbonyl at C-8 is 4,15-diacetoxy-verrucarol (XXXVIII) from Myrothecium species.[71] Deoxynivalenol (XXXIX), from Fusarium roseum is one exmple with a C-8 carbonyl.[72]

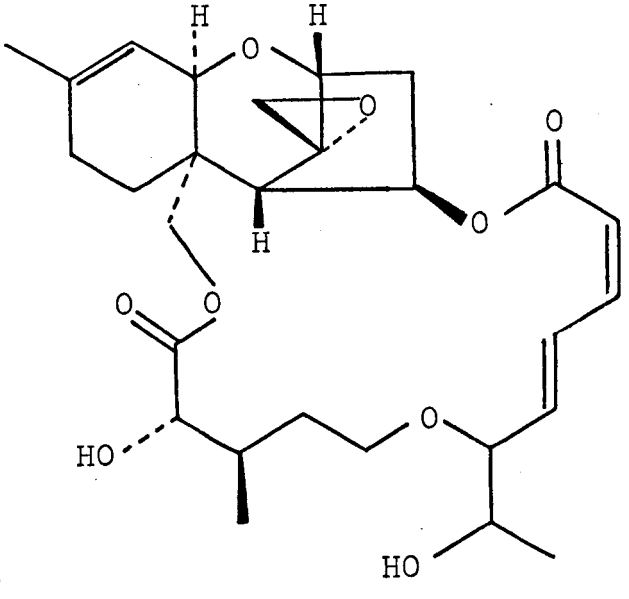


(XXXVIII)



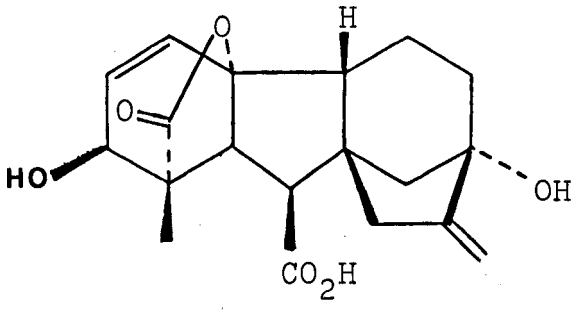
(XXXIX)

The other two groups, the roridins and verrucarins, contain a macrocyclic ester bridge between carbons 4 and 5. Roridin A[73] (XL), from Myrothecium verrucaria and M. roridum, is a macrocyclic diester of verrucarol, whereas the verrucarins are triesters of verrucarol.



(XL)

The gibberellins comprise another group of terpenoids that has been widely investigated. They can act as either phytotoxins or as growth regulators, depending on their concentration in the injected plant. Some of the gibberellins, such as gibberellic acid (XLI), are natural plant hormones, acting as growth regulators.



(XLI)

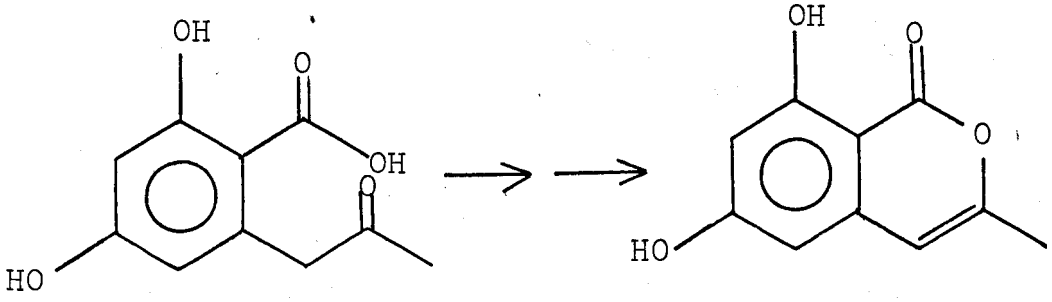
It is over twenty years since the gibberellins were characterised as metabolites of the rice pathogen Gibberella fujikuroi[74] but, although gibberellin-like substances have been obtained from other microorganisms, only recently has production by another fungus, the cassava pathogen, Sphaceloma manihiticola,[75] been conclusively established. Production of other plant hormones by plant pathogens is known, such as abscisic acid by Cercospora rosicola, and the cytokinins by Corynebacterium fascians and Agrobacterium tumefaciens.

Many other gibberellins exist as minor metabolites of G. fujikuroi. Due to their low concentrations, some

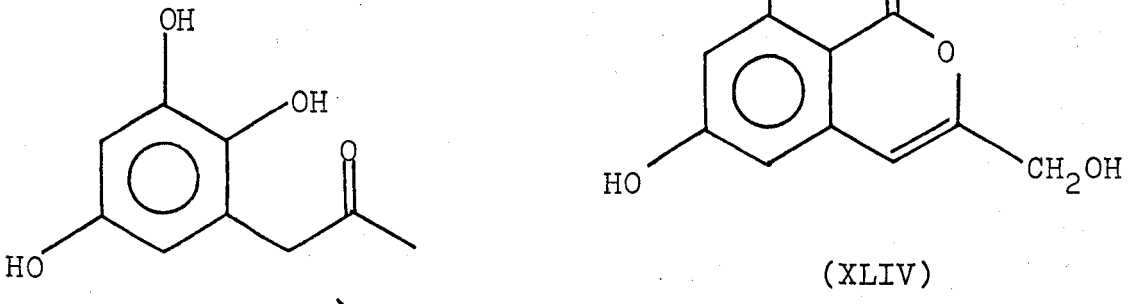
have been identified by gc/ms methods[76] and many natural gibberellins are now known; in 1980, fifty-eight naturally occurring gibberellins had been identified.[77]

Many fungal toxins are also known to be produced via the polyketide pathway. Classification is based mainly on structural considerations, but in the last ten years much biosynthetic work has taken place, utilizing improved ^{13}C-nmr methods.[78] Claydon[79] found a number of polyketide acids and their lactol derivatives in Ceratocystis ulmi. For example, 2,4-dihydroxy-6-acetonyl benzoic acid (XLII) and its lactol have been identified. The lactols can be converted into isocoumarins, such as 6,8-dihydroxy-3-methyl-1H-2-benzopyran-1-one (XLIII), and the 3-hydroxymethyl derivative (XLIV), and these have been found in diseased tissue in the host. Claydon also found a correlation between the amount of these pentaketides produced and the virulence of C.ulmi isolates, but toxicity of these metabolites to the plant has not been established.[80] Phialophora asterus produced chlorinated antibiotics from pentaketide acids.[81] Two of these, cryptosporiopsin (XLV) and furasterin (XLVI), have fungistatic activity, but their phytotoxicity has not been studied.

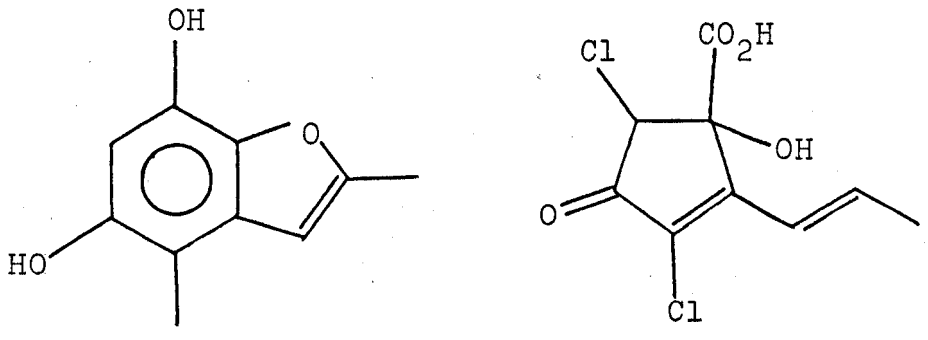
Cephalosporium gregatum synthesises several phytotoxic antibiotics called gregatins.[82] Gregatin A (XLVII) causes wilting, vascular browning and death of leaves of soybean. Graminin A, which is produced by C.gramineum and is highly toxic to wheat is identical to gregatin A except for two additional reduced carbons on the short side-chain.[83]



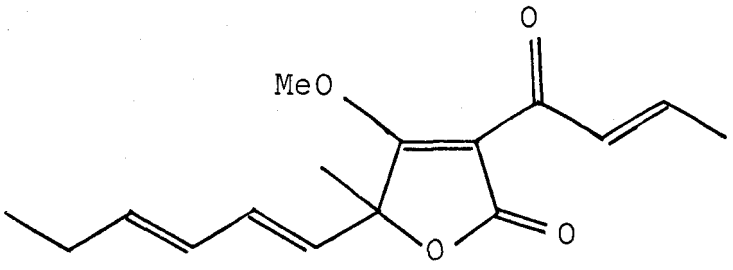
(XLII) (XLIII)



(XLIV)

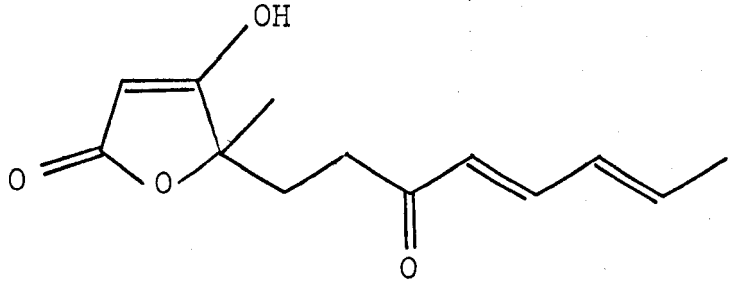


(XLV) (XLVI)



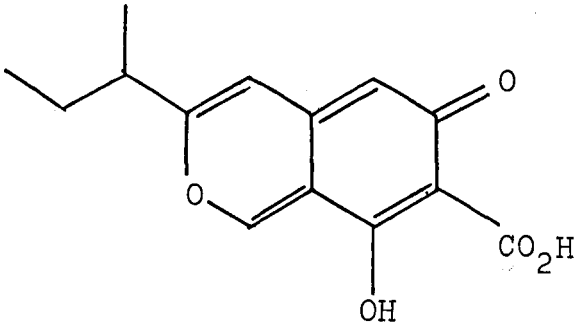
(XLVII)

Several other structurally similar metabolites are known; the aspertetronins[84] from Aspergillus rugulosus are identical to the gregatins apart from their being optical antipodes. The tetronic acids and their derivatives, a large group of secondary metabolites, have a similar skeleton, as with vertinolide (XLVIII),[85] from Verticillium intertexum.

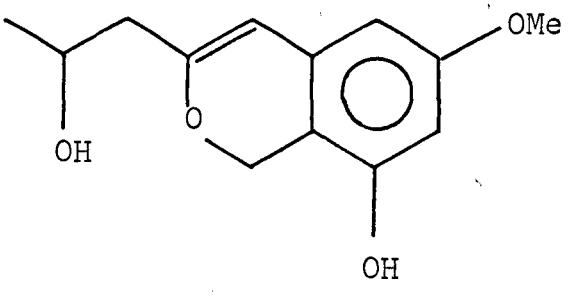


(XLVIII)

Asochyta fabae, a broad bean pathogen, produces the hexaketide ascochitine,[86] the structure of which (XLIX) has been deduced by degradative studies.[87] Diaporthin (L), a metabolite of Endothia parasitica,[88] has a similar structure.

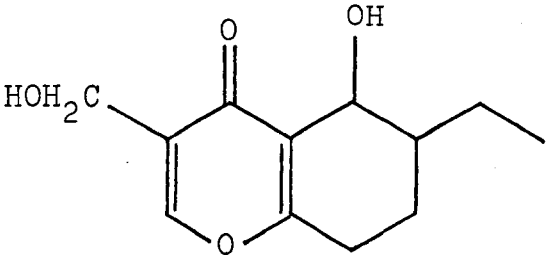


(XLIX)



(L)

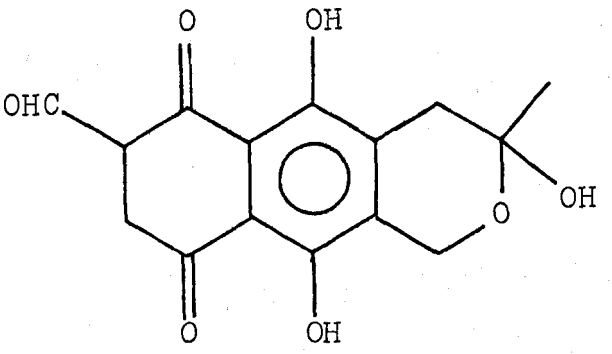
Diplosporin (LI), isolated from Diplodia macrospora[89] is a pentaketide with phytotoxic properties.



(LI)

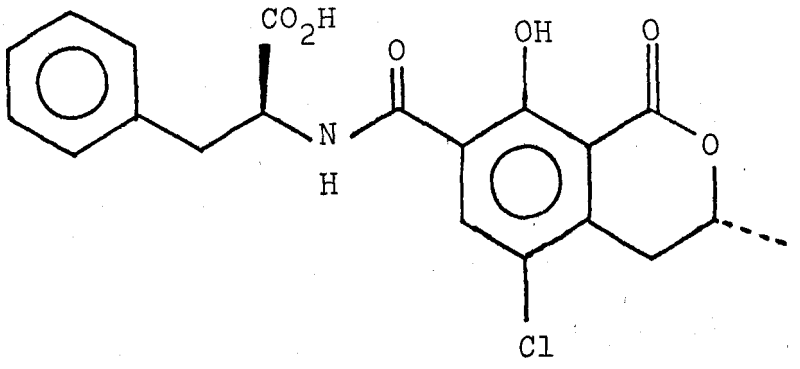
The structural variety of the polyketides is great and is dependent upon many biosynthetic functional group interconversions.

Fusarium solani, from fibrous roots of blight diseased citrus trees, produces several isolates which are phytotoxic, the main inhibitory effects being due to the presence of naphthoquinone derivatives.[90] The phytotoxicity was found to be mainly due to fusarubin (LII), first isolated from F.javanicum.[91]



(LII)

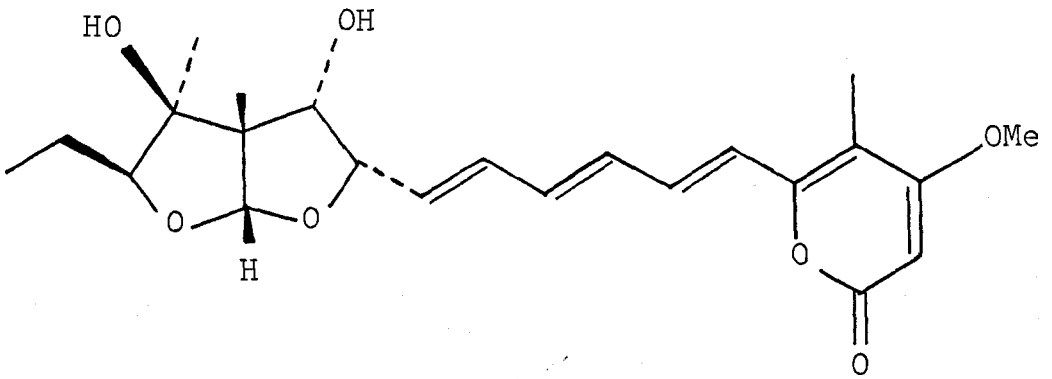
Many secondary metabolites are products of two or more pathways, usually thought of as being entirely separate. Ochratoxin A (LIII), isolated from



(LIII)

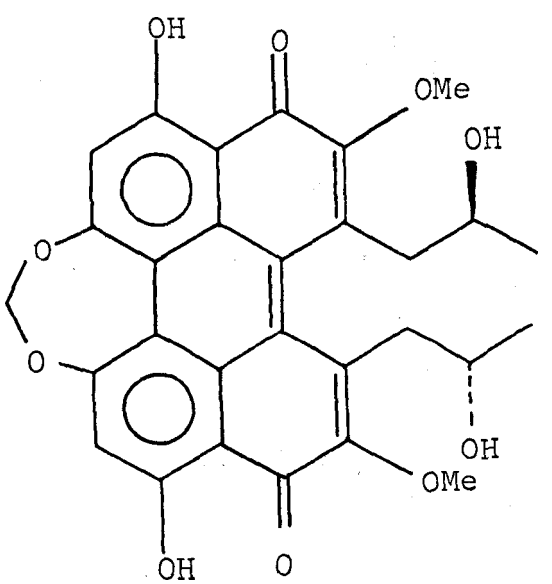
Aspergillus ochraceus,[92] is a potent nephrotoxin in mammals. It has been shown to consist of a pentaketide derived dihydroisocoumarin part linked to L-β-phenylalanine from the shikimate pathway.

Asteltoxin (LIV), another potent mycotoxin, is a nonaketide isolated from A.stellatus.[93] Astelltoxin is structurally related to citreoviridin and aurovertin B.



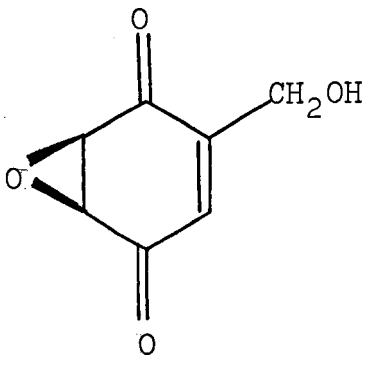
(LIV)

Another example of phytotoxic polyketides is cercosporin (LV), obtained from Cerospora nicotianae[94] and characterised in 1971.[95] Cercosporin is a polyketide derived perylenequinone and is produced by Cercospora species, which are pathogenic to many plants. Cercosporin exhibits photodynamic antibacterial activity, and is phytotoxic to tobacco plant cells.[96]

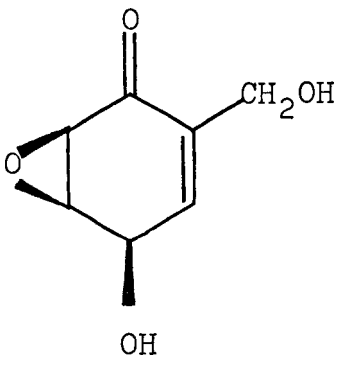


(LV)

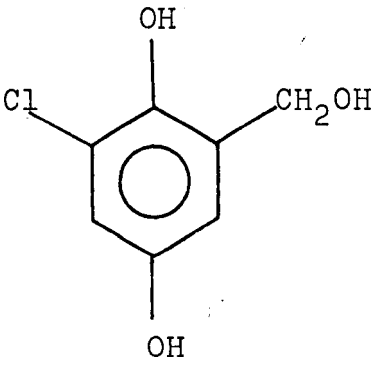
Three related compounds, phyllostine (LVI), phyllosinol (LVII), and chlorogentisyl alcohol (LVIII), have been isolated from an unspecified Phyllosticta fungus.[97]



(LVI)



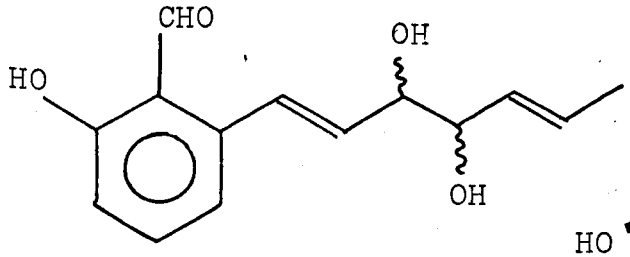
(LVII)



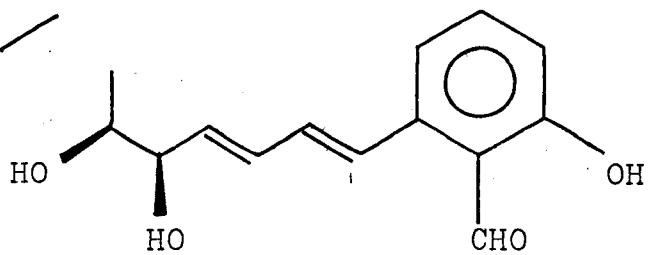
(LVIII)

These phytotoxins in the culture filtrates were shown to induce wilting and decolorisation of clover leaves.

Pericularia oryzae, the fungus responsible for rice blast, produces a number of toxins including pericularin,[98] α-picolinic acid, periculol[99] (LIX) and periculariol (LX).[100] The structure of pericularin is not known, but it has been found to be ten times more toxic to the organism that produces it than to the rice plant.



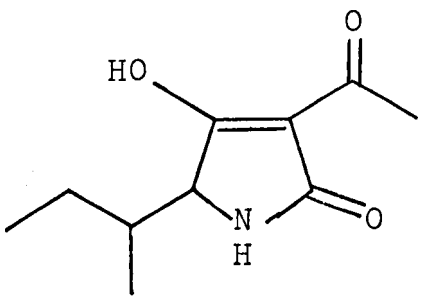
(LIX)



(LX)

This seemingly contradictory effect is possibly due to the binding of pericularin with a protein in the plant, rendering it less toxic.

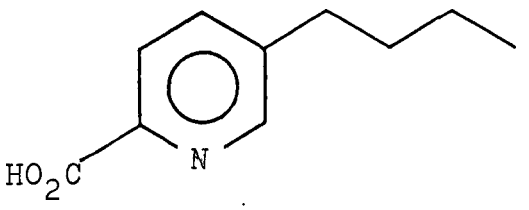
P.oryzae also produces tenuazonic acid[101] (LXI), which has been isolated from Alternaria tenius,[102] a fungus causing variegated seedling chlorosis of cotton, citrus and many other plants. Tenuazonic acid has been isolated from several other species of Alternaria.[103]



(LXI)

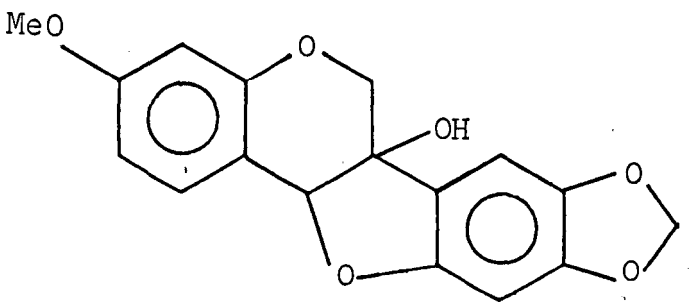
Tenuazonic acid inhibits protein and nucleic acid biosynthesis and exhibits conspicuous stunting on seedling growth of several species; it inhibits the growth of suspended cells of soybean and rice. Tenuazonic acid has been isolated as a vivotoxin in the host plant in tobacco brown-spot disease, caused by Alternaria longipes.[104]

Fusaric acid (LXII) is another example of a vivotoxin. It has been isolated from several wilt pathogens, including Fusarium lycopersici and F.vasinfectum.[105] Fusaric acid has been implicated in the disease syndrom of Fusarial wilt in tomatoes, cotton and watermelons, which is the result of a complex interaction of toxins.



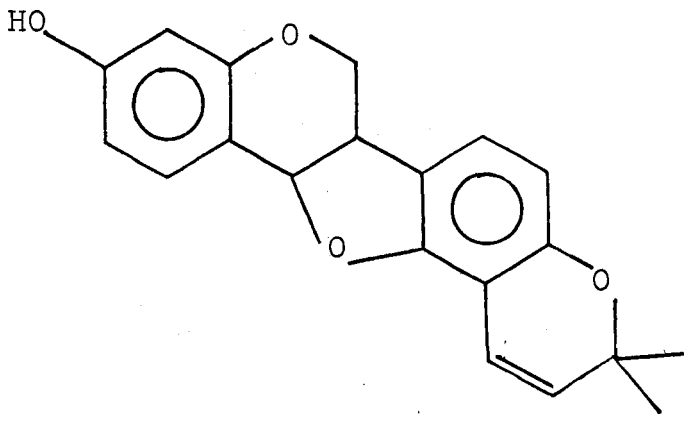
(LXII)

An example of toxins produced by the host plants themselves are the phytoalexins. Plants accumulate many different compounds as a result of infection or stress. Some of these (the phytoalexins), are toxic to micro-organisms and rapidly accumulate in resistant plants following infection. Early investigators were aware that many fungi were specialized for particular host plants. Ward[106] found that a common feature associated with failure to develop on non-hosts was rapid death of cells at sites of infection. The infection hyphae stopped growing among the dead cells, and a possible cause was release of a poison from the dead cells. However, it was not until 1962 that the antifungal compound pisatin (LXIV) from the pea plant Pisum sativum was subsequently characterised as



(LXIV)

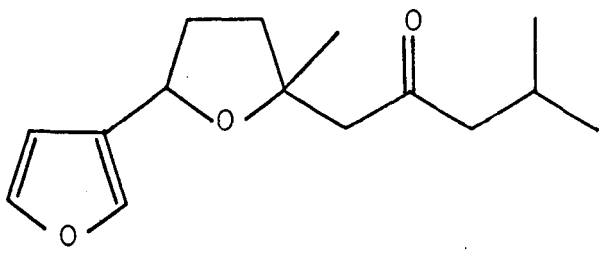
a pterocarpan.[107] Pisatin accumulates in the pea pod inoculated with fungi. It is a weak antibiotic with a broad spectrum. Fungi pathogenic to pea are generally insensitive to pisatin accumulating after infection, whereas nonpathogens are generally sensitive. Shortly after the discovery of pisatin, phaseollin (LXV), a similar



(LXV)

compound, was isolated from the green bean Phaseolus vulgaris.[108] Since then, many other active compounds have been isolated from the green bean. Ipomearone (LXVI) is a toxin produced by the sweet potato in response to two fungi, Ceratostomella Fimbriata[109] which causes black rot, and Helicobasidium mompa.[110]

Several other phytoalexins are produced by the sweet potato, and build up in the peel. Although the role of phytoalexins in resistance to plant disease is not fully

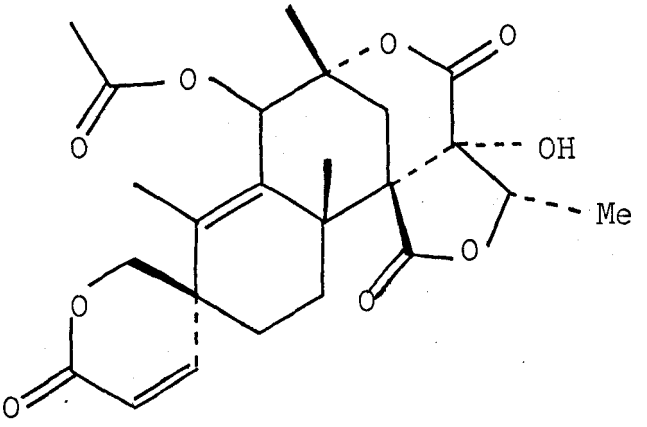


(LXVI)

understood, plant breeders developing new resistant varieties do select for plants which accumulate phytoalexins.

A continual problem in the isolation and characterization of natural products is the structural complexity and quantity of material available which, in the case of many secondary metabolites is often of the order of a few milligrams. Increasingly, spectroscopic methods have been used to greater effect; improved n.m.r. methods together with high resolution and fast atom bombardment mass spectrometry and X-ray crystallography means that compounds can be characterised with little or no chemical derivatization being required. Many complex metabolites are now routinely completely characterised by X-ray diffraction methods, which is particularly useful in determining the chirality of a multi-functional molecule, such as austin (LXVII), a toxic metabolite of Aspergillus ustus.[111] Characterization of such complex molecules would be extremely difficult using more traditional methods.

Mass spectroscopy has been increasingly used in structure elucidation and the recently introduced fast atom bombardment (FAB) ionization method is particularly



(LXVII)

useful as, for example, with the cyclic peptide toxins described earlier. The sequence of H.carbonum toxin was postulated from F.A.B./M.S. data,[18a] and the sequence of tentoxin[112] confirmed by the same method. In the work described in this thesis, spectroscopic and X-ray diffraction methods have played a large part in elucidation of the structures of several metabolites.

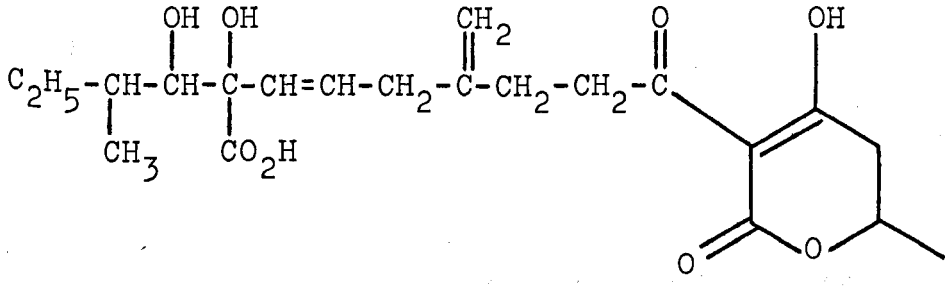
CHAPTER 1

METABOLITES OF ALTERNARIA (1)

INTRODUCTION

In addition to the compounds mentioned earlier, the Alternaria species produce a wide range of secondary metabolites. Although Alternaria species can be found on various agricultural products such as wheat, tobacco, corn, peanuts, sorghum, barley, oats, alfalfa and grasses, their toxin producing potential has not been exhaustively studied.[113] Studies to date have revealed the identities of several toxic metabolites, from a variety of structural classes. The toxicity of the Alternaria species is well known. Grains which had been infected with A.Humicola, A.tenuis Auct., and A.longipes[114] were believed to be the source of several outbreaks of moldy grain toxicosis in the U.S.S.R. during World War II.

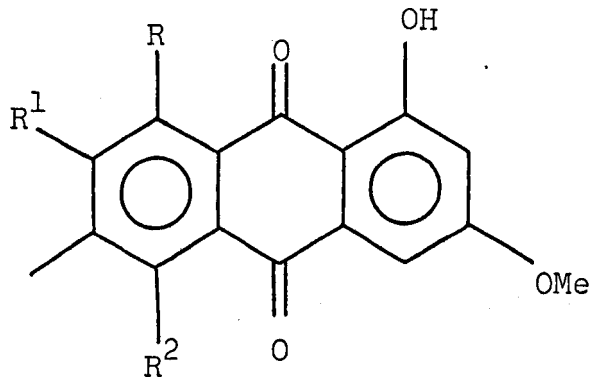
In 1949, alternaric acid was isolated from Alternaria solani,[115] a fungus which causes a devastating blight of tomato and potato plants. Eleven years later, the structure of alternaric acid (LXVIII) was reported.[116]



(LXVIII)

Alternaric acid has a low level of toxicity, and it is toxic to many plants not parasitized by A.solani. It causes wilting and death to seedlings of radish, cabbage, mustard and carrot. It also possesses some antifungal activity, inhibiting germination of Absidia, Myrothecium and Stachybotrys.

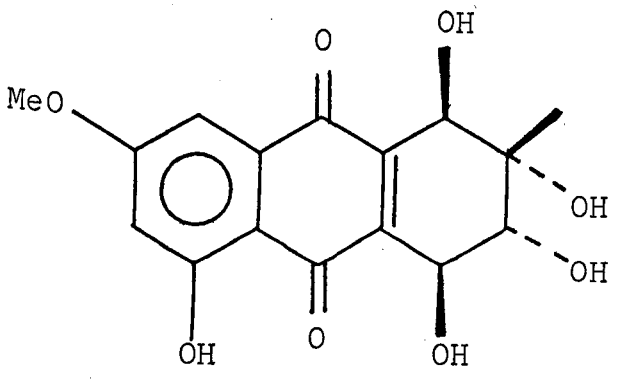
A second class of metabolites from A.solani are the anthraquinone pigments which were isolated by silica gel chromatography from chloroform extracts of the fungus.[117,118] Anthraquinones A(LXIX), B(LXX) and C(LXXI) are substituted xanthopurpurins. Anthraquinone C is idential to the previously reported macrosporin, a metabolite of Macrosporium porri.[119]



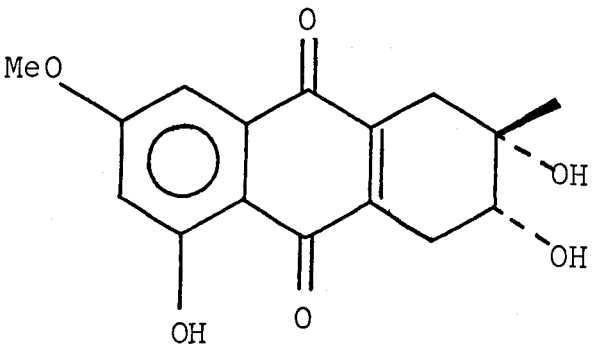
(LXIX)	R = H,	R^1 = H,	R^2 = H
(LXX)	R = OH,	R^1 = OH,	R^2 = H
(LXXI)	R = H,	R^1 = OH,	R^2 = H
(LXXII)	R = OH,	R^1 = H,	R^2 = H
(LXXIII)	R = OH,	R^1 = H,	R^2 = OH

The reddish-orange pigment, physcion (LXXII), which is also found in other fungi imperfecti, lichens and some higher plants,[120] has also been isolated from Alternaria porri.[121] Erythroglaucin[122] (LXXIII), another xanthopurpurin has been isolated from A.porri.[123]

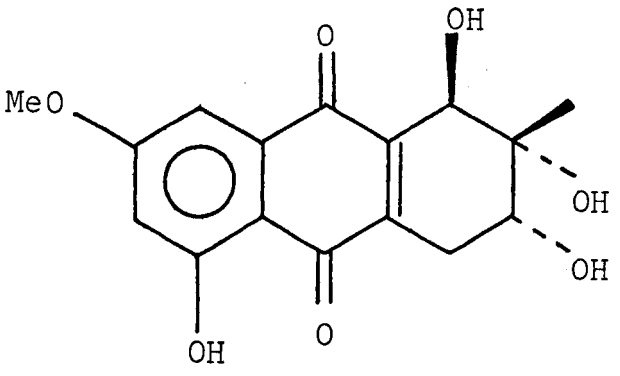
Altersolanols A (LXXIV), B (LXXV) and C (LXXVI)[124] are novel, partially reduced anthraquinones.



(LXXIV)



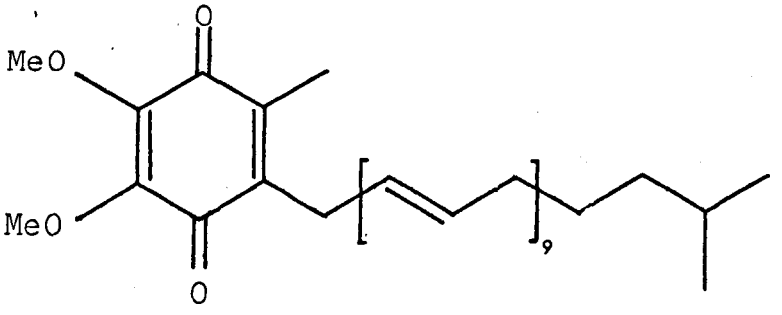
(LXXV)



(LXXVI)

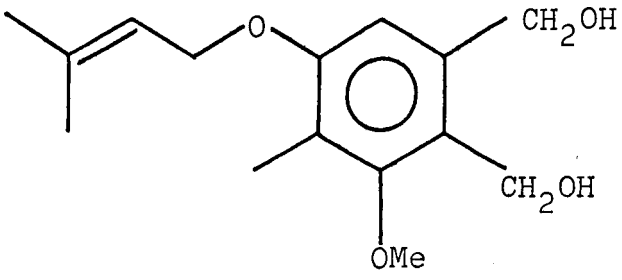
The altersolanols have also been obtained from Phomopsis juniperivora[125] and Dactylaria lutea.[126]

Another quinone metabolite, X-dihydro-ubiquinone-10 (LXXVII) has been isolated from A.solani.[127]

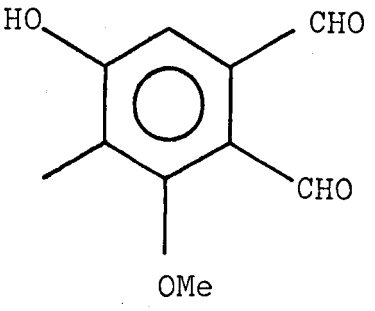


(LXXVII)

The fungus Alternaria zinniae is a pathogen which induces severe shrivelling of stems, browning of leaf veins and chlorosis of tissue in sunflowers and marigolds. The phytotoxic compound, zinniol (LXXVIII), has been isolated from A.zinniae,[128] and found to have wide-ranging activity against sunflowers, tomatoes and watermelons. Zinniol is an oil which was characterized by preparation of the diacetyl and dibenzoyl derivatives. It is similar in structure to quadrilineatin (LXXIX), an antifungal metabolite of Aspergillus quadrilineatus.[129]

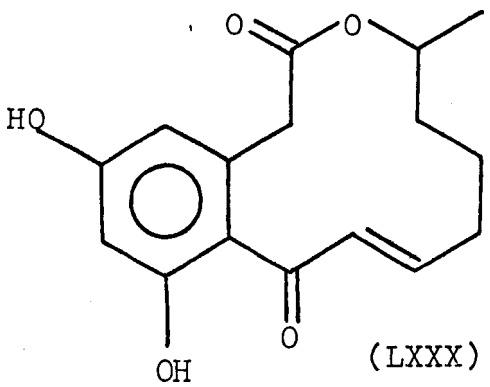


(LXXVIII)



(LXXIX)

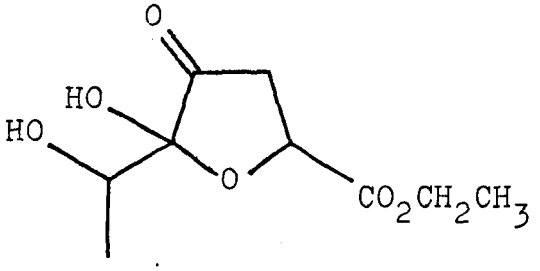
Alternaria cucumerina, the causative agent of leaf spot on cucurbits, particularly muskmelon and watermelon, has been examined, and the metabolite α,β-dehydrocurvularin (LXXX) has been isolated.[130] Dehydrocurvularin is also



(LXXX)

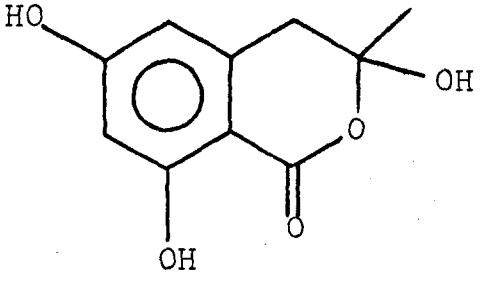
a metabolite of several Curvularia species.[131,132] The closely related metabolite, β-hydroxycurvularin, has been identified from Alternaria tomato.[133]

Alternaria kikuchiana Tanaka has been studied by several groups and, besides the host-specific A.K. toxins, other metabolites have been isolated. Altenin (LXXXI) is an optically active yellow liquid which was isolated



(LXXXI)

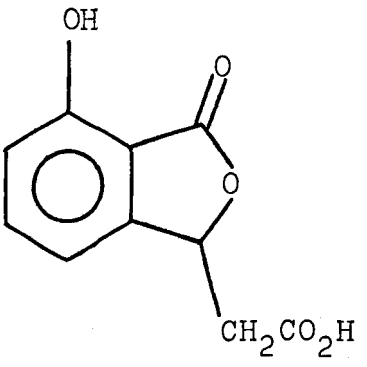
from the culture filtrate.[134] Altenin has been shown to cause black spots on susceptible pears. Further study of A.kikuchiana[135] revealed the presence of 6,8-dihydroxy-3-methylisocoumarin (LXXXII). This compound was found to



(LXXXII)

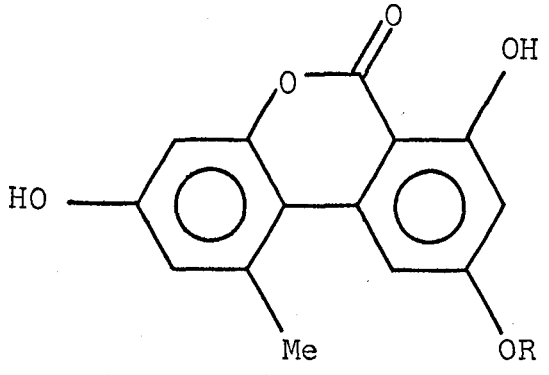
be weakly toxic, causing weak necrotic symptoms to pear leaves, and root elongation in rice and radish seedlings.

The same workers[136] went on to isolate the new compound, 3-carboxymethyl-7-hydroxyphthalide (LXXXIII), but no toxicity data were given.



(LXXXIII)

The most commonly occurring class of compounds isolated from Alternaria species are the dibenzo-pyrones and their derivatives. Alternariol (LXXXIV) and its monomethyl ether (LXXXV) were first detected by Raistrick[137] from a strain



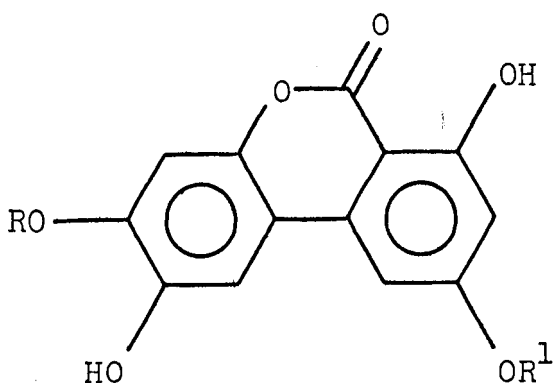
(LXXXIV) R = H

(LXXXV) R = Me

of Alternaria tenuis in 1953. Since then, they have been found in many other Alternaria species. They were finally

characterized by Thomas in 1961.[138]

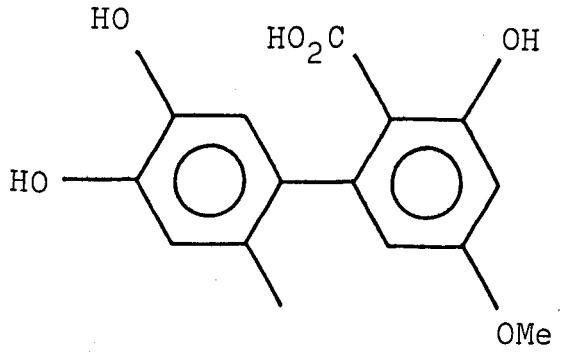
Also isolated by the same group of workers were several other metabolites derived from alternariol monomethyl ether.[102a] Altenusin ($C_{15}H_{14}O_6$), dehydroaltenusin ($C_{15}H_{12}O_6$), altertenuol ($C_{14}H_{10}O_6$), and altenuic acids I, II and III ($C_{15}H_{14}O_8$), were isolated, but their structures not fully elucidated. Thomas[138] postulated a structure (LXXXVI) for altertenuol based mainly on the empirical formula and biosynthetic arguments. However, Pero et al.,[139] isolated a compound, altenuisol, which was found to be identical to altertenuol. They proposed a different structure (LXXXVII) based on the fact that altenuisol did not react with a molybdate ion, a property common to orthodiphenols, but did react after demethylation with hydriodic acid.



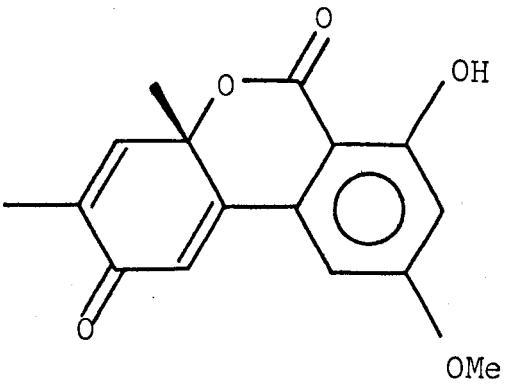
(LXXXVI) R = Me, R^1 = H

(LXXXVII) R = H, R^1 = Me

The structure of altenusin (LXXXIX) was finally solved in 1970[140] by detailed examination of its n.m.r. spectrum, and other spectroscopic properties. In the same paper, a structure was also suggested for dehydro-altenusin based mainly on its n.m.r. spectrum. This



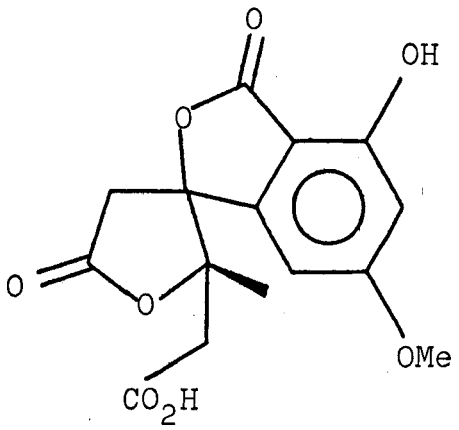
(LXXXIX)



(XC)

structure was later found to be incorrect after the X-ray crystal structure determination of dehydroaltenusin (XC) had been completed.[141]

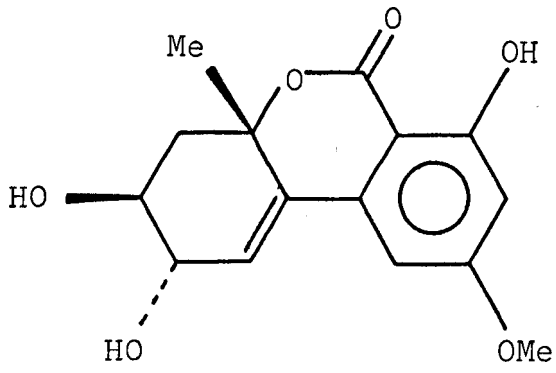
The altenuic acids are stereoisomers, but so far only the absolute structure of altenuic acid II (XCI) is known from X-ray diffraction studies.[142]



(XCI)

Altenuene ($C_{15}H_{16}O_6$) is another dibenzo-α-pyrone derivative, isolated from A.tenuis by Pero et al., in 1971.[143] The structure that they first assigned to altenuene on the basis of extensive spectroscopic studies

was again found to be incorrect, the correct structure (XCII) being elucidated by X-ray crystallography.[144]



(XCII)

Biosynthetically, it has been shown that alternariol is a heptaketide[145] arising from incorporation of ^{14}C acetate units into the molecule. Alternariol has also been synthesised by an enzyme complex isolated from A.tenuis by head to tail condensations of 1 mole of acetyl coenzyme A with six moles of malonate.[146] Alternariol methyl ether has been formed by the addition of S-adenosyl-methionine to the same enzyme complex.[147] It is reasonable to assume that the other dibenzo-pyrone metabolites are formed from alternariol monomethyl ether.[148]

There are several other Alternaria metabolites which are known, but their structures have not yet been elucidated. Altertoxin I ($C_{20}H_{16}O_6$) and II ($C_{20}H_{14}O_6$) are produced by strains of A.mali and A.tenuis.[149,150]

Phytoalternarins A, B and C have been isolated from culture filtrates and mycelial mats of A.kikuchiana Tanaka.[3c,151] Phytoalternarius A and C are colourless crystalline solids, whereas phytoalternarin B is a yellow oil. Investigation

of the phytoalternarins shows that they are probably low molecular weight proteins or peptides. Phytoalternarin A is host-specific to Japanese pear.

Brassicicolin is a colourless oil produced by A.brassicicola, and possesses anti-yeast and antibacterial properties.[152]

A phytotoxic substance has also been isolated from A.triticina, a wheat parasite.[153] The toxin inhibited germination and root elongation of seeds of both resistant and susceptible varieties. It induced similar symptoms to those of the fungus on leaves.

Some of the Alternaria metabolites are acutely toxic, cytotoxic and teratogenic. Both alternariol and alternariol monomethyl ether have been shown to be synergistic against bacteria. Other alternariol derivatives that have been examined also show high mammalian toxicity, as do the altertoxins. The toxicity of tenuazonic acid has been more extensively studied. Tenuazonic acid is toxic to many mammals,[154] and it also shows antiviral, antibacterial and antitumour activity.[155]

The majority of the Alternaria species cause leaf spot diseases, unlike the more extensively studied Fusarium speices, which are often responsible for wilting diseases, or rots. Alternaria longipes causes leaf spot disease of tobacco, and A.zinniae is responsible for leaf spot of zinnia, sunflower and marigold. Alternaria citri causes leaf spot diseases of several citrus fruit species, notably mandarin orange and tangerine. In the work

presented in this thesis, initial culturing experiments were undertaken with this fungus, using standard Czapek Dox growth medium. Treatment of the culture filtrate, and continuous extraction of the fungal mycelium yielded several secondary metabolites.

RESULTS AND DISCUSSION

The culture filtrates from Alternaria citri, strain 106.27, were found to be toxic to clover leaves (inserted into the aqueous broth), causing visible chlorosis after about two days, followed quickly by wilting and death. Other samples of clover were subjected to a drop of culture filtrate on the scratched leaf surface. These samples suffered no ill effects.

The culture filtrates of A.citri were acidified and extracted with ethyl acetate. On standing, a white solid precipitated from the ethyl acetate solution and was filtered off and found to be acidic. The ethyl acetate was evaporated from the filtrate to yield a dark brown viscous oil containing some solid. Thin-layer chromatography of the oil in chloroform showed several spots. The solid was separated by redissolving the oil in ethyl acetate and proved to be acidic. A third acidic fraction was obtained by extracting an ethyl acetate solution of the oil with sodium bicarbonate. Subsequent extraction of the ethyl acetate solution with cold aqueous sodium hydroxide yielded a negligible amount of material. Some neutral material remained in the ethyl acetate. A second

neutral extract was obtained from an identical extraction procedure of a later batch of A.citri cultures.

FIRST ACID FRACTION 1A

This was an amorphous white powder soluble in water from which small, needle-like crystals separated on standing (substance A). Accurate mass measurement on the molecular ion of the material (A) indicated a molecular weight of 191.0428, corresponding to a formula of $C_6H_9NO_6$; this was confirmed by elemental analysis. A partial element listing is shown in Table 1.1. A few metastable ions are apparent

TABLE 1.1

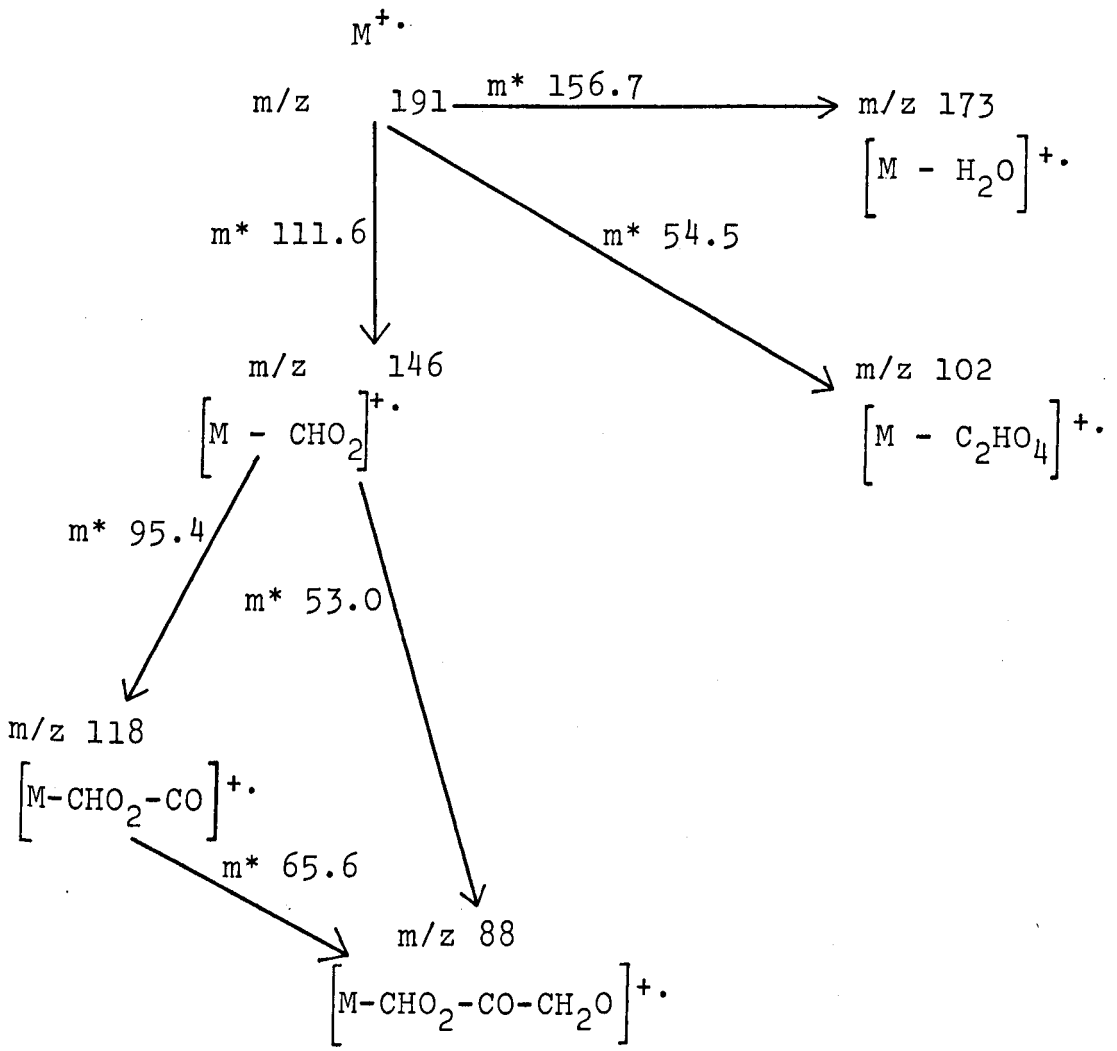
PARTIAL ELEMENT LISTING FOR THE MASS SPECTRUM OF METABOLITE A

M/Z	% RELATIVE ABUNDANCE	COMPOSITION	ERROR (p.p.m.)
191.0428	2.17	$C_6H_9NO_6$	0.1
173.0332	2.68	$C_6H_7NO_5$	0.8
146.0459	71.01	$C_5H_8NO_4$	0.6
128.0328	2.03	$C_5H_6NO_3$	1.9
118.0508	1.00	$C_4H_8NO_3$	0.4
102.0546	23.83	$C_4H_8NO_2$	0.9
100.0392	4.51	$C_4H_6NO_2$	0.7
88.0402	69.90	$C_3H_6NO_2$	1.6
86.0247	4.33	$C_3H_4NO_2$	0.5
60.0464	12.66	C_2H_6NO	1.5

in the spectrum and these were used to determine the major fragmentation pathways (Figure 1.1). Infrared absorption data of (A) showed the presence of hydroxyl and carbonyl

FIGURE 1.1

FRAGMENTATION PATHWAYS OF METABOLITE A



groups. Due to the insolubility of the compound, no data were available from ultraviolet or p.m.r. spectroscopy. Attempted methylation of this material with diazomethane also failed due to its insolubility in ether and other organic solvents. However, esterification with acidic ethanol yielded a pale yellow oil, the mass spectrum of which had a molecular ion at m/z 275 suggesting that it was the triethyl ester of the original material.

Accurate mass measurement confirmed the formula as $C_{12}H_{21}NO_6$, showing that 3 x C_2H_4 had been added during esterification. The infra-red spectrum of this tri-ester showed the presence of a carbonyl group, but no longer any OH peak. The ultra-violet spectrum showed only end-absorption below 240 nm.

The p.m.r. spectrum was simple and showed the methyl and methylene of an ethoxy group, and a singlet, in the ratio 3:2:2 respectively. As there are twenty-one protons in the ester, the molecule must possess symmetry. This was confirmed by the c.m.r. spectrum which had only a single peak in the carbonyl region and three other peaks. ^1H-Decoupling of these peaks showed two different methylene groups and one methyl group.

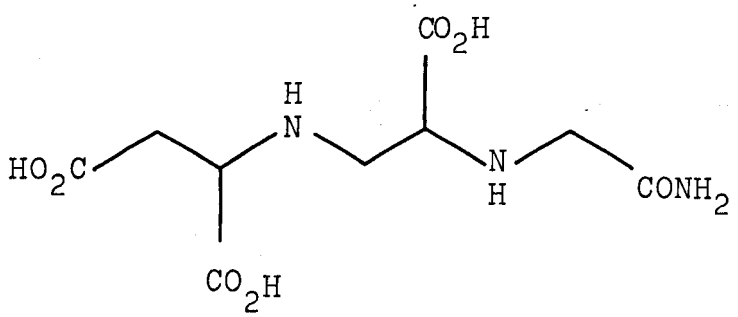
Only one structure (XCIII) fits the spectral data and the formula, $C_{12}H_{21}NO_6$, for the triester.

$N(CH_2CO_2CH_2CH_3)_3$ (XCIII)

$N(CH_2CO_2H)_3$ (XCIV)

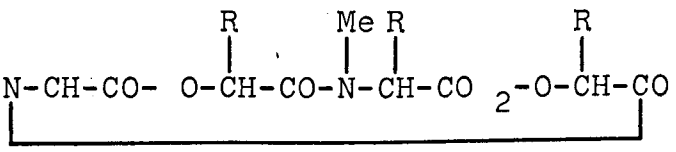
The original acid must be the known nitrilotriacetic acid (XCIV). This deduction was confirmed by comparison of the material with an authentic sample (melting point and spectroscopic properties). However, it is apparently the first time nitrilotriacetic acid has been isolated as a natural product.

Nitrilotriacetic acid (NTA) is well known for its ability to form complexes with metals and, in this respect, is very similar to ethylenediaminetetraacetic acid (EDTA). Complexing ability is not uncommon in secondary metabolites, but the stability constants for complexation of some cations are high.[156] Fusarium lycopersici produces fusaric acid (LXII)[105] and lycomarasmine (XCV),[157] both phytotoxic.



(XCV)

Their toxicity is probably due to their chelating ability. Fusaric acid strongly inhibits enzymes that depend on copper and iron ions for their activities.[158] Lycomarasmine forms a weak chelate with iron causing iron to accumulate to toxic levels in the plant. Fusarium oxysporum produces the cyclic depsipeptides, enniatin A (XCVI) and B (XCVII),[159] which are potent phytotoxins.



(XCVI) R = EtCH(Me)

(XCVII) R = Me_2CH

Their toxicity is apparently due to their ability to form complexes with the potassium cation. As a result, gradients of K^+ across the cellular or mitochondrial membranes are destroyed.[160]

Nitrilotriacetic acid has been shown to exhibit antibacterial, fungicidal, and herbicidal activity.[161] In a study of several chelating agents, including NTA and EDTA, it was found that the viability of gram negative bacteria was diminished by the presence of chelating agents.[162]

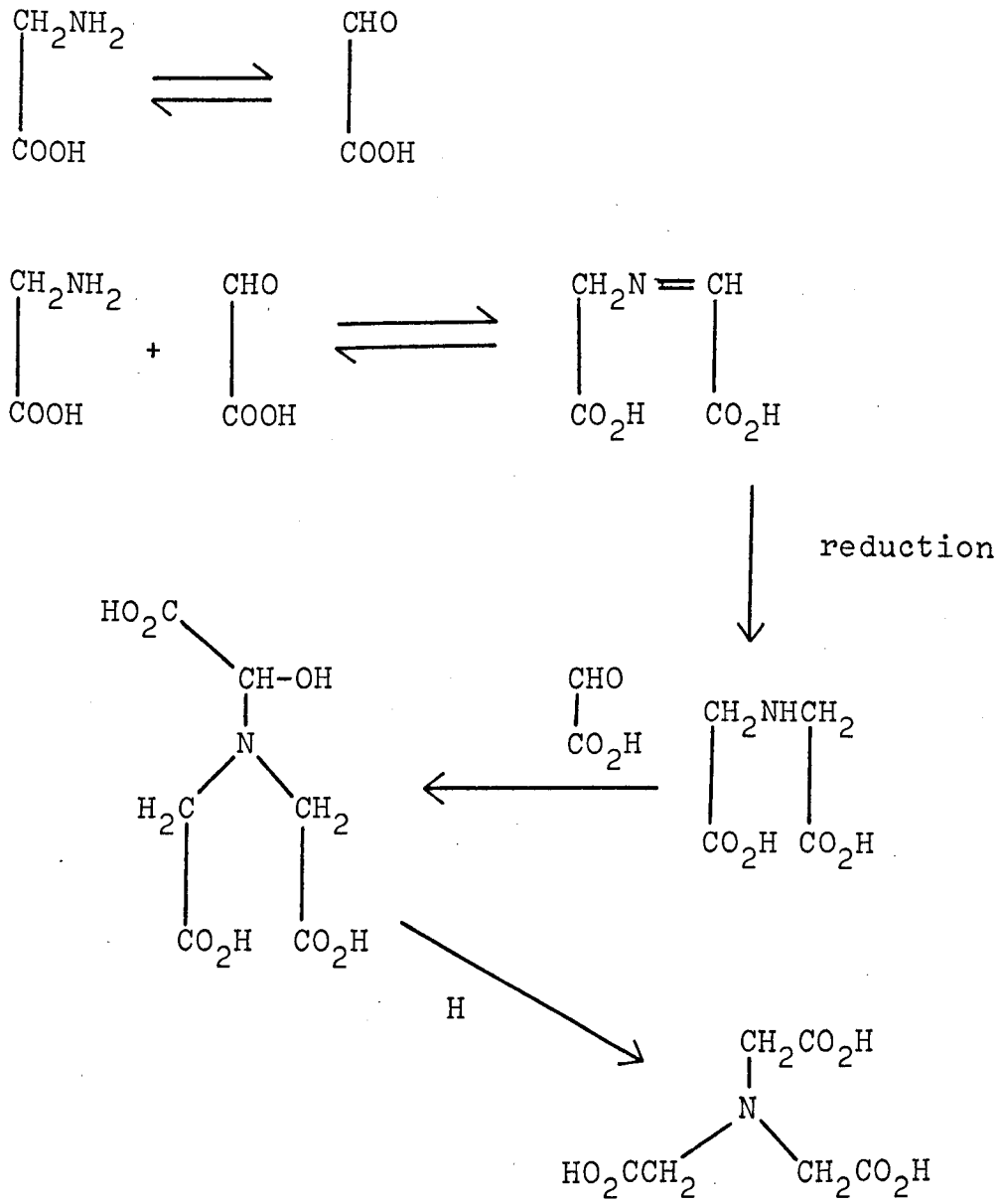
The activities of these compounds towards viability, cell lysis, and the release of intracellular materials was related to chelating ability.

NTA is also used to a large extent in complexometric titrations, along with other aminopolycarboxylic acids.[163] The anion of NTA has four atoms available for co-ordination to a metal cation in such a way that five-membered chelate rings are produced. The aminopolycarboxylic acids form complexes even with the rare-earth metals and with the alkaline earth metals and, to a slight extent, with Li^+ and Na^+.

Although no work has been done on the biosynthesis of NTA, it has been proposed[164] that NTA arises from the condensation of one molecule of glycine with two molecules of glyoxalic acid as in Figure 1.2

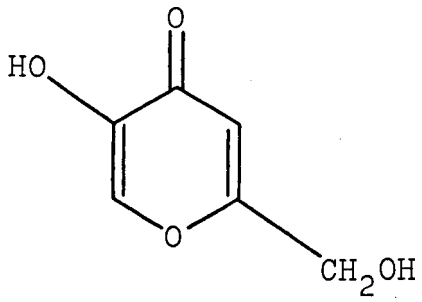
FIGURE 1.2

PROPOSED BIOSYNTHESIS OF NITRILOTRIACETIC ACID



SECOND ACID FRACTION

This was an oily orange solid. A crystalline free acid (B) was obtained quite easily by crystallization from methanol. The compound (B) was readily identified as kojic acid (XCVIII) by spectroscopic data and melting point.



(XCVIII)

Kojic acid was first isolated from the fungus, Aspergillus oryzae,[165] and was later found to be produced in high yield by many fungi, in particular A.flavus. The chemistry of kojic acid was studied by Yabuta,[166] who was also responsible for elucidating its chemical structure. Much of the early work on kojic acid was related to its antimicrobial properties, but it is of no commercial use due to its high mammalian toxicity.[167]

Kojic acid has also been found to be weakly phytotoxic.[168] The phytotoxicity of several fungi has been found predominantly to be due to the large quantities of kojic acid that accumulate in the fungi.

Early biosynthetic studies involving feeding of ^{14}C-labelled precursors, with subsequent degradation of the products, indicated that kojic acid is formed directly from D-glucose[169] which can be oxidised to 3-ketogluconic acid and then form kojic acid by enzymatic dehydration and reduction.[170,171] It has been shown that gluconic acid and gluconolactone both serve as precursors in kojic acid biosynthesis.[172]

THIRD ACID FRACTION

The $NaHCO_3$ extract of the liquor was a dark brown oil. After examination by t.l.c., the oil was chromatographed on a silica gel column using chloroform as the eluent. Left overnight, a small quantity of long needle-like crystals (C) formed on the sides of the collecting tubes. Concentration of the solvent yielded more solid (C). Preparative tlc of the solid (C) followed by crystallization from ethyl acetate gave a small quantity of colourless crystals, melting point 205 - 207°C. Accurate mass measurement on the molecular ion of the compound (C) indicated a molecular weight of 188.05804, suggesting a formula of $C_{10}H_8N_2O_2$. Micro-analysis confirmed the presence of nitrogen in the molecule. A partial element listing is shown in Table 1.2.

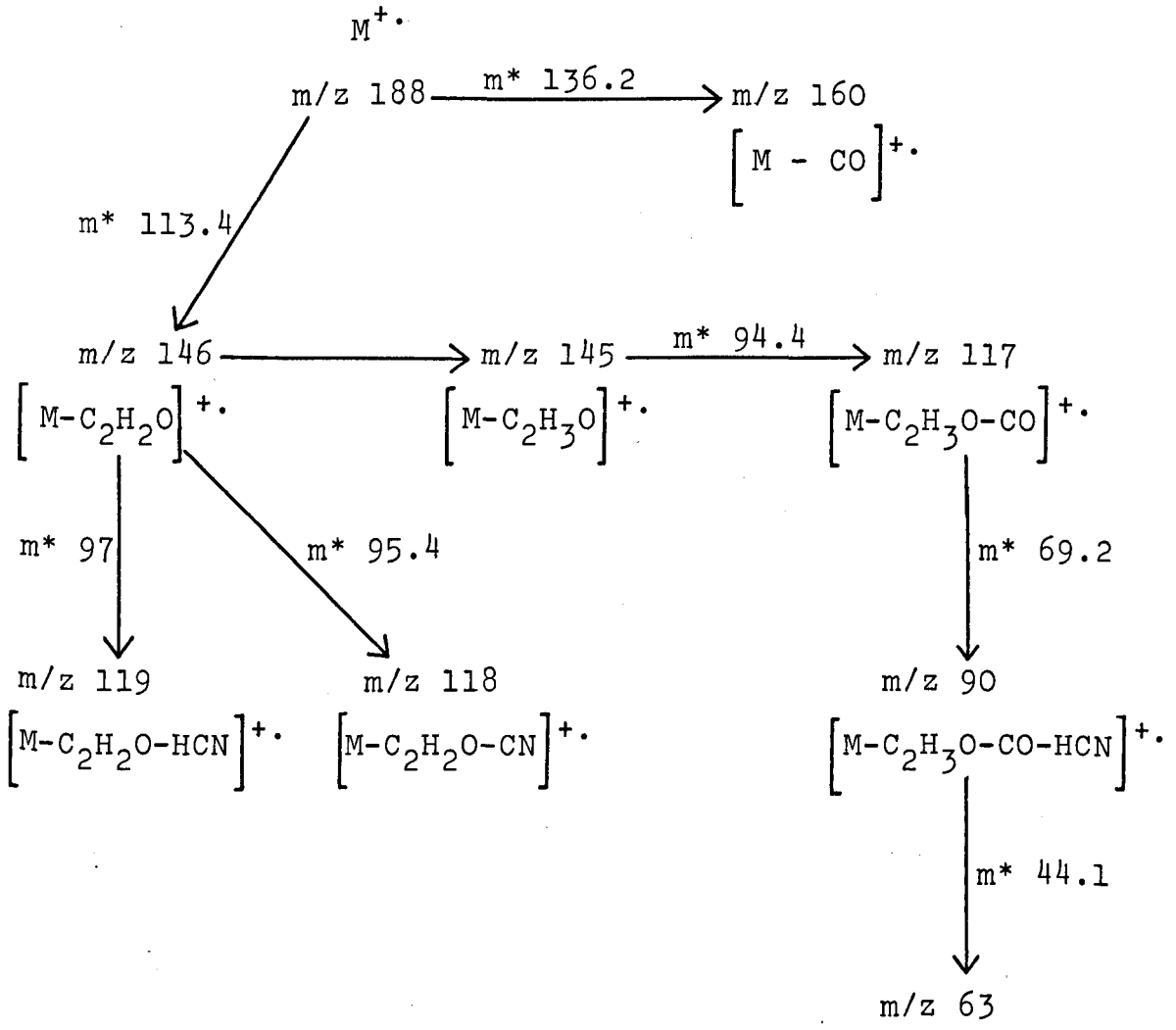
TABLE 1.2

PARTIAL ELEMENT LISTING FOR MASS SPECTRUM OF 2-ACETYL-QUINAZOLINONE

m/z	RELATIVE ABUNDANCE	COMPOSITION	ERROR (p.p.m.)
188.0587	100.0	$C_{10}H_8N_2O_2$	0.5
160.0640	29.4	$C_9H_8N_2O$	1.9
146.0489	71.1	$C_8H_6N_2O$	4.8
145.0415	40.4	$C_8H_5N_2O$	2.1
132.0444	2.2	C_8H_6NO	2.7
119.0372	48.3	C_7H_5NO	0.5
118.0538	37.5	$C_7H_6N_2$	3.7
117.0462	12.1	$C_7H_5N_2$	7.7
90.0361	41.7	C_6H_4N	9.4

FIGURE 1.3

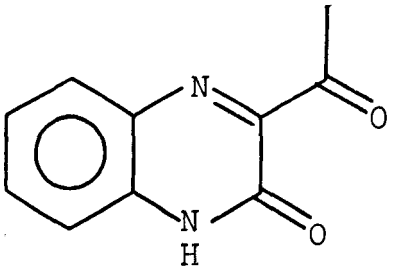
MAJOR FRAGMENTATION PATHWAYS OF 2-ACETYLQUINAZOLINONE



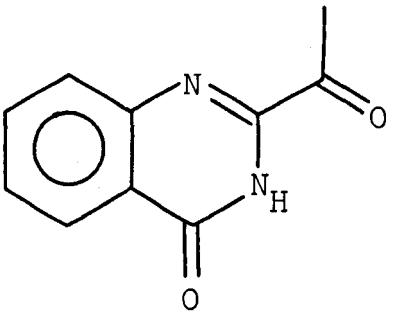
The major fragmentation pathways of compound (C) were determined from the meta stable ions present in the mass spectrum and are shown in Figure 1.3. An infrared spectrum indicated either NH or OH, and also carbonyl and unsaturated groups, whilst ultraviolet spectroscopy revealed a strong absorption band centred at 302 nm (ε = 11,500). The p.m.r. spectrum contained four strongly coupled, contiguous

aromatic hydrogens and a singlet methyl corresponding in chemical shift to that of a methyl in an acetyl group, and either an NH or OH hydrogen at δ 10.1. The mass spectrum also indicated the presence of an acetyl group by the loss of 43 mass units from the molecular ion.

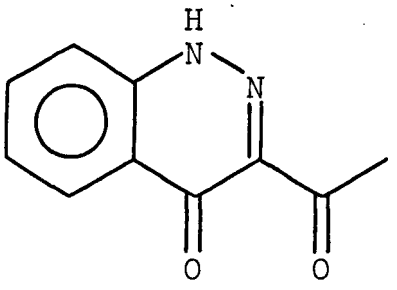
At this stage, a definite structure could not be assigned to the molecule, although a few candidate structures could be proposed (structures XCIX - CIV).



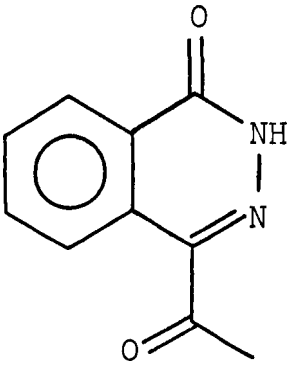
(XCIX)



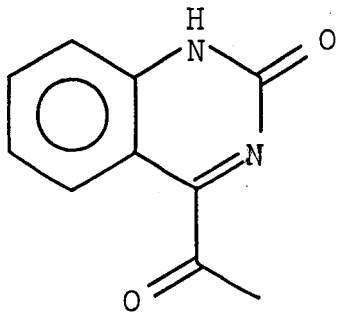
(C)



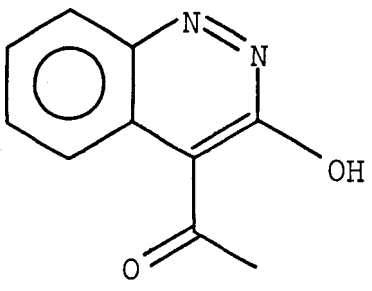
(CI)



(CII)



(CIII)



(CIV)

The supply of compound (C) was inadequate for degradative studies; 68 mg of crude acid (C) were obtained initially. However, compound (C) could be obtained in well-formed crystals from slow evaporation of its solution in ethyl acetate, so it was decided to undertake an X-ray crystallographic study[218] of the compound to determine its structure.

Precession X-ray photographs were taken on a Charles Supper Co. precession camera using a Philips PW 1720 X-ray generator. Weissenberg photographs were taken on a Stoe camera using an identical X-ray source to the precession camera. Agfa Gevaert X-ray film was used, and the films were developed in the chemistry department. Care was taken at all times to ensure that the photographs were fully developed and that the photographs remained free of scratches and abrasions, as this would diminish the accuracy provided by densitometer measurements.

A single crystal of compound (C) was cut with a new razor blade, mounted on a glass capillary using shellac glue, and attached to a goniometer head with plasticine.

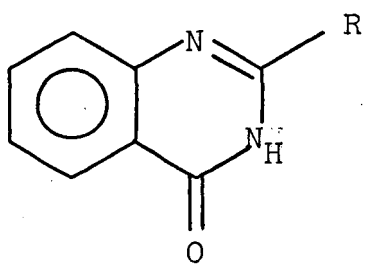
Initial aligning photographs were taken on the Weissenberg camera using the double oscillation technique.[173] The crystal was aligned accurately along its axes on the precession camera. When it had been accurately aligned, the crystal was transferred back to the Weissenberg camera, and data photographs were taken of the different levels of the crystal lattice using a film pack of five films; the film closest to the X-ray source being the most exposed, and also shielding the films behind it. This was done so that a full range of intensities of the spots in the X-ray diffraction pattern was available.

The density of the crystal was measured by floating the crystal on carbon tetrachloride, and adding petroleum ether 40 - 60 with mixing until the crystal remained suspended in the liquid. A sample of this liquid was placed in a pre-weighed accurate measuring vessel, and the vessel reweighed.

The X-ray data reported are as follows. Initial photographs showed that the crystals (C) were triclinic with a = 8.44(2), b = 10.19(2), c = 5.25(2)Å, α = 102.4(1), β = 90.0(1), γ = 101.2(1)°, U = 431.3Å^3, Z = 2, Dx = 1.49 gcm^{-3}. Intensity measurements were made with Cu-Kα radiation (λ = 1.5418Å) out to θ = 37.45°. 822 Unique reflections were observed, the intensities being measured[219] by a microdensitometer from Weissenberg photographs of layers h0 l to h8 l for a crystal of dimensions 0.3 x 0.2 x 0.2 mm. Cell dimensions were determined from

precession photographs and interlayer scale factors. The structure was solved in the space group $P\bar{1}$ by direct methods using MULTAN[174] with some difficulty experienced because of the problem of locating reasonable structural fragments, but misplaced in the cell by the MULTAN programme, being repeatedly encountered. Success was finally achieved by insertion of one such fragment as a group of known orientation, but random position, into the programme and manipulation of the parity group scale factors. The structure was refined satisfactorily on the F values by full-matrix least squares using the XRAY system.[175] At an intermediate stage, the positions of the H atoms were located from a difference map and subsequently refined with isotropic temperature factors; all other atoms were refined using anisotropic thermal parameters. In the final stages the interlayer scale factors were refined individually and an extinction correction ($g = 7.04 \times 10^{-3}$) was applied. At convergence, R was 0.044.

The compound (C) was found to be 2-acetyl-4(3H)-quinazolinone (C). The atomic numbering system is shown in Figure 1.4, and the crystal structure is depicted in



(C) R = $COCH_3$

(CV) R = Ph

(CVI) R = 2-C_5H_4N

(CVII) R = $CH(OH)CH_3$

FIGURE 1.4

ATOMIC NUMBERING SYSTEM FOR 2-ACETYLQUINAZOLINONE.

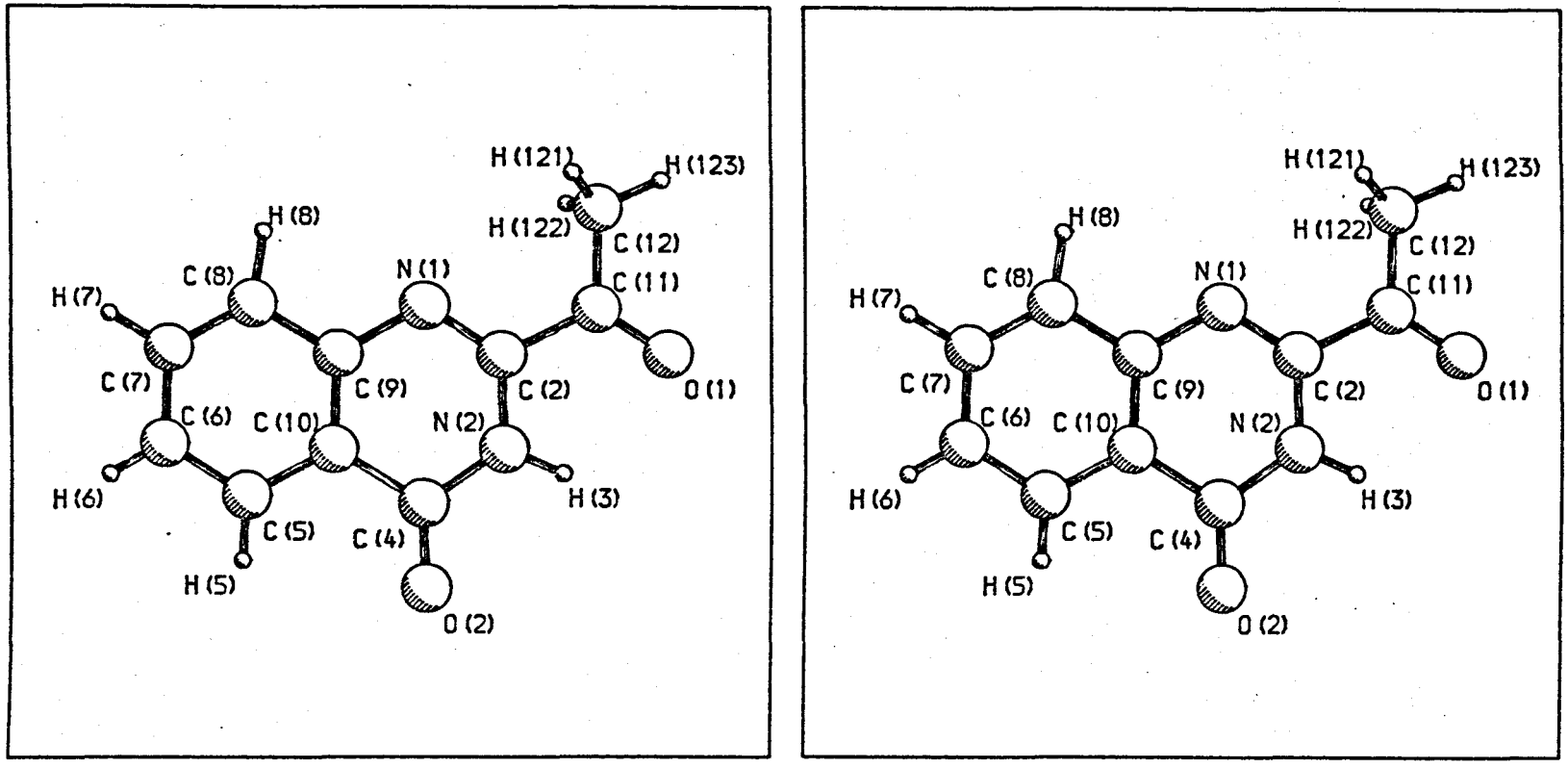


Figure 1.5. The bond lengths, bond angles and torsion angles (not involving H) are listed in Figure 1.6. A search[220] of a crystallographic database revealed 2-acetyl-4(3H)-quinazolinone to be the simplest quinazolinone system for which accurate geometric data are available. Other simple, substituted derivatives whose structures are known include the 2-phenyl analogue[176] (CV) and the 2-pyridyl analogue[177] (CVI) (in chelation with Cu^{II}). The structure of the aromatic nucleus found for compound (C) is closely comparable with the aromatic parts of the structures of compound (CV) and (CVI); in all cases, the ten-membered, bicyclic ring system is close to planarity.

There is considerable bond-length variation around the heterocyclic ring indicating substantial bond-length fixation. However, a close comparison of the structures of compounds (C), (CV) and (CVI) reveals small, but significant trends in bond-length variation which presumably reflect differing degrees of π-electron delocalization.

The carbonyl group of the acetyl substituent in the metabolite (C) is disposed in a transoid sense with respect to the N(1)-C(2) double bond. This is perhaps not surprising in view of the preference of the simple molecule methyl vinyl ketone for adopting such a transoid configuration.[178] The structure determination definitely establishes that atom N(2) bears an H atom, i.e., that the structure is as shown in Figure 1.4, and not one of the alternative tautomeric forms (4-hydroxyquinazoline (CVIII) or 4(1H)-quinazolinone (CIX)).

FIGURE 1.5

CRYSTAL STRUCTURE OF 2-ACETYLQUINAZOLINONE

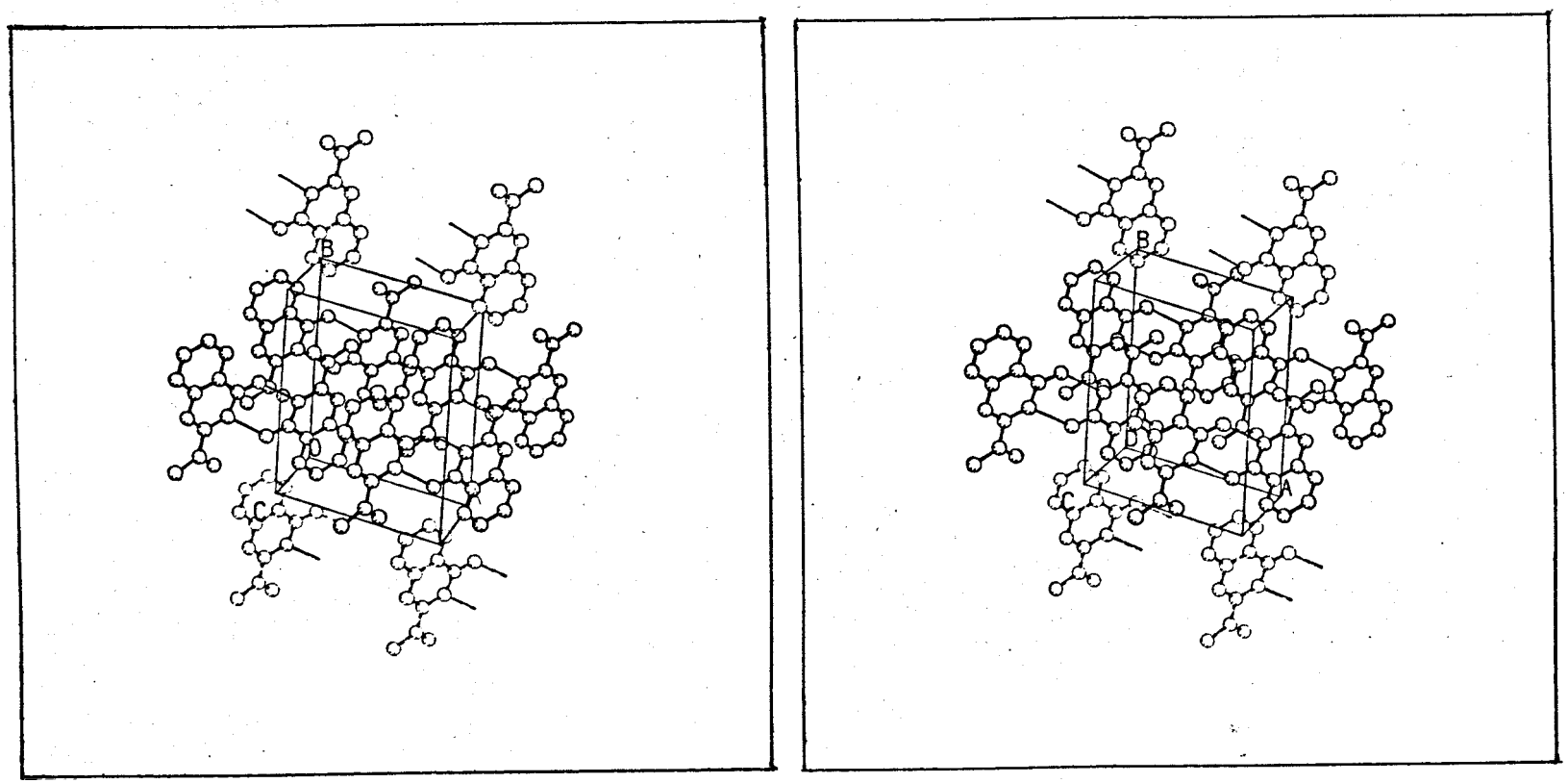
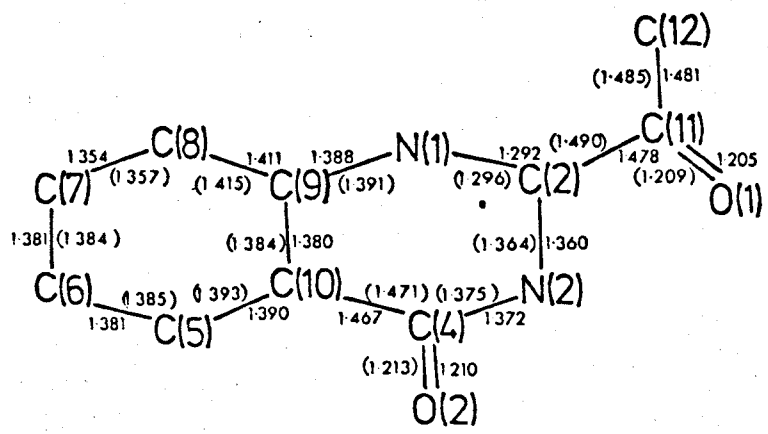
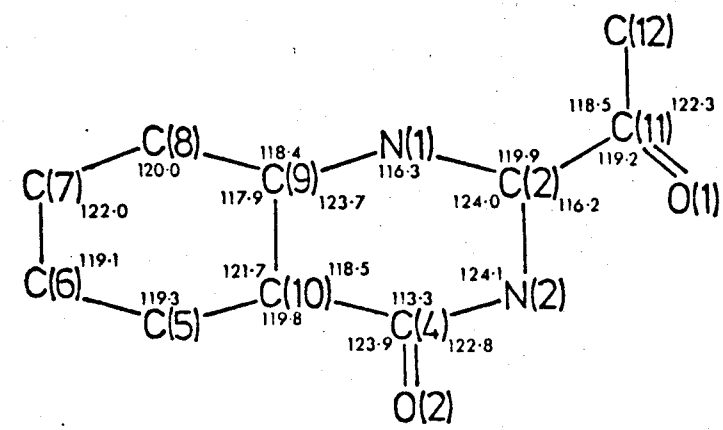


FIGURE 1.6

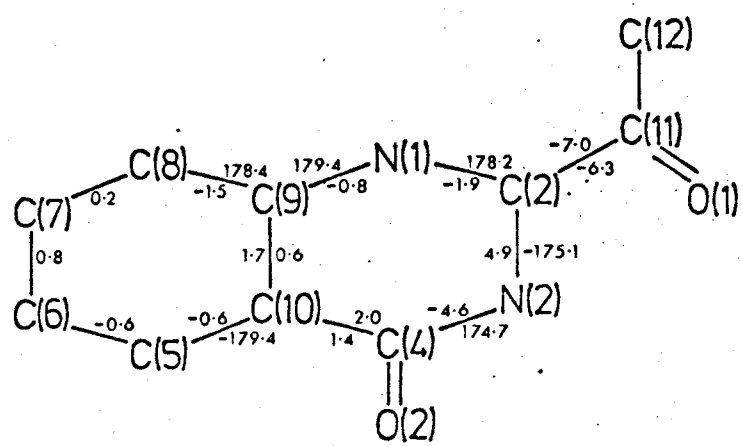
BOND LENGTHS, BOND ANGLES AND TORSION ANGLES FOR 2-ACETYLQUINAZOLINONE



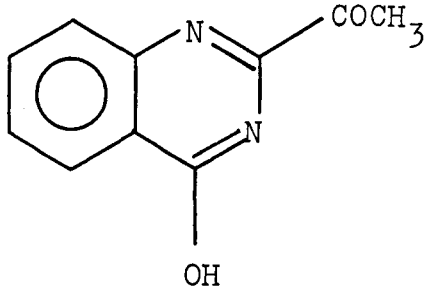
N(2)-H(3)	0.894
C-H	0.902 - 1.038



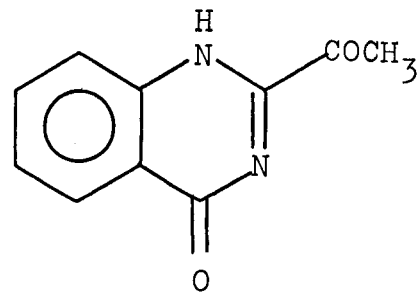
H(3)-N(2)-C(2)	114.4
H(3)-N(2)-C(4)	121.5
H-C-H	99.2 - 117.4
Aromatic C-H	112.4 - 127.6



N(1)-C(9)-C(10)-C(5)	-178.1
N(1)-C(2)-C(11)-O(1)	173.7
N(2)-C(4)-C(10)-C(5)	-179.3
N(2)-C(2)-C(11)-C(12)	173.0
O(2)-C(4)-C(10)-C(9)	-177.3
C(8)-C(9)-C(10)-C(4)	-179.6



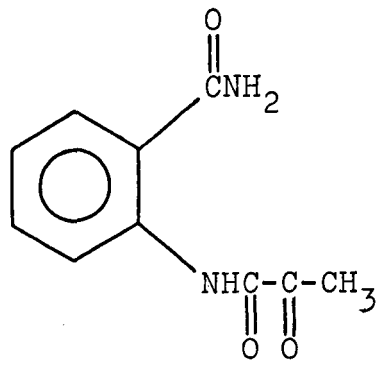
(CVIII)



(CIX)

In the crystal, the amide system undergoes intermolecular hydrogen bonding with a neighbouring molecule (N(2)...O(2) 2.899Å) so that dimers are present.

After the structure of 2-acetyl-4(3H)-quinazolinone had been determined, it was found that the compound had been isolated previously from the fungus Fusarium culmorium.[179] It has also been described as a transformation product of chrysogine[180] (CVII) from Penicillium chrysogenum. The compound 2-pyruvoylaminobenzamide (CX) from P.chrysogenum,[181] P.notatum and Collectotrichum lagenarium[182] shows a potential biosynthetic relationship to 2-acetylquinazolinone.



(CX)

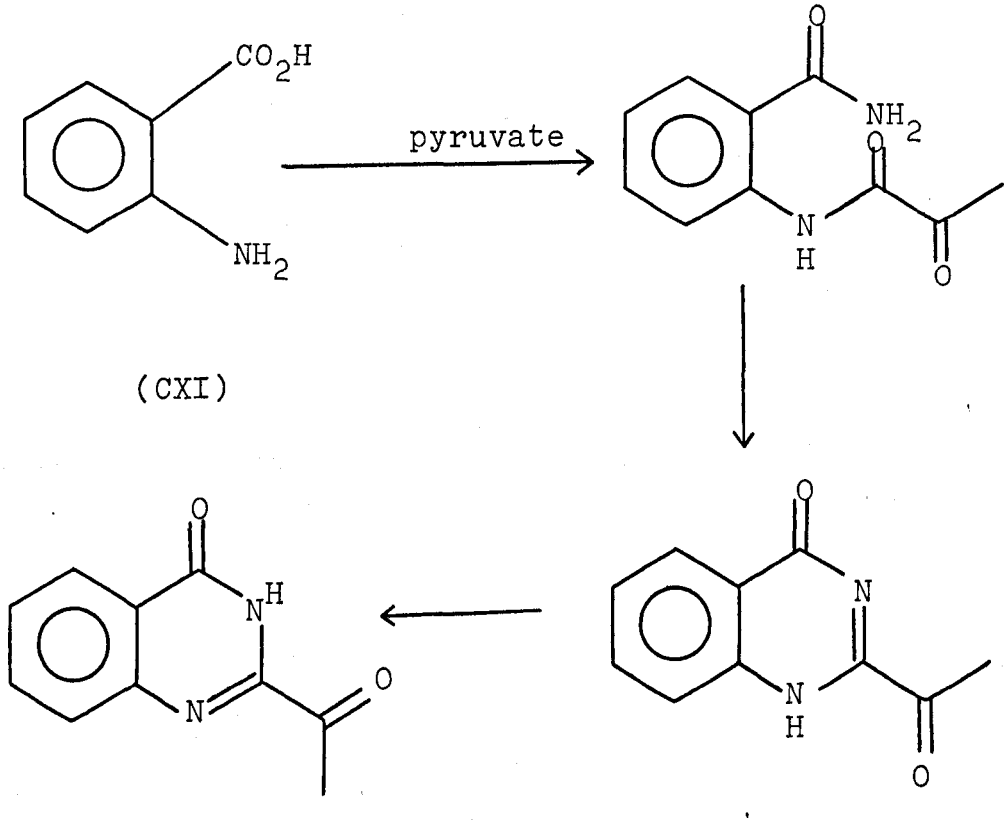
Although 2-acetylquinazolinone was recovered from the P.chrysogenum fermentation, it was considered to be an

artifact[181] of the isolation procedure.

Biosynthesis of 2-acetylquinazolinone and chrysogine is possibly in a 2-pyruvoylaminobenzamide (CX) formed from anthranilic acid (XCI) by the route shown in Figure 1.7.

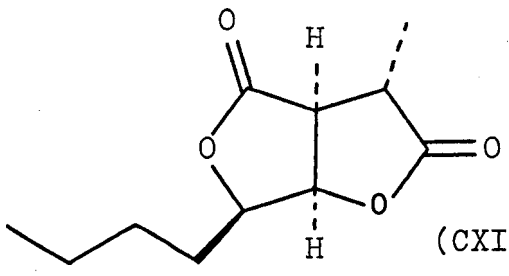
FIGURE 1.7

PROPOSED BIOSYNTHESIS OF 2-ACETYL-4(3H)-QUINAZOLINONE FROM ANTHRANILIC ACID.



NEUTRAL FRACTION

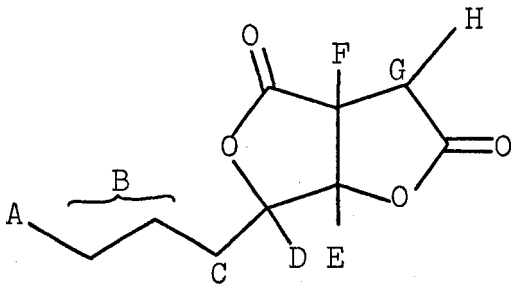
On evaporation of the fraction that was not soluble in acid or base by the previously described extraction procedures, a dark brown oil remained. The oil was chromatographed on a silica-gel column using chloroform and an increasing proportion of methanol as eluents. With 3% methanol in chloroform as the eluent, a band separated on the column and was collected. The solvent was removed *in vacuo* leaving a pale orange oily solid which was purified further by preparative thin-layer chromatography on silica-gel in dichloromethane. A colourless oily solid was obtained following soxhlet extraction of the silica-gel and evaporation of the solvent. This solid (D) crystallized in plates from methanol. Accurate mass measurement of the purified material (D) indicated a molecular weight of 212.10294, corresponding to a molecular formula of $C_{11}H_{16}O_4$ which agreed with the results of elemental analysis. Infrared absorption spectra showed the presence of C=O and C-O groups, but no hydroxyls or alkenes. The ultra-violet spectrum exhibited only end-absorption below 240 nm. P.m.r. and c.m.r. spectra were obtained and are summarised in Tables 1.3 and 1.4 respectively. Comparison of the data available for metabolite (D) with those contained in published reports in dihydrocanadensolide (CXII) revealed the two compounds to be identical.



(CXII)

TABLE 1.3

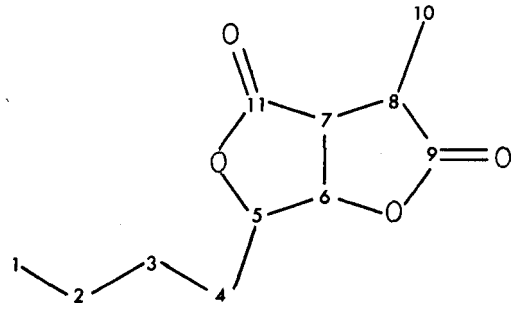
P.M.R. SPECTRUM OF DIHYDROCANADENSOLIDE



CHEMICAL SHIFT (δ)	INTEGRAL	MULTIPLICITY	SPLITTING (Hz)	ASSIGNMENT
5.17	1	doublet of doublets	4.0,6.0	E
4.60	1	doublet of triplets	4.0,7.5	D
3.2	1	doublet of doublets	6.0,1.3	F
3.05	1	doublet of quartets	1.3,7.5	G
1.87	2	multiplet		C
1.40	4	multiplet		B
1.40	3	doublet	7.5	H
0.92	3	triplet		A

TABLE 1.4

C.M.R. SPECTRUM OF DIHYDROCANADENSOLIDE



CHEMICAL SHIFT (ppm)	MULTIPLICITY WITH OFF RESONANCE DECOUPLING	ASSIGNMENT
13.82	quartet	1
17.05	quartet	10
22.40	triplet	2 - 4
27.47	triplet	
28.48	triplet	
38.36	doublet	7 - 8
48.93	doublet	
78.30	doublet	5 - 6
82.29	doublet	
174.60	singlet	9 - 11
176.64	singlet	

A partial element listing is shown in Table 1.5. Some metastable ions were present in the mass spectrum and were used to determine the major fragmentation pathways (Figure 1.8).

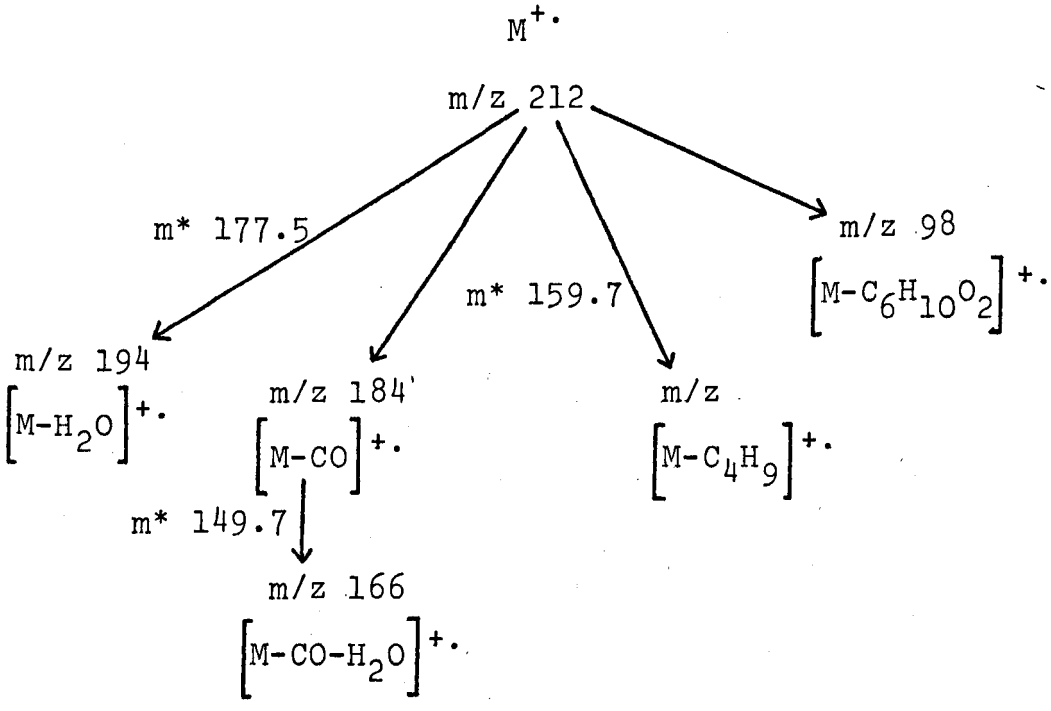
TABLE 1.5

PARTIAL ELEMENT LISTING FOR MASS SPECTRUM OF DIHYDRO-CANADENSOLIDE

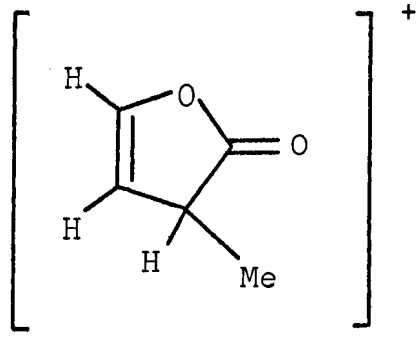
m/z	RELATIVE ABUNDANCE	COMPOSITION	ERROR (p.p.m.)
212.10298	30.93	$C_{11}H_{16}O_4$	8.5
194.09432	5.15	$C_{11}H_{14}O_3$	0.1
184.10991	6.19	$C_{10}H_{16}O_3$	0.1
166.09937	19.59	$C_{10}H_{14}O_2$	4.2
155.03432	17.53	$C_7H_7O_4$	7.0
139.07598	21.65	$C_8H_{11}O_2$	1.3
125.02388	49.48	$C_6H_5O_3$	1.1
98.03692	100.00	$C_5H_6O_2$	3.8

FIGURE 1.8

MAJOR FRAGMENTATION PATHWAYS OF DIHYDROCANADENSOLIDE



Mass spectrometrically, the most favoured cleavage of γ- or δ- lactones is the rupture of the bond between a side-chain and the γ- or δ- carbon atom.[183] The ion at m/z 155 could arise from dihydrocanadensolide by loss of C_4H_9 (the n-butyl side-chain). Similar cleavage with respect to the other lactone ring, followed by a second bond fission could result in the formation of an ion such as that shown in structure (XCIII) at m/z 98.

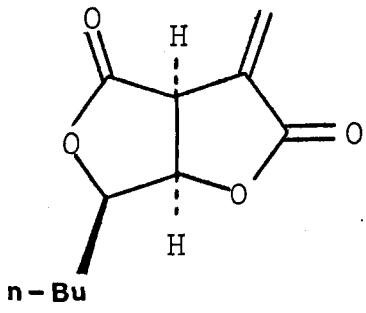


(XCIII)

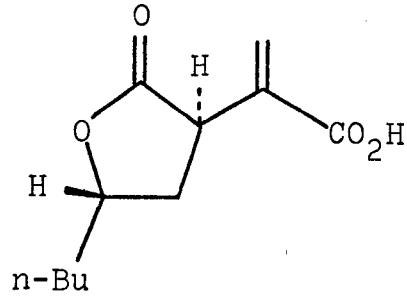
Dihydrocanadensolide was first isolated from Penicillium canadense and was characterized by McCorkindale et al.[184] Shortly afterwards, it was isolated from the liquors of Aspergillus indicus.[185] Dihydrocanadensolide has been found to possess antifungal[186] and weak antibiotic activity, as well as antiulcerous properties.[187]

Several other fungal metabolites with related structures are known: canadensolide (CXIV), from P.canadense[184]

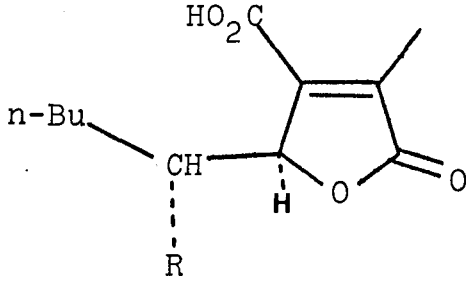
and A.flavus,[188] and canadensic acid (XCV), isocanadensic acid (XCVI), and hydroxyisocanadensic acid (XCVII) and dihydroisocanadensic acid (CXVIII) from P.canadense[189] are all biosynthetically related. The compound



(CXIV)

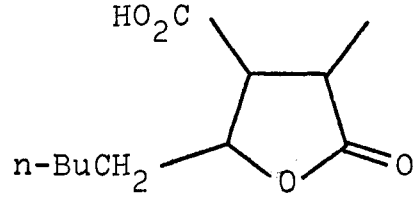


(CXV)



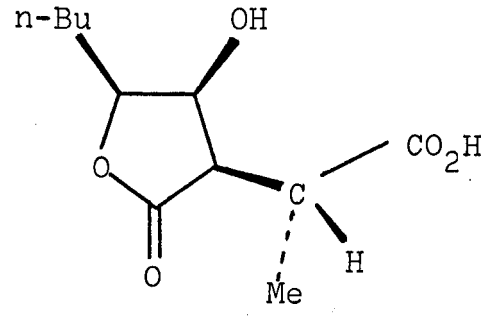
(CXVI) R = H

(CXVII) R = OH



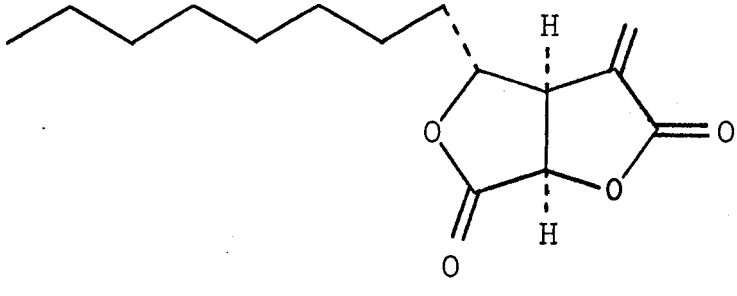
(CXVIII)

(+)-2-(5β-n-butyl-4β-hydroxy-2-oxotetrahydrofuran-3β-yl)-2α-methylacetic acid (CXIX)[190] has also been isolated from P.canadense.

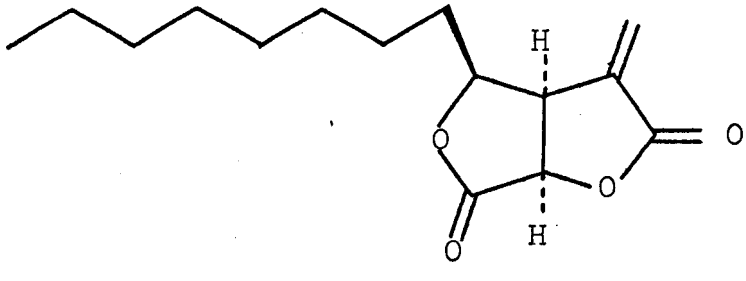


(CXIX)

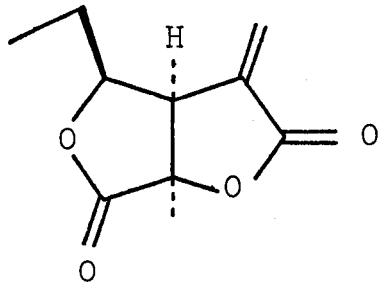
Metabolites with a dilactone skeleton include avenaciolide[191] (CXX) and 4-isoavenaciolide[192] (CXXI) from Aspergillus avenaceus, and ethisolide (CXXII)[192] from Penicillium ACC 3903 and P.decumbens.



(CXX)



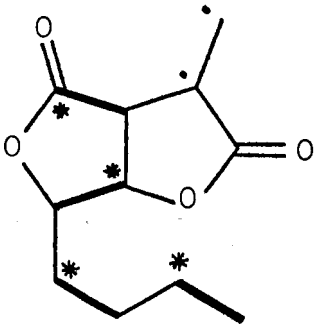
(CXXI)



(CXXII)

The stereochemistry and stereoselective synthesis of canadensolide and dihydrocanadensolide have received much attention,[193-198] the absolute stereochemistry being determined as (-)-canadensolide (CXIV) by stereoselective synthesis from D-glucose.[198] The relative stereochemistry of the methyl group of dihydrocanadensolide can be suggested from its p.m.r. spectrum. The magnitude of J_{FG}, (see Table 1.3), 1.3 Hz necessitates that these protons are trans-related since a cis-relationship would imply an eclipsed interaction, and therefore a much larger J value,[199] given the electronegativities of the groups around these hydrogens.

Biosynthetically, it has been shown for dihydrocanadensolide[200] that ^{14}C -acetate was incorporated specifically into the fatty acid portion of the molecule, and ^{13}C -acetate was incorporated as per the labelling pattern indicated in structure (CXXIII). This is in accord with intact incorporation into the fatty acid part of the molecule of an alkylcitric acid precursor. However, succinic acid was incorporated as shown into the C_3 unit of dihydro-canadensolide. These results strongly favoured hexylcitric



(CXXIII)

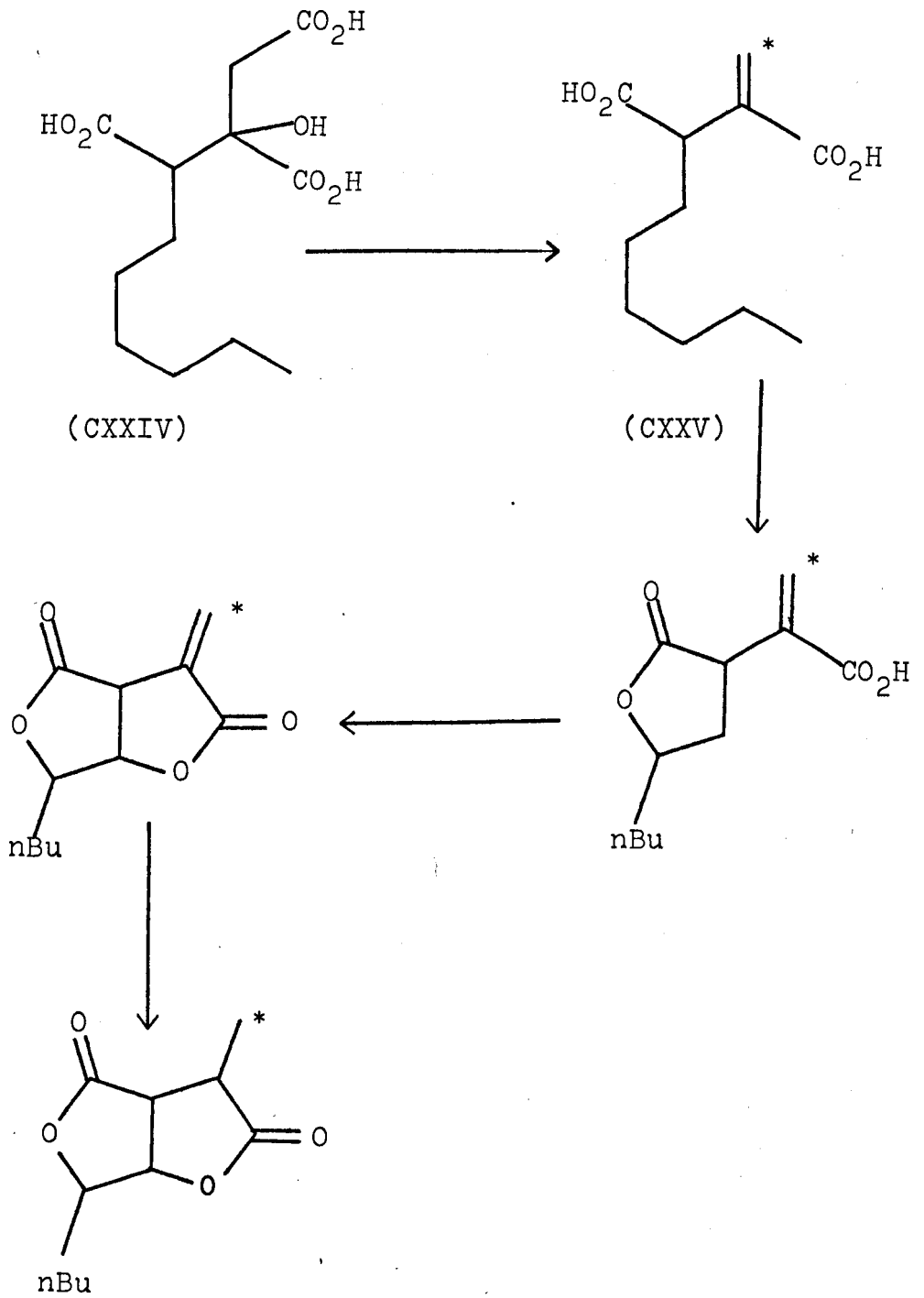
• : ^{13}C label from 2,3-$^{13}C_2$ succinic acid

*: from ^{14}C -CH_3CO_2Na

acid (CXXIV) as a precursor and it also seemed likely that the first intermediate having the same carbon sekeleton as metabolites (CXII), (CXIV) and (CXV) would he hexylitaconic acid (CXXV). This supposition has been veritifed by the efficient incorporation of a radioactively labelled sample of compound (CXXV) into the three metabolites. The same workers also proposed a biosynthetic pathway for ethisolide via butylitaconic acid, and isolated several possible intermediates in this pathway. It seems likely that the biosyntheses of the dilactone metabolites which have been mentioned are closely related.

FIGURE 1.9

PROPOSED BIOSYNTHETIC PATHWAY TO DIHYDROCANADENSOLIDE



* : ^{14}C label on hexylitaconic acid.

SECOND NEUTRAL FRACTION

In an attempt to extract other metabolites related to dihydrocanadensolide, a fresh batch of the liquors of *A.citri* were collected and examined. After initial extraction with base, the remaining liquors were chromatographed on a silica-gel column using chloroform and then chloroform/methanol as eluents. Collection of fractions from the column gave, on evaporation of the solvent, two different materials (E) and (F). The second material (F) was crystallized from ethyl acetate to give discoloured needles. Examination of compound (F) by tlc in chloroform/methanol indicated that only one component was present. Accurate mass measurement on the molecular ion of compound (F) indicated a molecular weight of 190.0741 corresponding to $C_{10}H_{10}N_2O_2$. This agreed with results from elemental analysis. A partial element listing for the mass spectrum of the metabolite is given in Table 1.6. Figure 1.10 shows the major fragmentations of compound (F) based on the metastable ions appearing in the routine mass spectrum. The infrared absorption spectrum revealed the presence of an hydroxyl group and an α,β-unsaturated ketone system, whilst ultraviolet spectroscopy indicated a strong absorption band at 266 nm (ε = 7,450). The p.m.r. spectrum revealed four aromatic protons, a methyl doublet at δ 1.65 and a methine quartet at δ 4.90. The chemical shifts and couplings of the aromatic protons were very similar to those found in the

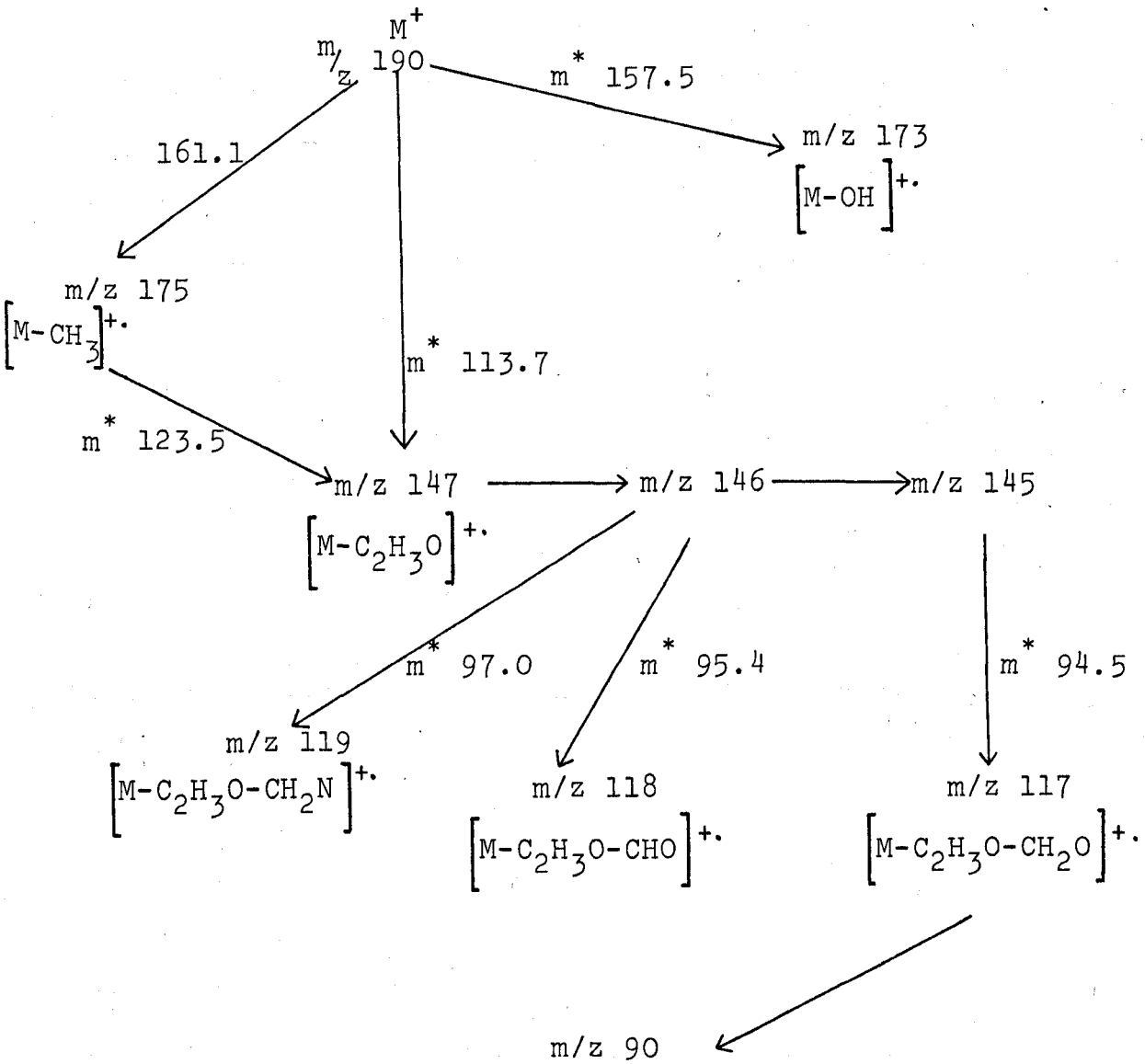
TABLE 1.6

PARTIAL ELEMENT LISTING FOR MASS SPECTRUM OF CHRYSOGINE

m/z	RELATIVE ABUNDANCE	COMPOSITION	ERROR (p.p.m.)
190.0741	100.00	$C_{10}H_{10}N_2O_2$	0.1
189.0659	11.52	$C_{10}H_9N_2O_2$	0.5
188.0538	6.37	$C_{10}H_8N_2O_2$	0.2
175.0499	53.38	$C_9H_7N_2O_2$	0.8
173.0718	75.98	$C_{10}H_9N_2O$	0.3
161.0701	4.61	$C_9H_9N_2O$	1.4
147.0549	61.93	$C_8H_7N_2O$	0.9
146.0476	19.87	$C_8H_6N_2O$	0.4
145.0397	17.00	$C_8H_5N_2O$	0.5
130.0288	9.45	C_8H_4NO	0.3
119.0384	39.47	C_7H_5NO	1.3
118.0518	10.66	$C_7H_6N_2$	1.3
117.0457	5.59	$C_7H_5N_2$	0.4
90.0355	32.06	C_6H_4N	1.2
76.0309	6.02	C_6H_4	0.4

FIGURE 1.10

MAJOR FRAGMENTATION PATHWAYS OF CHRYSOGINE (2-HYDROXYETHYL-QUINAZOLINONE).



p.m.r. spectrum of 2-acetylquinazolinone; their mass spectra were also similar. Comparison of data from this metabolite (F) with those contained in published reports on chrysogine (CVII), the reduced form of 2-acetylquinazolinone, showed the two to be identical. References to chrysogine, and the proposed biosynthesis are contained in the section on 2-acetylquinazolinone. Although there are no data on the biological activities of 2-acetylquinazolinone or chrysogine, it has been found that their possible biosynthetic precursor 2-pyruvoylaminobenzamide in some cases acts as an antiauxin-like substance.[201]

The other band from the neutral liquor extract, material (E), yielded a pale yellow solid on evaporation of solvent. After crystallization from ethyl acetate this solid was still impure. The material was purified by repeated preparative t.l.c. on silica gel in ether, and recrystallized from ethyl acetate to give colourless cubes. Accurate mass measurement of the molecular ion of compound (E) indicated a molecular weight of 398.17114, suggesting a molecular formula of $C_{23}H_{26}O_6$. A partial element listing for the mass spectrum of metabolite (E) is given in Table 1.7. The large peak at m/z 105.0366, corresponding to C_7H_5O suggests the presence of a benzoyl group in the molecule. The infrared absorption spectrum indicated the presence of carbonyl groups and an alkene, but no hydroxyl. Substance (E) was optically active, and the ultraviolet spectrum showed maxima at 243 nm (ε = 4,500) and 275 nm (ε = 950).

TABLE 1.7

PARTIAL ELEMENT LISTING FOR MASS SPECTRUM OF PARASITICOLIDE A

m/z	RELATIVE ABUNDANCE	COMPOSITION	ERROR (p.p.m.)
398.1716	42.30	$C_{23}H_{26}O_6$	1.3
288.1367	11.27	$C_{17}H_{20}O_4$	0.6
276.1342	20.30	$C_{16}H_{20}O_4$	2.0
275.1273	29.07	$C_{16}H_{19}O_4$	1.0
233.1172	13.06	$C_{14}H_{17}O_3$	0.6
228.1133	7.22	$C_{15}H_{16}O_2$	1.7
216.1143	62.49	$C_{14}H_{16}O_2$	0.8
215.1052	19.98	$C_{14}H_{15}O_2$	2.0
204.1124	7.09	$C_{13}H_{16}O_2$	2.6
203.1071	24.80	$C_{13}H_{15}O_2$	0.1
173.0590	5.64	$C_{11}H_9O_2$	1.3
161.0586	7.40	$C_{10}H_9O_2$	1.7
148.0500	15.03	$C_9H_8O_2$	2.4
106.0395	6.98	C_7H_6O	2.3
105.0366	100.00	C_7H_5O	2.6
77.0410	23.09	C_6H_5	1.9

The p.m.r. spectrum was complex and is summarised in Table 1.8. The c.m.r. spectrum is summarised in Table 1.9. The splitting pattern of the aromatic protons confirmed the presence of a benzoyl group. Also present were three methyl groups, two possibly adjacent to carbonyl groups. The infrared spectrum indicated the presence of

TABLE 1.8

P.M.R. SPECTRUM OF PARASITICOLIDE A

CHEMICAL SHIFT (δ)	INTEGRAL	MULTI-PLICITY	SPLITTING (Hz)	ASSIGNMENT
1.15	3	singlet		13
1.90	3	singlet		CH_3CO_2
2.11	3	singlet		CH_3CO_2
3.99	1	doublet	12	7
4.38	1	doublet	12	7
4.88	1	doublet	11	11
4.97	1	doublet	11	11
5.95	1	multiplet		6
7.47	2	triplet	8	4' 6'
7.57	1	triplet	8	5'
8.02	2	doublet	8	3' 7'

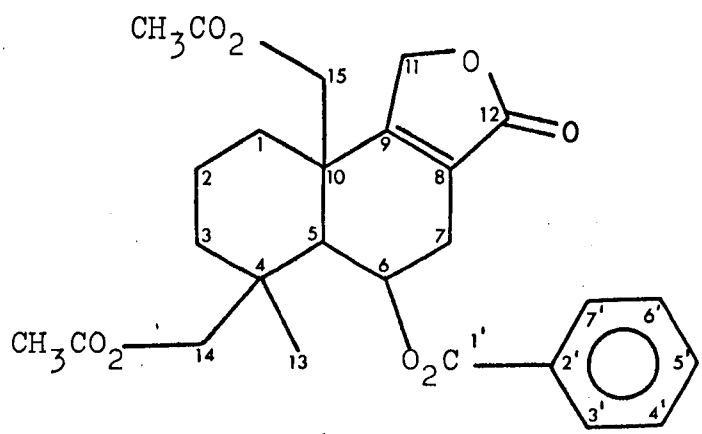


TABLE 1.9

C.M.R. SPECTRUM OF PARASITICOLIDE A

CHEMICAL SHIFT (δ)	MULTIPLICITY WITH OFF RESONANCE DECOUPLING	ASSIGNMENT
17.82	t	2
20.43	q	$\underline{C}H_3CO$
20.84	q	$\underline{C}H_3CO$
27.74	q	13
29.06	t	7
31.79	t	1
37.07	t	3
37.72	s	4
40.47	s	10
54.23	d	5
65.93	t	11
66.42	d	6
66.88	t	14
71.14	t	15
122.86	s	8
128.69	d	4' 6'
129.58	d	3' 7'
133.46	d	5'
151.59		
165.45	s	9
170.01	s	$CH_3\underline{C}O$

carbonyl groups. It was difficult to assign a structure on the limited data available, and the supply of material (E) was inadequate for degradative studies. However, the compound could be obtained in well-formed crystals, so it seemed a likely candidate for X-ray crystallography.

A suitable crystal was selected and cut to approximately 0.3 x 0.3 x 0.3 mm^3. The crystal was initially aligned roughly using a Stoe Weissenberg camera and then transferred to the precession camera to be aligned accurately. Problems were encountered at this stage, due to the number of diagonal axes that were present in the crystal lattice. Alignment along the correct crystal axes was finally achieved by remounting the crystal at approximately 45° to a crystal face.

The data collected are as follows:
Initial photographs showed that the crystals were orthorhombic with a = 14.428, b = 11.056, c = 15.062Å, V = 2399.3$Å^3$, Dx = 1.3273 gcm^{-3}. From the systematic absences in the precession and Weissenberg photographs it was found that the space group for the crystal was $P2_12_12_1$. For this space group, the number of molecules per unit cell (Z) = 4. However, using the equation

$$\rho = \frac{M \times 1.6604}{V}$$

where

M = molecular weight of compound ^{xZ}

ρ = density of compound

V = volume of unit cell,

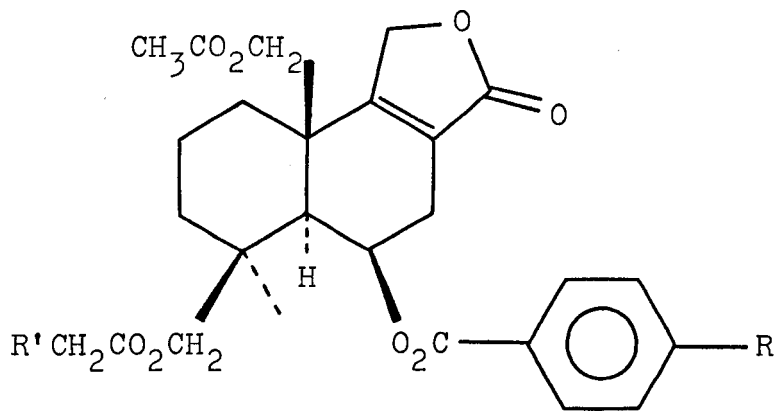
it was found that the calculated density (1.1017 gcm^{-3}) did not agree with the measured density. Using the measured density, M was calculated as 1918, so the molecular weight of the compound was approximately 479.

A fast atom bombardment (FAB) mass spectrum of compound (E) was obtained to determine whether the "molecular ion" revealed by EI mass spectroscopy was or was not in fact the molecular ion. In the FAB spectrum, peaks were present at m/z 503, 493, 479, 473, together with the largest at m/z 471, indicating a possible molecular weight of 470 (addition of a proton to compound (E) by the glycerol used as solvent for the FAB spectrum). Other significant ions were at m/z 459, 445, 434, 423, 417 and 399.

Some more of compound (E) was obtained from the mycelium of A.citri, and a micro analysis became possible. The analysis for C, H suggested a molecular formula of $C_{23}H_{30}O_8$, molecular weight 470. A search of the literature revealed that a compound, parasiticolide A (CXXVI), exhibited identical physical and spectroscopic to those of compound (E).

Parasiticolide A was first isolated from Aspergillus parasiticus Speare,[202] which also produces the aflatoxins.

In this work, the molecular weight of parasiticolide A was also determined by X-ray crystallographic means. The structure was first assigned on the basis of chemical and spectroscopic data and was confirmed by the X-ray diffraction method using the mono-bromo derivative (CXXVII).[203]



(CXXVI) R = H, R' = H

(CXXVII) R = H, R' = Br

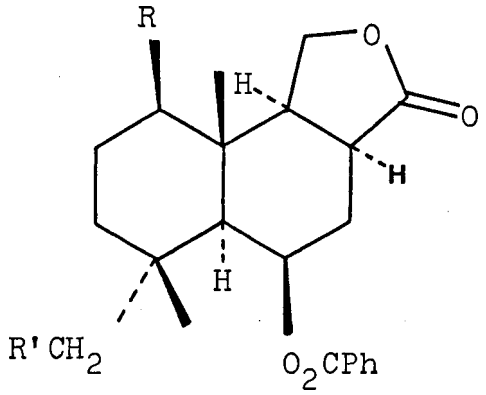
(CXXVIII) R = OH, r' = H

ParasiticolideA has been isolated also from Aspergillus variecolor,[204] together with its phenolic derivative (CXXVIII). These compounds were renamed astellolide A and B respectively. The structures were again determined by X-ray diffraction methods, the initial crystal data on cell dimensions, space group and density agreeing very closely with the results described in this thesis.

The c.m.r. assignments in Table 1.9 follow from a study of the various assignments.[204]

Several other similar metabolites with a drimane sesquiterpenoid skeleton have been isolated. Pebrolide

(CXXIX), desacetylpebrolide (CXXX) and 1-deoxypebrolide (CXXXI)[205,206] have been isolated from the fungus Penicillium brevi-compactum.

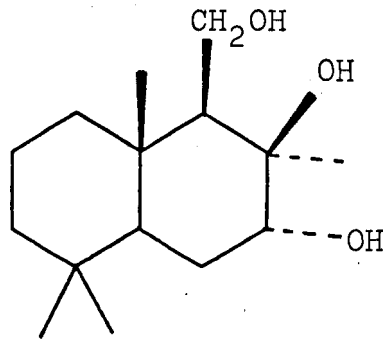


(CXXIX) R = OH, R' = Ac

(CXXX) R = Oh, r' = H

(CXXXI) R = H, R' = Ac

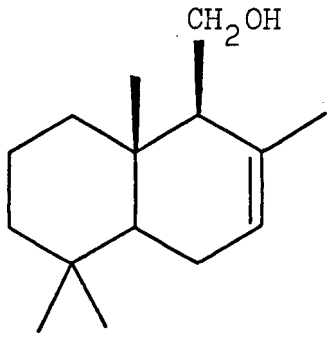
7α,8β,11-Trihydroxydrimane (CXXXII), from Fomes Annosus [207] has a similar skeleton.



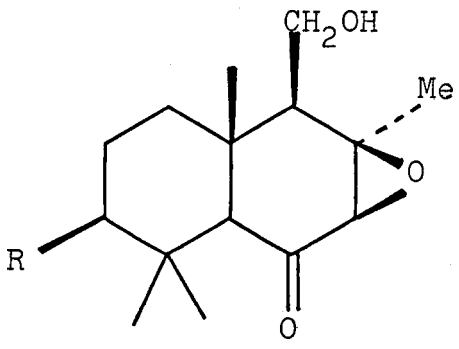
(CXXXII)

Three drimane sesquiterpenes, drimenol (CXXXIII), uvidin A (CXXXIV) and B (CXXXV) have been isolated from the mushroom Lactarius uvidus Fries.[208]

The biosynthesis of pebrolide has been studied.[209] A biosynthetic pathway was suggested based on the pattern of



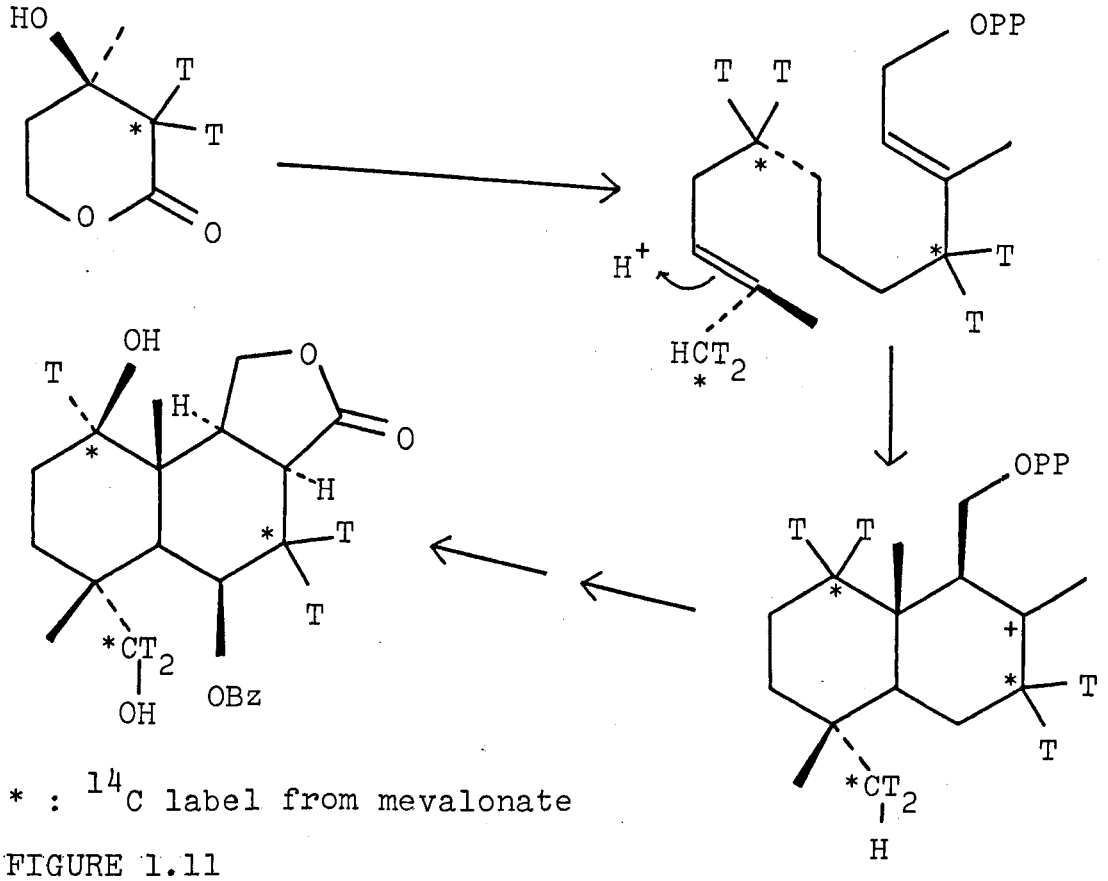
(CXXXIII)



(CXXXIV) R = H

(CXXXV) R = OH

incorporation of ^{3}H and ^{14}C from mevalonate. The results were consistent with the biosynthetic scheme indicated in Figure 1.11.



* : ^{14}C label from mevalonate

FIGURE 1.11

PROPOSED BIOSYNTHETIC PATHWAY TO PEBROLIDE

The scheme in Figure 1.11 shows the formation of a bicyclofarnesyl cation (CXXXVI) by stereospecific cyclization of farnesyl pyrophosphate (CXXXVII).

It seems likely that the biosynthetic pathway for the formation of parasiticolide A follows that in Figure 1.11 very closely.

METABOLITES FROM THE MYCELIUM OF ALTERNARIA CITRI

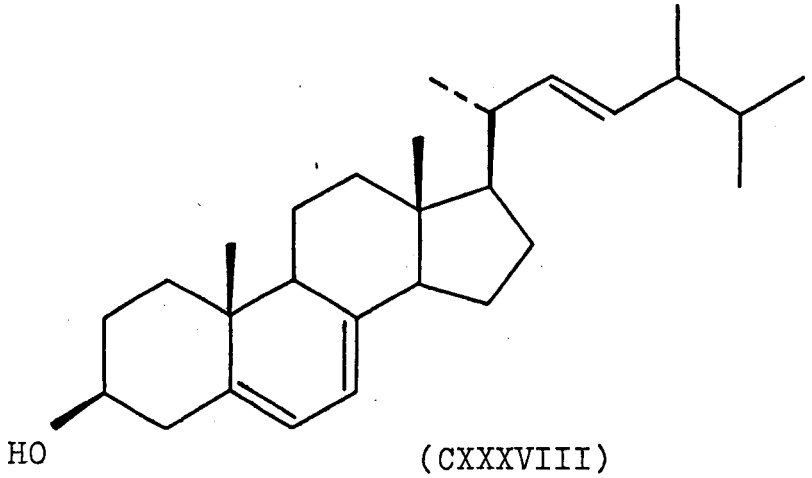
The mycelium of Alternaria citri was examined. The mycelium grew on the surface of the growth medium as a grey-green mat. The aqueous growth medium was filtered off, and the mycelium was dried in a vacuum oven overnight. After drying, the mycelium was ground to a powder and poured into a large cloth bag. The bag was placed in a Soxhlet extractor, and the mycelium was first extracted with petroleum ether (b.p. 40 - 60°) for forty-eight hours to remove oils and fatty acids. The petrol solution was pale orange after extraction and, on cooling, a white solid (G) precipitated. This was filtered off and the petrol extract was concentrated to yield an orange oil which was discarded because it consisted mostly of fatty acids, esters and glycerides.

The mycelium was further extracted with ethyl acetate for forty-eight hours; the solvent was dried (Na_2SO_4), filtered and evaporated *in vacuo* to leave a dark brown oil.

(i) PETROLEUM ETHER EXTRACT

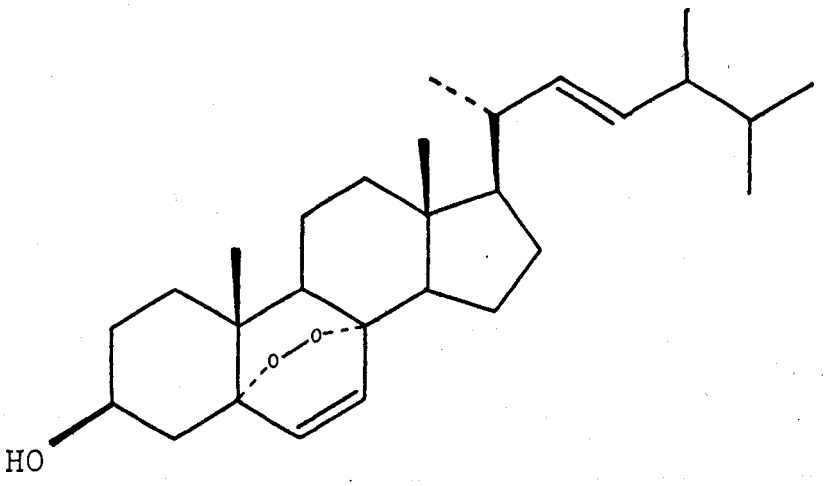
The solid material (G) isolated from the petrol extract was recrystallized from ethanol. Accurate mass measurement on the molecular ion of material (G) indicated a molecular weight of 396.33923, corresponding to a formula of $C_{28}H_{40}O$. An elemental analysis of compound (G) agreed with the accurate mass figure. Infrared absorption data showed the presence of an OH group and also a C=C double bond, while ultraviolet absorption spectroscopy showed strong absorbance at 263, 273, 283 and 294 nm. Compound (G) was optically active.

The p.m.r. spectrum was complex, most of the peaks being above δ 2.5, suggesting a mainly saturated structure. Other peaks were present as multiplets at δ 3.6, 5.16, 5.35 and 5.53 with integrals of 1:2:1:1 respectively. The spectroscopic data coupled with the molecular formula suggested that compound (G) had a sterol skeleton. A search of the literature revealed that compound (G) was identical to the fungal sterol ergosterol (CXXXVIII).

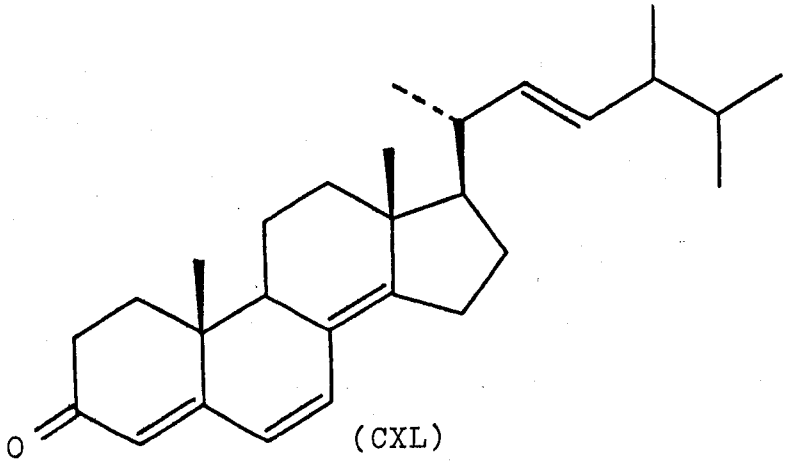


(CXXXVIII)

Ergosterol is a very common secondary metabolite of many species of fungi and has previously been identified as a metabolite of Alternaria alternata[210] and other Alternaria species.[211,212] Two other metabolites related to ergosterol have been isolated from Alternaria species; ergosterol peroxide (CXXXIX) from Alternaria dianthicola[213] and ergostatetraenone (CXL) from Alternaria alternata.[214]



(CXXXIX)



(CXL)

These compounds were previously isolated from Aspergillus fumigatus,[215] and Fomes officinalis[216] respectively. There is some doubt whether ergosterol peroxide is a true metabolite or an artifact formed by the atmospheric oxidation of the diene group of ergosterol.

Ergosterol has been used in an assay to indicate invasion by Alternaria species in grains such as sorghum, wheat and corn.[212]

(ii) ETHYL ACETATE EXTRACT

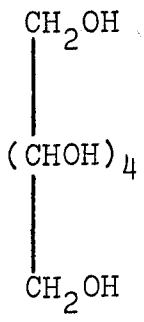
The dark brown oil was dissolved in acetone and adsorbed onto silica-gel. This mixture was loaded onto a column, which was eluted with chloroform followed by chloroform/methanol. One yellow band quickly separated and was collected after elution through the column. Long needle-like crystals (compound (H)) formed on evaporation of the solvent. Accurate mass measurement on the molecular ion of material (H) indicated a molecular weight of 428.32116 corresponding to a molecular formula of $C_{28}H_{44}O_3$. Infrared absorption data showed the presence of a hydroxyl group and the p.m.r. spectrum showed two doublets at δ 6.52 and 6.24, and two multiplets at δ 5.17 and 3.96, with integrals of 1:1:2:1 respectively. The rest of the p.m.r. spectrum was complex and confined between δ 2.4 and 0.5. The ultraviolet spectrum of compound (H) showed only end-absorbtion below 240 nm. It seemed probable that compound (H) was another fungal

sterol, and comparison of its physical and spectroscopic properties with those of ergosterol peroxide (CXXXIX) showed the two compounds to be identical. Ergosterol peroxide has been discussed above.

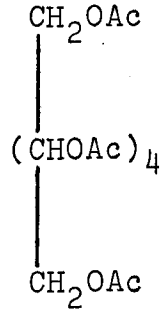
After addition of 5% methanol to the column, another coloured band separated and was collected. After further purification by preparative thin-layer chromatography on silica-gel using ether as the eluent, well-formed cubic crystals were obtained from ethyl acetate. Spectroscopic data showed this compound to be identical to parasiticolide A (CXXV), previously isolated from the neutral liquor extract of A.citri.

(iii) METHANOLIC EXTRACT OF THE MYCELIUM OF ALTERNARIA CITRI.

Soxhlet extraction of the solid mycelium of A.citri with methanol yielded an orange solution, from which a white solid (I) precipitated on cooling. This compound was crystallized from ethanol to give colourless flaky crystals with a melting point of 163 - 165°C. A mixed melting point with an authentic sample showed compound (I) to be mannitol (CXLI), an extremely common fungal metabolite. The identity of compound (I) was confirmed by formation of the hexaacetate from acetic anhydride and sodium acetate. The physical and spectroscopic data for this compound were identical to those of authentic mannitol hexaacetate.



(CXLI)



(CXLII)

EXPERIMENTAL

ANALYTICAL PROCEDURES AND CHARACTERIZATION

This section applies to all the experimental work described in this thesis.

SPECTRA

Proton n.m.r. spectra were recorded on the following spectrometers: Perkin-Elmer R34 (220 MHz), Bruker WM 250 (250 MHz) and Bruker WM 360 (360 MHz); carbon-13 n.m.r. spectra were recorded on a Varian XL-100A (25.2 MHz) and a Bruker WM 360 (90.52 MHz). Deuteriochloroform solutions were used, unless otherwise stated, with tetramethylsilane (TMS) as the internal standard.

Infrared spectra were recorded as liquid films (l), nujol mulls (nujol), carbon tetrachloride solutions (CCl_4) or in potassium bromide discs (KBr), using a Pye-Unicam SP200 or a Perkin-Elmer 157G spectrophotometer.

Low resolution mass spectra were recorded on an AEI MS12 mass spectrometer and high resolution mass spectra were recorded on an AEI MS902 instrument coupled to an Argos 900 computer. Fast atom bombardment data were obtained on a VG 7070 mass spectrometer.

Ultraviolet spectra were obtained with a Unicam SP 8000 spectrophotometer. The solvent used was ethanol unless otherwise stated.

CHROMATOGRAPHY

Thin-layer plates were coated with Kieselgel GF 254 nach Stahl, ex E. Merck A G, 0.2 mm thick.

Preparative plates were coated with Kieselgel PF 254, 1 mm thick.

High performance liquid chromatography data were obtained using a Spectra Physics SP 8700 solvent delivery system with an SP 8440 U.V. detector and a Whatman Partisil PXS 10/25 ODS.2 reverse phase column.

Flash chromatography was performed with Whatman K6 silica gel.

Column chromatograms were prepared using Fluka GF254 Kieselgel.

MELTING POINTS

Melting points were determined on a Kofler hot stage apparatus and are uncorrected.

ABBREVIATIONS

In the text, the words "evaporated" or "evaporation" refer to evaporation of the solvent under reduced pressure (10 - 20 mm of mercury) using a rotary film evaporator. The words "drying" or "dried" refer to the removal of water by treatment with anhydrous sodium or magnesium sulphate of a solution of a compound, followed by filtration to remove the partially hydrated inorganic salt.

The symbol (M^+) is used to indicate the molecular ion (determined by mass spectroscopic measurements) and %RA

refers to percentage relative abundance of peaks in the mass spectrum of a compound. In n.m.r. data, the symbols s, d, t, q, m, and br refer to the appearance of singlet, doublet, triplet, quartet, multiplet and broad signals respectively. I refer to proton coupling constants between identifiable signals.

FUNGAL CULTURE

Alternaria citri, strain 106.27 obtained from the Centraalburea voor Schimmelcultures, Baarn, Netherlands, was grown for twenty-eight days at 25°C in static culture on Czapek Dox medium consisting of $NaNO_3$ (50g), KCl (12.5g), $MgSO_4$ (12.5g), $FeSO_4$ (0.25g), K_2HPO_4 (25g), sucrose (750g), tap water (25 l), 500 ml of growth medium per pan. The usual sterile techniques were observed during the preparation of the growth medium and innoculation of the fungal spores.

EXTRACTION OF LIQUORS FROM ALTERNARIA CITRI

The mycelium of A.citri was filtered off and dried, and the aqueous filtrate was concentrated to approximately four litres. The filtrate was acidified to approximately pH 2 with concentrated hydrochloric acid and extracted four times, each time with half the volume (of filtrate) of ethyl acetate. The extracts were combined and, on leaving to stand for twenty-four hours, a white solid (A) precipitated from solution. The solid (A) was filtered off and air dried.

The ethyl acetate extract of the liquors was dried and evaporated to yield a dark brown viscous oil containing some solid material. The oil was redissolved in ethyl acetate, leaving a pale yellow solid (B).

This ethyl acetate solution (S) of the remaining oil was extracted with sodium bicarbonate solution (8% w/v, 4 x 500 ml), which was then acidified to pH 4 - 5 with dilute hydrochloric acid. The acidified aqueous solution was re-extracted with ethyl acetate (4 x 500 ml). The organic layer was separated, dried and the solvent evaporated to yield a dark brown oil. The oil was pre-adsorbed onto silica-gel and chromatographed on a silica-gel column ($CHCl_3$ and then a mixture, $CHCl_3$:MeOH; 90:10) to afford the product (C).

The ethyl acetate extract (S) of the liquors of A.citri which was not extracted into sodium bicarbonate solution was dried and the solvent evaporated to yield a viscous brown oil. The oil was pre-adsorbed onto silica-gel and chromatographed on a silica-gel column ($CHCl_3$ changing to a mixture, $CHCl_3$:MeOH; 90:10), to afford the product (D).

The liquors from a separate culture of A.citri were examined. The ethyl acetate extract of the culture fluid, after removal of acids by washing with sodium bicarbonate (4 x 500 ml), was dried and evaporated to leave a residual brown oily solid which was chromatographed on silica-gel to give two separate solid fractions (E, F). The solid (E) which eluted first from the column was

purified by mutliple elution preparative thin-layer chromatography. The second component (F) that eluted from the column was purified by crystallization from ethyl acetate.

IDENTIFICATION OF COMPOUND (A)

After drying, compound (A) was recrystallized from water to yield colourless prisms (1.03g). Compound (A) was identified as nitrilotriacetic acid (XCIV), m.p. 249 - 252°C (lit. 246°).[235] Found: C, 37.44, H, 4.88, N, 7.33%; $C_6H_9NO_6$ requires C, 37.70, H, 4.75, N, 7.33%; ν_{max} (nujol): 2600 - 2100 (v. br.), 1728, 1434, 1332, 1010 cm^{-1}. M^+ 191.0428; $C_6H_9NO_6$ requires 191.0432 m/z (%R.A.):191 (2.17), 173 (2.68), 146 (71.01), 102 (23.83), 88 (69.9), 60 (12.7).

NITRILOTRIACETIC ACID TRIETHYL ESTER (XCIII)

Nitrilotriacetic acid (A), (132 mg) was refluxed for ten hours in absolute ethanol (15 ml) and sulphuric acid (four drops). After cooling, ethyl acetate (20 ml) was added, and the mixture was washed with an 8% weight/volume solution of sodium bicarbonate (2 x 20 ml). The organic layer was washed with water (10 ml) and dried. The solvent was evaporated to leave the product, nitrilotriacetic acid triethyl ester, as a pale yellow oil (128 mg, 76%) which was not purified further.

Found: C, 53.13, H, 7.63, N, 4.74%; $C_{12}H_{21}NO_6$ requires C, 52.35, H, 7.69, N, 5.09%; $\nu_{max.}$ (1): 1755, 1460, 1200, 1036 cm^{-1}; δ ($CDCl_3$):1.27 (9H, t, CH_3), 3.68

(6H, s, CH_2), 4.19 (6H, q, CH_2). ^{13}C (ppm):14.19(q), 55.13 (t), 56.12(t), 170.62(s); M^+ 275.13842; $C_{12}H_{21}NO_6$ requires 275.13688; m/z (% R.A.), 275 (13.2), 231 (17.6), 202 (80.9), 181 (50.0), 161 (45.6), 131 (61.8), 119 (58.8), 116 (66.2), 88 (41.2), 69 (100.0).

IDENTIFICATION OF COMPOUND (B)

Compound (B) was crystallized from methanol, yielding small sandy coloured crystals (2.30g). Compound (B) was identified as Kojic acid (2-hydroxy-5-hydroxymethyl-4-pyrone (XCVIII)), m.p. 153 - 154° (lit. 154 - 155°).[217] Found: C, 50.64, H, 4.27%; $C_6H_6O_4$ requires C, 50.71, H, 4.26%. $\nu_{max.}$ (nujol):3400 - 2900 (v.br.), 1660, 1610, 1580 cm^{-1}; $\lambda_{max.}^{EtOH}$ nm (log ε):272 (3.91). δ (d_6 acetone): 2.7 - 3.3 (1H, v.br., OH), 4.47 (2H, s, CH_2), 6.46 (1H, s, CH), 7.45 - 7.65 (1H, br., OH), 7.99 (1H, s, CH). M^+ 142.02683; $C_6H_6O_4$ requires 142.02661. m/z (% R.A.), 142 (35.4), 129 (6.3), 116 (55.7), 99 (67.1), 98 (100.0), 69 (49.4).

IDENTIFICATION OF COMPOUND (C)

Compound (C) was purified by preparative thin-layer chromatography (silica-gel, $CHCl_3$) and crystallized from ethyl acetate as colourless needles (68 mg). Compound (C) was identified as 2-acetyl-4-(3H)-quinazolinone (C), m.p. 201 - 203° (lit. 202 - 205°C).[181] Found: C, 63.10, H, 4.45, N, 14.34%; $C_{10}H_8N_2O_2$ requires C, 63.82, H, 4.29,

N, 14.89%; $\nu_{max.}$ (KBr), 3165 (br.), 3065 (br.), 1710, 1670, 1600 cm^{-1}; $\lambda_{max.}^{EtOH}$ nm (log ε); 302 (3.93); δ ($CDCl_3$); 2.75, (3H, s, CH_3), 7.4 - 8.3 (4H, m, ArH), 9.7 (1H, br, NH). ^{13}C (ppm); 23.98 (q), 123.4 (s), 126.9 (d), 129.1 (d), 129.3 (d), 138.4 (d), 147.7 (s), 160.6 (s), 194.2 (s), 212.5 (s). M^+ 188.05804, $C_{10}H_8N_2O_2$ requires 188.05857. M/z (% R.A.); 188 (100), 146 (71.1), 145 (40.4), 119 (48.3), 118 (37.5), 90 (41.7).

IDENTIFICATION OF COMPOUND (D).

Compound (D) was purified by preparative thin-layer chromatography (silica-gel, CH_2Cl_2) and crystallized from methanol as colourless plates (84 mg). Compound (D) was identified as dihydrocanadensolide (3-methyl-6-butyltetrahydrofuro- 3,4-b -furan-2,4-dione (CXII)), m.p. 94 - 96° (lit. 94 - 96°).[184] α_D^{25} -42.6°. Found: C, 59.92, H, 7.80%; $C_{11}H_{16}O_4$ requires C, 62.25, H, 7.60%; $\nu_{max.}$ (CCl_4), 1802, 1785, 1180, 905; δ ($CDCl_3$), 0.92 (3H, t, CH_3), 1.40 (3H, d, CH_3), 1.40 (4H, m, CH_2CH_2), 1.87 (2H, m, CH_2), 3.05 (1H, dq, CH), 3.2 (1H, dd, CH), 4.60 (1H, dt, CH), 5.17 (1H, dd, CH). ^{13}C (ppm), 13.82 (q), 17.05 (q), 22.40 (t), 27.47 (t), 28.48 (t), 38.36 (d), 48.93 (d), 78.30 (d), 82.99 (d), 174.60 (s), 174.64 (s). M^+ 212.10298; $C_{11}H_{16}O_4$ requires 212.10485. M/z (% R.A.), 212 (30.9), 194 (5.2), 166 (19.6), 155 (17.5), 139 (21.7), 125 (49.5), 98 (100.0).

IDENTIFICATION OF COMPOUND (E)

Compound (E) was purified by multiple elution preparative thin-layer chromatography (silica-gel, diethyl ether) and crystallized from ethyl acetate in colourless cubes (47 mg). Compound (E) was identified as parasiticolide A (astellolide A (CXXVI)), m.p. 212 - 214° (lit. 214 - 216).[202] α_D^{25} -10°. Found: C, 66.33; H, 6.53%; $C_{26}H_{30}O_8$ requires, C, 66.37, H, 6.43%; $\nu_{max.}$ (KBr), 1762, 1744, 1734, 1726, 1678 cm^{-1}; $\lambda_{max.}^{CHCl_3}$ nm (log ε), 243 (3.65), 274 (2.97), 282 (2.87); δ ($CDCl_3$), 1.15 (3H, s, CH_3), 1.90 (3H, s, CH_3), 2.11 (3H, s, CH_3), 3.99 (1H, d, CH), 4.38 (1H, d, CH), 4.88 (1H, d, CH), 4.97 (1H, d, CH), 5.95 (1H, m, CH), 6.02 (2H, d, ArH), 7.47 (2H, t, ArH), 7.57 (1H, t, ArH). ^{13}C p.p.m., 20.5 (q), 20.9 (q), 27.7 (q), 29.0 (t), 17.9 (t), 31.6 (t), 37.0 (t), 37.7 (s), 40.5 (s), 53.9 (d), 65.9 (t), 66.6 (d),66.6 (t), 71.3 (t), 122.5 (s), 128.6 (d), 129.5 (s), 129.5 (d), 133.3 (d), 165.6 (s), 165.9 (s), 170.0 (s), 170.4 (s), 173.0 (s). M^+ 470. M/z (% R.A.), 398 (42.3), 275 (29.1), 216 (62.5), 203 (24.8), 105 (100.0), 77 (23.1).

IDENTIFICATION OF COMPOUND (F)

Compound (F) was crystallized from ethyl acetate to give colourless prisms (83 mg). Compound (F) was identified as chrysogine (2(1-hydroxyethyl)-3-(3H)-quinazolinone (CVII)), m.p. 174 - 177°, (lit. 190 - 191°).[181] α_D^{25} -15.6°. Found: C, 63.19, H, 5.42, N, 14.45%; $C_{10}H_{10}N_2O_2$ requires C, 63.15, H, 5.30, N, 14.73%; $\nu_{max.}$ (nujol); 3440 (br.),

3100 - 2800 (br), 1680, 1610 cm^{-1}; $\lambda_{max.}$ EtOH (log ε), 228 (4.38), 266 (3.87), 305 (3.61); δ ($CDCl_3$), 1.65 (3H, d, CH_3), 4.90 (1H, q, CH), 7.4 - 7.9 (3H, m, ArH), 8.23 (1H, d, ArH). ^{13}C (p.p.m.), 22.48, 67.40, 120.75, 126.31, 126.68, 126.95, 134.76, 148.35, 157.83, 162.77. M^+ 190.0741, $C_{10}H_{10}N_2O_2$ requires 190.0742. M/z (% R.A.), 190 (100.0), 175 (53.4), 173 (76.0), 147 (61.9), 119 (39.5), 90 (32.1).

EXTRACTION OF MYCELIUM FROM ALTERNARIA CITRI

The mycelium of A.citri was dried in a vacuum oven overnight, and ground to a fine powder. The powder was placed in a Soxhlet extractor and extracted with petroleum ether (b.p. 40 - 60°C) for forty-eight hours, followed by extraction with ethyl acetate and then methanol for forty-eight hours each. The petroleum ether solution was left overnight at 0 - 5°C. A white precipitate (G) formed, which was filtered from the solution.

The ethyl acetate extract of the mycelium of A.citri was dried and the solvent evaporated to leave a brown oil. The oil was dissolved in acetone and adsorbed onto silica-gel prior to being chromatographed on a silica-gel column. The solid (H) which eluted first from the column was purified by crystallization from methanol. The second component (F) to be eluted from the column was crystallized from ethyl acetate.

After being extracted with petroleum ether and ethyl acetate, the mycelium of A.citri was extracted with methanol

for forty-eight hours. A white solid (I) precipitated from solution on cooling and was recrystallized from ethanol.

IDENTIFICATION OF COMPOUND (G)

Compound (G) was filtered from the petroleum ether extract and crystallized from ethanol in plates (4.92 g). Compound (G) was identified as ergosterol (CXXXVIII), m.p. 158 - 160° (lit. 168 - 170°).[236] $[\alpha]_D^{25}$ -79.4°. Found: C, 79.79, H, 11.68%; $C_{28}H_{44}O$ requires C, 84.78, H, 11.18%; $\nu_{max.}$ (KBr), 3420 (br), 1640, 1598, 1466 cm^{-1}; $\lambda_{max.}^{CHCl_3}$ nm (log ε), 263 (3.85), 273 (3.94), 283 (3.96), 430 (3.75); δ ($CDCl_3$), 0.5 - 2.5 (m, 38H), 3.6 (1H, m, CH), 5.16 (2H, m, CH=CH), 5.35 (1H, m, CH), 5.53 (1H, m, CH). M^+ 396.33842, $C_{28}H_{44}O$ requires 396.33920. M/z (% R.A.), 396 (67.2), 363 (45.9), 337 (16.4), 143 (32.8), 91 (36.1), 69 (100.0).

IDENTIFICATION OF COMPOUND (H)

Compound (H) was crystallized from methanol to give small needles (46 mg). Compound (H) was identified as ergosterol peroxide (CXXXIX), m.p. 165 - 170° (lit. 177 - 178°).[213] Found: C, 73.41, H, 9.48%, $C_{28}H_{44}O_3$ requires, C, 78.45, H, 10.35%; $\nu_{max.}$ (KBr), 3505, 3300 (br), 1450, 1040 cm^{-1}; δ ($CDCl_3$), 0.5 - 2.5 (m, 38H), 3.96 (1H, m, CH), 5.17 (2H, m, CH=CH), 6.24 (1H, d, CH), 6.52 (1H, d, CH). M^+ 428.32116, $C_{28}H_{33}O_3$ requires 428.32903. M/z (% R.A.), 428 (20.0), 410 (60.2), 393 (49.1), 363 (52.7), 337 (63.6), 69 (100.0).

IDENTIFICATION OF COMPOUND (F)

Compound (F) was crystallized from ethyl acetate in colourless cubes (53 mg) and identified as parasiticolide A (astellolide A (CXXVI)), m.p. 212 - 214°, by comparison of its physical and spectroscopic data with a sample from the liquors of A. citri.

IDENTIFICATION OF COMPOUND (I)

Compound (I) was crystallized from ethanol to give colourless crystals (3.23g) which were identified as mannitol (CXLI), m.p. 163 - 165° (lit. 165 - 166°).[237] Found: C, 39.56, H, 7.76%, $C_6H_{14}O_6$ requires C, 39.56, H, 7.75%; $\nu_{max.}$ (nujol), 3500 - 3200 (br), 1512, 1230, 1098 cm^{-1}.

MANNITOL HEXAACETATE (CXLII)

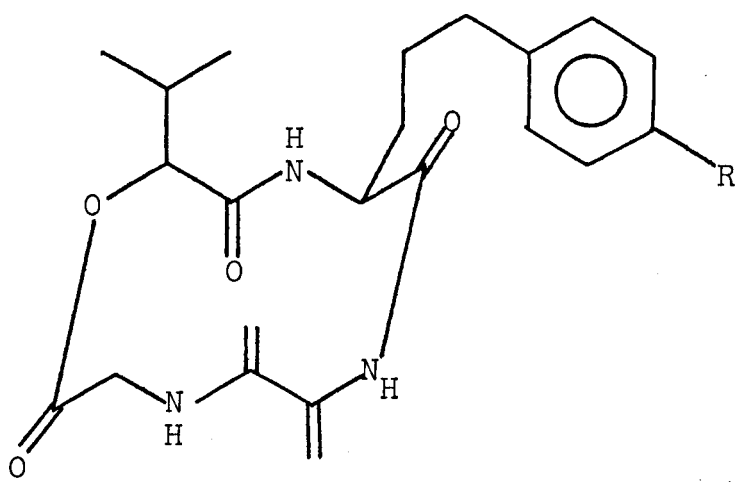
Mannitol (100 mg), acetic anhydride (4 ml) and anhydrous sodium acetate (100 mg) were refluxed for one hour and then poured into water (10 ml). Mannitol hexaacetate precipitated out of solution and was filtered and air dried (175 mg, 74%), m.p. 120 - 122°C (lit. 119 - 122°).[238] Found: C, 49.97, H, 5.63%; $C_{18}H_{24}O_{12}$ requires C, 50.00, H, 5.60%; $\nu_{max.}$ (nujol), 1750, 1465, 1375, 1230, 1033 cm^{-1}.

CHAPTER TWO

METABOLITES OF ALTERNARIA (2)

INTRODUCTION

Alternaria mali, the second organism to be investigated, has been mentioned earlier in this thesis. A.mali (Roberts) produces the cyclic tetrapeptide host-specific toxins, A.M. toxin I (VII), II (VIII) and III (IX).



(VII) R = OCH_3

(VIII) R = H

(IX) R = OH

Alternaria mali causes leaf spot disease of apple and is a serious pest on several varieties. In the work presented in this thesis, culturing experiments were undertaken with potato dextrose growth medium because the fungus grew badly on Czapek Dox growth medium.

Alternaria mali was found to produce several secondary metabolites, notably some of polyketide origin related

biosynthetically to alternariol. One of these polyketide-derived metabolites has not previously been isolated and this compound was characterized by spectroscopic means and X-ray crystallography. Throughout the purification stages of the work described in this thesis, much use was made of preparative thin-layer chromatography. Two useful techniques that were employed were multiple and continuous elution procedures. Multiple elution of a t.l.c. plate, using a solvent of lower polarity than would normally be used, is a way of effectively increasing the number of theoretical plates available in a given length of support and so improving the separation of close-running spots.

The behaviour of a spot during multiple elution is amenable to simple mathematical treatment and examples have appeared in the literature.[221] The method has also been used for the paper chromatography of carbohydrates.[222]

Even greater resolving power is available from continuous elution thin-layer chromatography, which requires specially adapted apparatus. Several different designs have appeared over the years. The first to be described[223] used horizontal development in which the solvent was fed to the chromatogram by a paper wick. An overlaid glass plate prevented evaporation of solvent from the support until the whole plate had been traversed. Later workers[224] used ascending development in an open chamber, with the lower part lined with solvent-soaked paper. This is only suited to single solvents, as

differential evaporation changes the composition of mixtures.

Ascending development in a closed chamber has been described.[225] At the top of the chromatogram the solvent was taken up in absorbent held in a trough fastened to the plate. Another method is to leave the top of the chromatogram out of an enclosed chamber, and leave the solvent to evaporate. This simple approach was the one used in continuous elution preparative t.l.c. mentioned in this thesis.

RESULTS AND DISCUSSION

EXTRACTION OF METABOLITES FROM THE MYCELIUM OF ALTERNARIA MALI

The mycelium from fifty pans of Alternari mali Roberts, strain 106.24, ATCC 13963 obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands, grown on aqueous potato dextrose medium for twenty-eight days was filtered from the growth medium and dried. The mycelium was ground to a powder which was initially extracted with petroleum ether to remove oils and fatty acids, followed by ethyl acetate. The petrol extract was dried and the solvent removed in vacuo to leave a pale orange oil. Flash column chromatography of the oil on silica-gel using petroleum ether (b.p. 40 - 60°) as eluent yielded a yellow oil. Leaching of the column with dichloromethane gave an oily yellow solid (G). The yellow oil was not investigated further.

The ethyl acetate extract of the mycelium of A.mali was dried and the solvent evaporated to yield a dark brown viscous oil. Thin-layer chromatography of this oil in chloroform:methanol (9:1) showed many spots. The oil was redissolved in ethyl acetate and washed with 4 x 250 ml aliquots of Na_2CO_3 solution to extract acids, and NaOH solution to extract the phenolic fraction. The phenolic fraction was neutralized with dilute HCl, and back-extracted into ethyl acetate. The ethyl acetate was dried and evaporated to give a brown oily solid mixture. Two major bands separated from the baseline on preparative t.l.c. of this solid mixture on silica-gel in chloroform:

methanol (9:1). The two bands were extracted from the silica-gel using a Soxhlet apparatus. The band with the smallest r.f. fluoresced bright blue under ultraviolet irradiation and, on extraction, yielded a solid, compound (J).

The band with the largest r.f. value on the preparative t.l.c. plate also fluoresced blue under ultraviolet irradiation. On extraction of the silica-gel with acetone using a Soxhlet apparatus, a pale yellow solid (K) was obtained after removal of solvent.

PETROLEUM EXTRACT OF THE MYCELIUM OF ALTERNARIA MALI

The yellow solid (G) was found to have the same r.f. value as ergosterol when investigated by thin-layer chromatography. Crystallization of compound (G) from ethanol yielded flaky crystals which were shown to be ergosterol by physical and spectroscopic data, and by comparison with an authentic sample.

ETHYL ACETATE EXTRACT OF THE MYCELIUM OF ALTERNARIA MALI

After Soxhlet extraction from the silica-gel, compound (J) was crystallized from methanol to give a colourless solid. Accurate mass measurement of the molecular ion of compound (J) indicated a molecular weight of 292.09618 corresponding to a molecular formula of $C_{15}H_{17}O_6$. Infrared analysis of compound (J) indicated the presence of C=O at 1635 cm^{-1} and a broad peak at

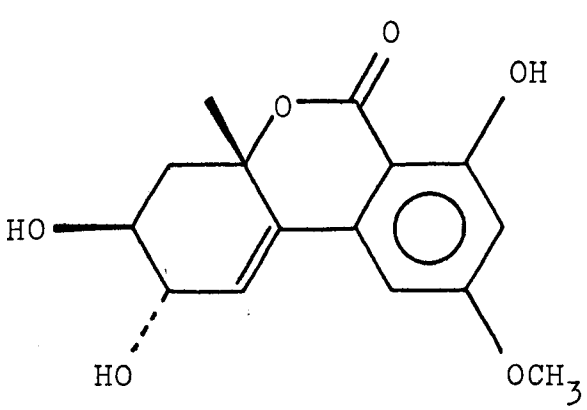
3400 cm^{-1} corresponding to an OH group.

The ultraviolet spectrum of compound (J) showed strong absorption at 320, 278 and 240 nm.

The p.m.r. spectrum is given in Table 2.1.

The peaks at δ 1.50 and δ 3.88 correspond to methyl and methoxyl groups respectively, δ 3.88 being typical of aromatic methoxyl shifts. Doublets at δ 6.66 and δ 6.48 indicate the presence of two aromatic protons, and the low coupling constant demonstrates that they are meta to each other. Crystals of compound (J) produced an intense purple colour on addition of ethanolic $FeCl_3$, indicating the presence of a carbonyl group adjacent to a hydroxyl group. The p.m.r. spectrum shows another peak at δ 6.23 indicating further unsaturation in the molecule.

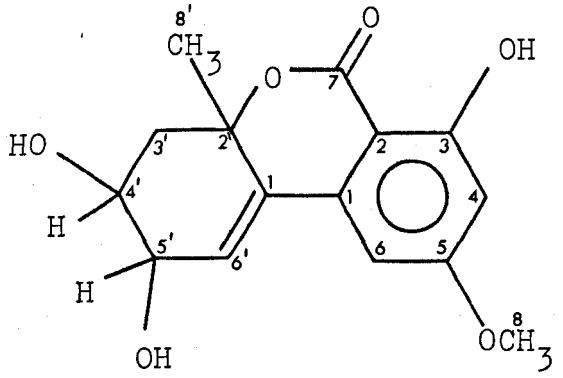
A search of the literature, and comparison of the data available showed that compound (J) was altenuene (XCII), previously isolated from Alternaria tenuis.[143]



(XCII)

TABLE 2.1

P.M.R. SPECTRUM OF ALTENUENE IN d_4-METHANOL



CHEMICAL SHIFT (δ)	INTEGRAL	MULTIPLICITY	SPLITTING (Hz)	ASSIGNMENT
6.66	1	doublet	2.5	6
6.48	1	doublet	2.5	4
6.23	1	doublet		6'
4.08	1	multiplet		5'
3.88	3	singlet		8
3.80	1	multiplet		4'
2.40	1	multiplet		3a'
1.98	1	multiplet		3b'
1.50	3	singlet		8'

Altenuene has been mentioned earlier in this thesis. The correct structure was determined by X-Ray diffraction studies[144] after it had earlier been isolated and an incorrect structure assigned to it.

Compound (K) was crystallized from ethanol to give colourless needles and, on addition of ethanolic $FeCl_3$, a purple solution was formed. Accurate mass measurement of the molecular ion of the material gave a molecular weight of 272.06736, indicating a molecular formula of $C_{15}H_{12}O_5$. This formula was in agreement with results from micro-analysis of the compound.

The infrared absorption spectrum of compound (K) showed the presence of OH at 3300 cm^{-1}, and C=O at 1650 cm^{-1}.

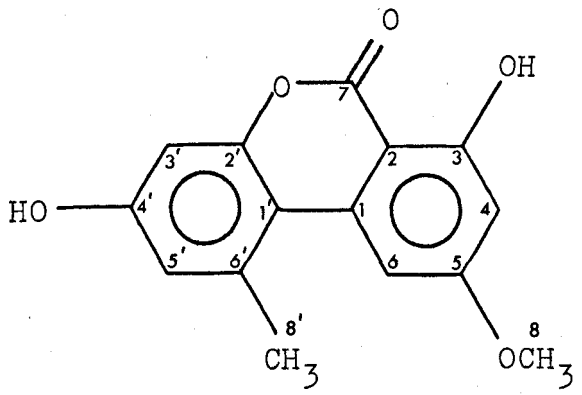
The ultraviolet spectrum showed absorption bands at 217, 259, 297, 303 and 336 nm.

The p.m.r. spectrum is summarised in Table 2.2 .

The p.m.r. spectrum of compound (K) shares similarities with that of altenuene; the peaks at δ 2.80 and 3.99 correspond to an aromatic methyl and a methoxyl group respectively. However, four aromatic protons are present, indicating two separate aromatic rings. Spin decoupling experiments showed that the two hydrogens with peaks at δ 7.34 and δ 6.62 were attached to the same aromatic ring. Examination of the coupling constants of both sets of peaks indicated that the hydrogens were positioned meta to each other. A hydroxyl hydrogen was

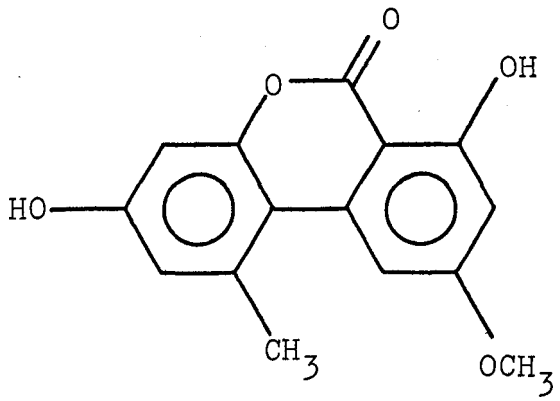
TABLE 2.2

P.M.R. SPECTRUM OF ALTERNARIOL MONOMETHYL ETHER IN d_6-ACETONE



CHEMICAL SHIFT (δ)	INTEGRAL	MULTIPLICITY	SPLITTING (Hz)	ASSIGNMENT
11.97	1	singlet		OH
7.34	1	doublet	2.5	3'
6.86	1	doublet	2.5	6
6.77	1	doublet	2.5	5'
6.62	1	doublet	2.5	4
3.99	3	singlet		8
2.80	3	singlet		8'

present at δ 11.97. The physical and spectroscopic data obtained for compound (K) were identical to that of alternariol monomethyl ether (LXXXV).



(LXXXV)

Ergosterol, altenuene and alternariol methyl ether were the only secondary metabolites to be characterized from the mycelium of Alternaria mali. However, the liquor extracts of A.mali were also examined.

EXTRACTION OF METABOLITES FROM THE LIQUORS OF ALTERNARIA MALI

The crude liquor filtrate (25 litres) from fifty growth pans of A.mali were concentrated to approximately four litres and extracted with light petroleum (b.p. 40 - 60°) to remove fatty acids, esters and glycerides, followed by extraction with diethyl ether. The ether extract was dried and the solvent evaporated to yield a brown oily solid (L).

After extraction of the liquors with diethyl ether, the liquors were acidified to approximately pH 2 with dilute

hydrochloric acid and extracted with 4 x 500 ml aliquots of ethyl acetate. The organic layer was separated and dried, and the ethyl acetate concentrated. After leaving at 0°C overnight, a crystalline solid and an oil separated out on the bottom of the flask. The solvent was decanted off and the residual solid and oil were washed repeatedly with 25 ml aliquots of cold ethyl acetate to leave a pale orange solid (M).

DIETHYL ETHER EXTRACT

The oily solid (L) was crystallized from ethanol, yielding small pale yellow crystals with sharp melting point and showing only one component on t.l.c.

Accurate mass measurement of the molecular ion of compound (L) gave a molecular weight of 288.0643 indicating a formula of $C_{15}H_{12}O_6$. Microanalysis of the solid confirmed this formula. An element listing of the mass spectrum of compound (L) was obtained and is shown in Table 2.3. Some metastable ions were also identified and were used to determine the fragmentation pathways shown in Figure 2.1.

The p.m.r. spectrum of compound (L) is shown in Table 2.4 and the c.m.r. spectrum in Table 2.5.

The infrared absorption spectrum showed the presence of C=O and OH in the molecule. The ultraviolet spectrum gave maxima at 219, 249 and 294 nm. On addition of ethanolic $FeCl_3$ to a solution of compound (L) in ethanol, a brown colour was formed.

TABLE 2.3

PARTIAL ELEMENT LISTING FOR THE MASS SPECTRUM OF DEHYDROALTENUSIN.

M/z	% RELATIVE ABUNDANCE	COMPOSITION	ERROR (ppm)
288.0643	100.0	$C_{15}H_{12}O_6$	3
273.0421	10.80	$C_{14}H_9O_6$	8
260.0687	39.98	$C_{14}H_{12}O_5$	1
259.0624	20.26	$C_{14}H_{11}O_5$	7
243.0656	12.23	$C_{14}H_{11}O_4$	1
232.0726	26.55	$C_{13}H_{12}O_4$	4
217.0501	25.44	$C_{12}H_9O_4$	0
177.0561	17.95	$C_{10}H_9O_3$	5

FIGURE 2.1

MAJOR FRAGMENTATION PATHWAYS OF DEHYDROALTENUSIN

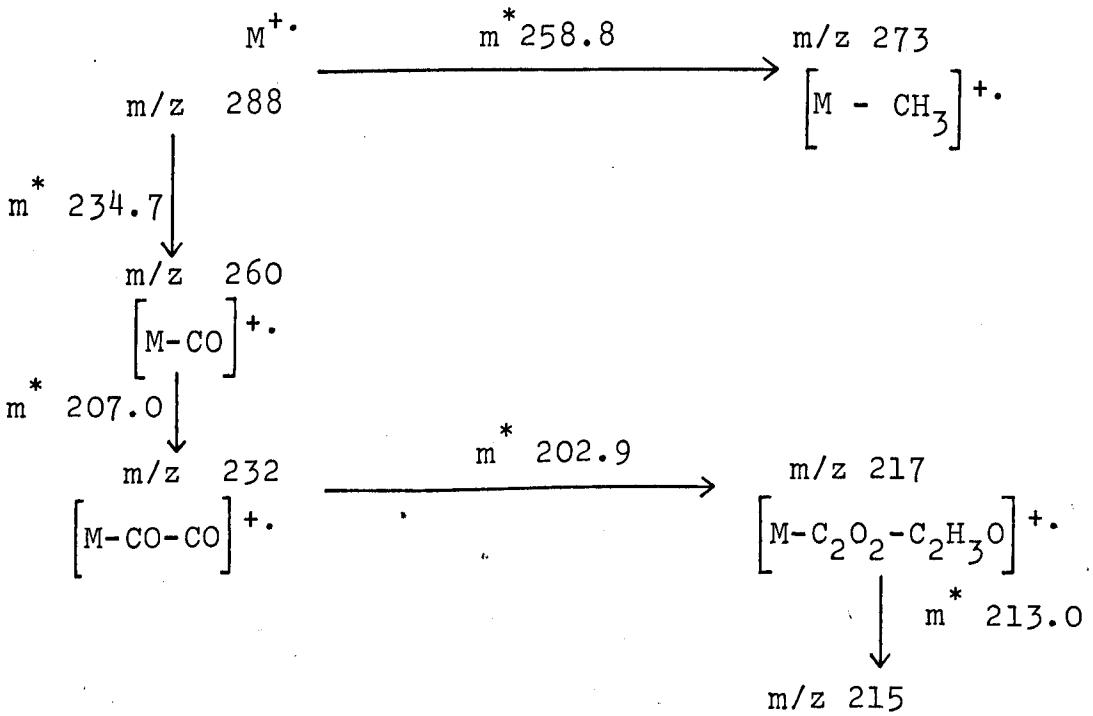
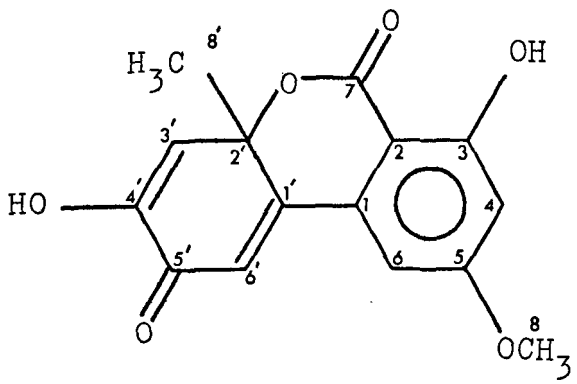


TABLE 2.4

P.M.R. SPECTRUM OF DEHYDROALTENUSIN



CHEMICAL SHIFT (δ)	INTEGRAL	MULTIPLICITY	SPLITTING (Hz)	ASSIGNMENT
11.26	1	singlet		OH
6.74	1	doublet	2.5	6
6.69	1	singlet		6'
6.62	1	doublet	2.5	4
6.28	1	singlet		3'
3.89	3	singlet		8
1.71	3	singlet		8'

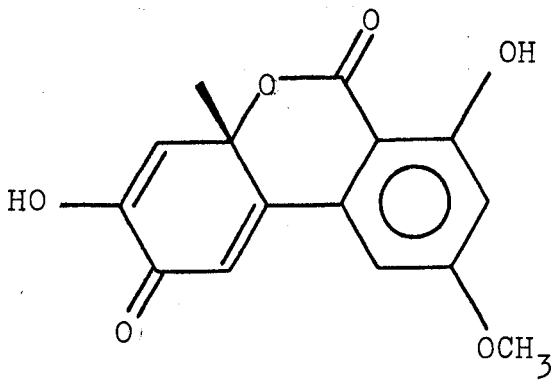
TABLE 2.5

C.M.R. SPECTRUM OF DEHYDROALTENUSIN

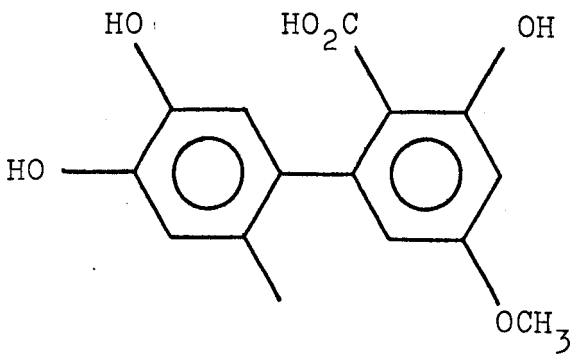
CHEMICAL SHIFT (δ)	MULTIPLICITY WITH OFF RESONANCE DECOUPLING
29.41	q
55.91	q
79.01	s
99.72	d
103.58	s
104.20	s
116.06	d
120.69	d
146.04	s
152.89	d
164.51	s
166.18	s
167.15	s
180.61	s
235.20	s

It can be seen from the p.m.r. spectrum of compound (L) that it has a pair of meta-coupled doublets similar to those present in altenuene and alternariol methyl ether. The singlets at δ 6.28 and δ 6.69 can be assigned to protons on a separate ring of compound (L). A methyl absorption occurs at δ 1.71 and a methoxyl group is present at δ 3.89.

The compound dehydroaltenusin (XC) was found to fit the physical and spectroscopic data for compound (L).



(XC)

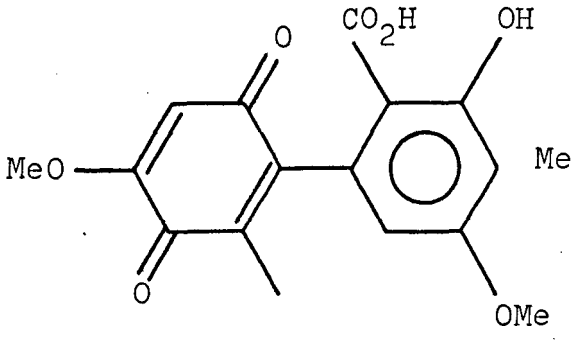


(LXXXIX)

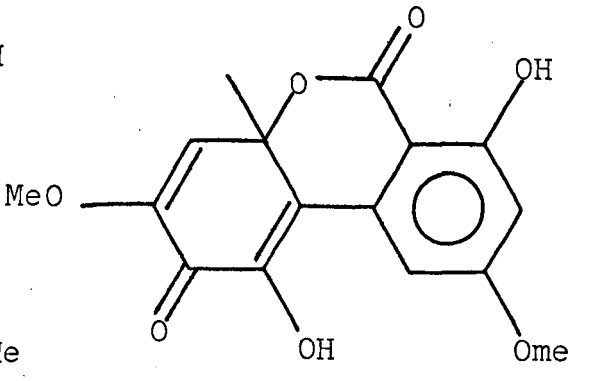
The identity of compound (L) was confirmed by reduction with sodium dithionite, breaking the lactone ring and giving rise to altenusin (LXXXIX). On reaction with ethanolic $FeCl_3$, altenusin gave a purple colour which quickly turned brown as it was oxidised to dehydroaltenusin.

Dehydroaltenusin was originally isolated in 1957,[102a] and a partial structure was later assigned to it by Thomas.[138] However, it was not until 1971 that the structure of dehydroaltenusin was correctly determined by X-ray crystallography.[141]

The altenariol derivatives are unique to the Alternaria species of fungi, and are biosynthetically related to each other. However, botrallin, a compound structurally very similar to dehydroaltenusin has been isolated from the fungus Botrytis allii.[226] The structure (CXLIII) was initially assigned to botrallin. On further examination, and also comparison with the structure of



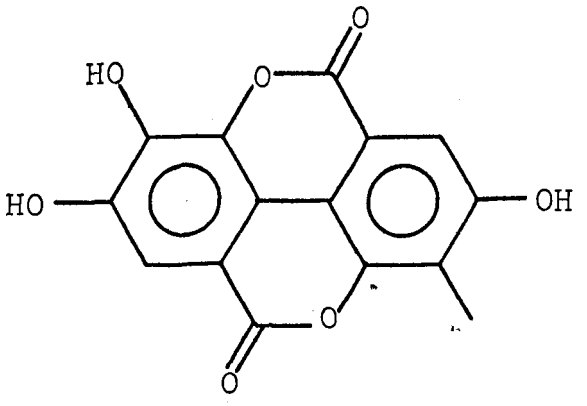
(CXLIII)



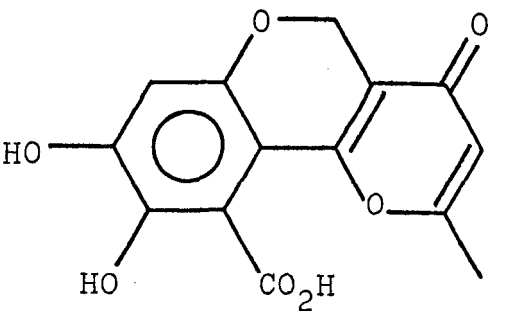
(CXLIV)

dehydroaltenusin obtained from X-ray diffraction studies, a new structure (CXLIV) was assigned to (±)-botrallin.[227]

Other dibenzopyrones are known as secondary metabolites of fungi. Ellagic acid (CXLV)[228] is apparently formed by a similar biosynthetic pathway to alternariol. It has been suggested[229] that citromycetin (CXLVI)[230] has a common biosynthetic precursor with alternariol. This



(CXLV)



(CXLVI)

seems probable since it has been found that the carboxylic acid group of citromycetin is derived biogenetically via oxidation of an acetate methyl group.[231]

ACIDIC EXTRACT OF LIQUORS

The solid (M) was recrystallized from methanol to give large colourless cubes. On complete removal of solvent, the cubes turned opaque and on examination under a microscope, they were found to consist of microcrystals.

The infrared absorption spectrum of compound (M) indicated the presence of strongly hydrogen bonded hydroxyl and carbonyl groups. Ultraviolet spectroscopy showed absorbance maxima at 220, 260 and 305 nm. The p.m.r. spectrum is summarised in Table 2.6, and the c.m.r. spectrum in Table 2.7. On addition of ethanolic $FeCl_3$, a solution of compound (M) turned purple.

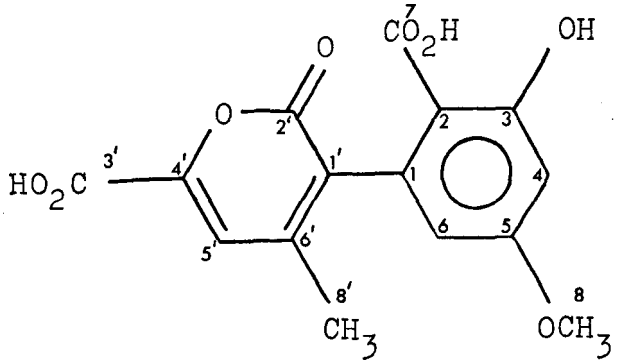
Accurate mass measurement of the molecular ion of compound (M) gave a molecular weight of 320.0486, indicating a formula of $C_{15}H_{12}O_8$. Microanalysis of the compound agreed with this formula. An element listing of the mass spectrum was obtained and is summarised in Table 2.8.

Compound (M) was found to be insoluble in petroleum ether, ether and dichloromethane. It was slightly soluble in cold ethyl acetate and water and soluble in ethyl acetate, acetone, methanol and tetrahydrofuran. Compound (M) was found to be optically inactive.

The p.m.r. spectrum showed the presence of aromatic meta-coupled doublets at δ 6.35 and δ 6.52. A one proton singlet from a separate ring was present in the aromatic region. Two singlet methyl groups were present, the peak at δ 3.86 being typical of an aromatic methoxyl

TABLE 2.6

P.M.R. SPECTRUM OF ALTERNARIAN ACID IN d_4-METHANOL



CHEMICAL SHIFT (δ)	INTEGRAL	MULTIPLICITY	SPLITTING (Hz)	ASSIGNMENT
2.06	3	singlet		8'
3.86	3	singlet		8
6.35	1	doublet	2.5	4
6.52	1	doublet	2.5	6
7.15	1	singlet		5'

TABLE 2.7

C.M.R. SPECTRUM OF ALTERNARIAN ACID IN d_6-ACETONE

CHEMICAL SHIFT (δ)	MULTIPLICITY WITH OFF RESONANCE DECOUPLING
19.82	quartet
56.15	quartet
101.76	doublet
105.32	singlet
110.87	doublet
115.05	doublet
132.99	singlet
139.41	singlet
147.09	singlet
147.79	singlet
160.61	singlet
160.96	singlet
165.65	singlet
166.84	singlet
172.74	singlet

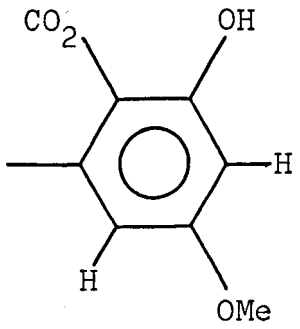
TABLE 2.8

PARTIAL ELEMENT LISTING FOR MASS SPECTRUM OF ALTERNARIAN ACID

M/Z	% RELATIVE ABUNDANCE	COMPOSITION	ERROR (ppm)
320.0486	41.37	$C_{15}H_{12}O_8$	11.1
303.0457	15.90	$C_{15}H_{11}O_7$	6.1
302.0438	100.00	$C_{15}H_{10}O_7$	3.0
276.0592	68.27	$C_{14}H_{12}O_6$	8.3
275.0494	65.95	$C_{14}H_{11}O_6$	2.2
274.0419	21.64	$C_{14}H_{10}O_6$	1.8
248.0665	64.62	$C_{13}H_{12}O_5$	7.3
231.0645	36.24	$C_{13}H_{11}O_4$	5.2
219.0649	24.51	$C_{12}H_{11}O_4$	3.7
203.0649	24.51	$C_{12}H_{11}O_4$	3.7
203.0724	51.45	$C_{12}H_{11}O_3$	7.9
175.0745	35.52	$C_{11}H_{11}O_2$	1.1

group. The p.m.r. spectrum of compound (M) showed that only nine protons were bonded directly to carbon, the remaining three protons presumably being attached to oxygen as hydroxyl or carboxylic acid functions. This was confirmed by the c.m.r. spectrum.

The limited data available from the n.m.r. spectra combined with a positive $FeCl_3$ test suggested that compound (M) was another derivative of alternariol with the partial structure (CXLVII).



(CXLVII)

However, the structure of the rest of the molecule could not be determined, mainly due to the lack of information from n.m.r. studies.

On methylation, using diazomethane in diethyl ether, compound (M) dissolved to give a pale yellow solution. Evaporation of the ether yielded a yellow viscous oil which, on preparative thin-layer chromatography in dichloromethane, gave a crystalline solid. The mass spectrum of the solid (M1) gave a molecular ion at m/z 348, suggesting that this was the dimethyl ester of the original material. Accurate mass measurement confirmed the formula of compound (M1) as $C_{17}H_{16}O_8$, showing that 2 x CH_2 had been added during esterification.

The infrared spectrum still showed the presence of a hydrogen-bonded OH in the molecule as well as carbonyl and olefinic groups.

The ultraviolet spectrum showed little change from that of the original acid and a solution of compound (M1) gave a brown colour on addition of ethanolic $FeCl_3$.

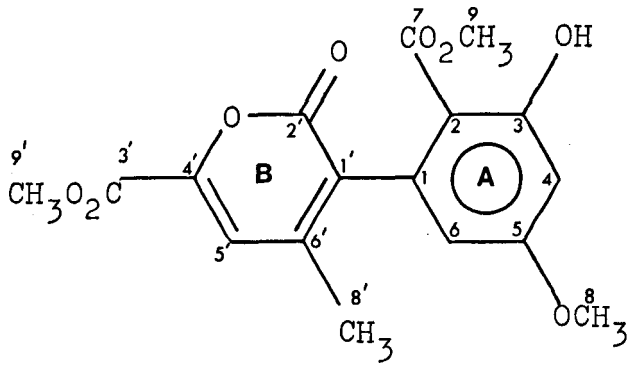
The p.m.r. spectrum of compound (M1) is summarised in Table 2.9.

The appearance of a phenolic OH peak at δ 11.53 indicates that the carboxyl group on the A ring had been methylated and was initially a free acid. The brown coloration formed during the $FeCl_3$ test also indicates weaker hydrogen bonding in the ortho-hydroxy carbonyl system. However, the composition of the rest of the molecule could not be determined. It seemed probable that, as well as containing a methyl and a carboxymethyl group, a pyrone ring was present. Several permutations were possible, but the spectroscopic data were inadequate for a conclusive assignment of structure. A long-range decoupling c.m.r. was obtained for (M1),[232] but this was still ambiguous.

The dimethyl ester (M1) seemed a likely candidate for an X-Ray crystallographic study, since it was available in well-formed crystals from ethyl acetate. Unfortunately on further examination, these crystals were found to be thinner than required being less than 0.1mm thick. In order to get thicker crystals, several methods of crystallization were tried. The most successful method was slow diffusion crystallization. This method was adopted using ethyl acetate and light petroleum (b.p. 40 - 60°); a sample of (M1) was dissolved in ethyl acetate in a 5ml conical flask. The flask was placed in a screw top jar, the bottom of which had been filled with petroleum ether (b.p. 40 - 60), and the lid was replaced. The jar

TABLE 2.9

P.M.R. SPECTRUM OF ALTERNARIAN ACID DIMETHYL ESTER



CHEMICAL SHIFT (δ)	INTEGRAL	MULTIPLICITY	SPLITTING (Hz)	ASSIGNMENT
1.98	3	singlet		8'
3.66	3	singlet		9
3.79	3	singlet		8
3.93	3	singlet		9'
6.18	1	doublet	2.5	4
6.49	1	doublet	2.5	6
7.06	1	singlet		5'
11.53	1	singlet		OH

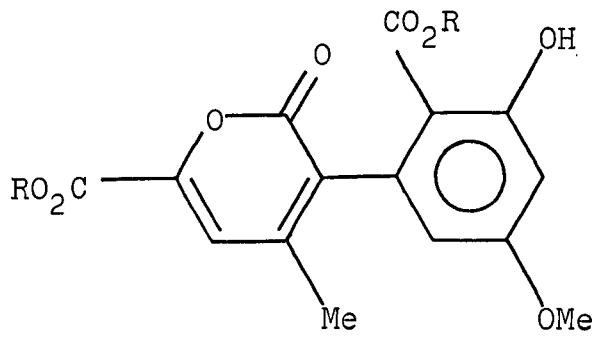
was left in a fridge overnight, and the light petroleum diffused into the conical flask. As the concentration of light petroleum increased, crystals of the dimethyl ester slowly formed on the bottom of the flask. The solvent was then removed to leave crystals of sufficient thickness for diffraction studies.

A crystal was selected and attached to the goniometer head and aligned in a similar manner to that described earlier in this thesis. The crystal data were as follows:

Initial photographs showed that the crystal of (M1) was monoclinic with a = 8.42(2), b = 13.42(3), c = 14.64(3)Å, $\beta = 104.7(1)^{\circ}$, V = 1601.6Å^3, Z = 4, Dx = 1.46 gm^{-3}. Intensity measurements were made with Cu-Kα radiation (λ = 1.5418 Å). 1970 Unique reflections were observed and the intensities measured from Weissenberg photographs of layers $\underline{h}0\underline{l}$ to $\underline{h}11\underline{l}$ for a crystal 0.3 mm in edge. Cell dimensions were determined from precession photographs and interlayer scale factors, initially, from exposure times. The structure was solved by direct methods[174] and refined on F values by full-matrix least squares.[175] At an intermediate stage, positions for all hydrogen atoms except for the hydroxyl hydrogen were located geometrically, the latter being found from a difference map. These were allowed to refine subsequently, with two common, isotropic temperature factors, one for the methyl hydrogens and the other for all other hydrogens by depending on the associated heavy atoms with appropriate bond-length constraints. In the final stages of refinement, interlayer

scale factors were refined individually and an extinction correction was applied. The R factor was finally refined to 0.071.

Compound (M1) was found to have the structure (CXLVIII) shown, identifying the B ring as an α-pyrone. This structure was unsystematically named alternarian acid dimethyl ester.



(CXLVIII) R = CH_3

(CXLIX) R = H

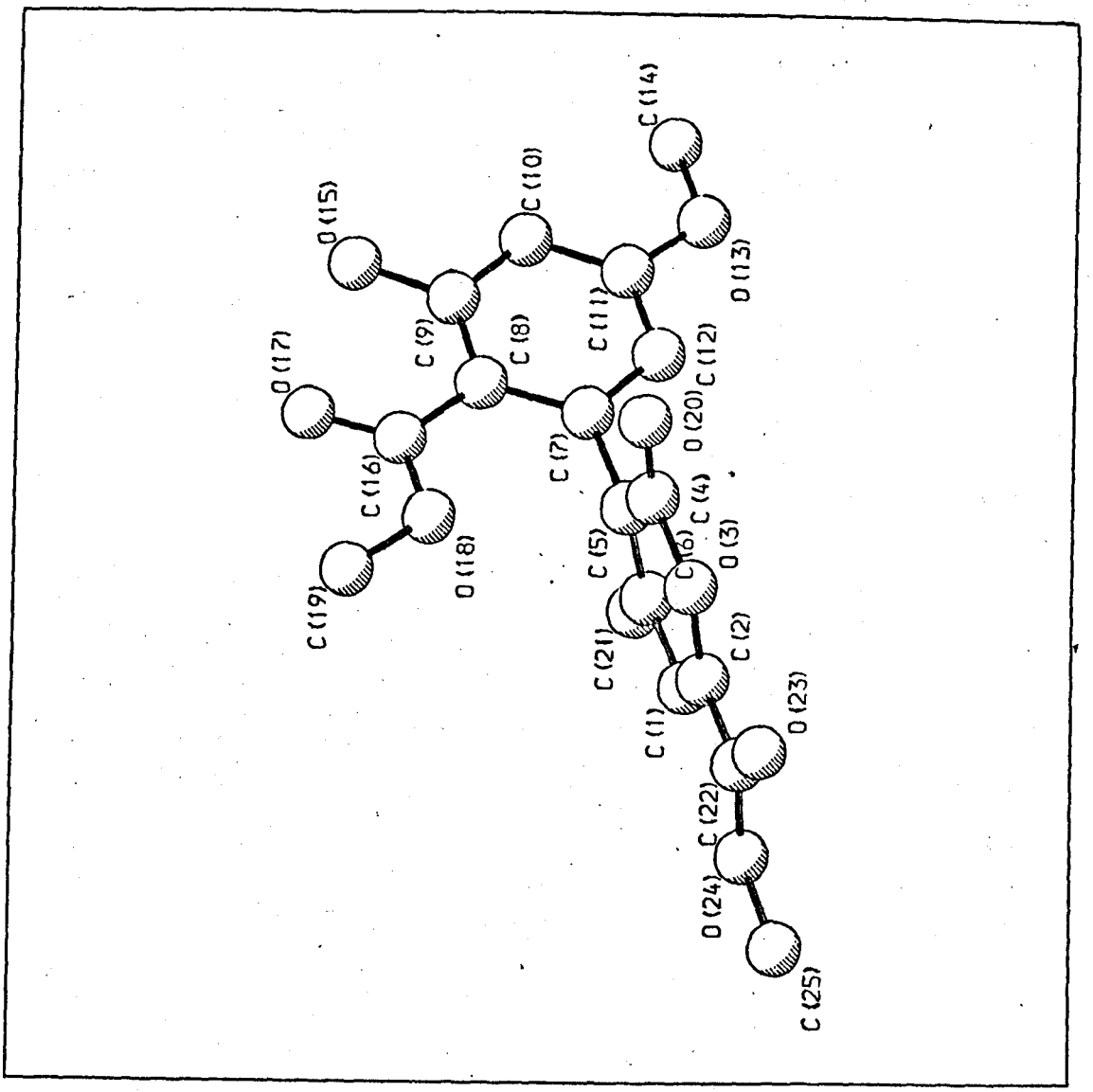
It follows, therefore, that alternarian acid (CXLIX) is compound (M).

The atomic numbering system and molecular structure of alternarian acid dimethyl ester is depicted in Figure 2.2.

A search of the Cambridge Crystallographic Database for structures containing the α-pyrone moiety not fused to another ring yielded twenty compounds. In three[233] of these, the pyrone unit bears only one alkyl-type substituent, which would not be expected to perturb the ring geometry greatly. The data from these structures have been averaged

FIGURE 2.2

ATOMIC NUMBERING SYSTEM AND MOLECULAR STRUCTURE OF ALTERNARIAN ACID DIMETHYL ESTER.



to yield a 'standard' α-pyrone ring geometry (Figure 2.3a) with which the geometry found for the current structure (Figure 2.3b) may be compared. Both the structures shown in Figure 2.3 display the expected pattern of bond length variation reflecting substantial bond fixation.

Conjugation between the π-systems of the pyrone and benzene rings of alternarian acid dimethyl ester would seen to be limited since the angle between their mean least-squares planes is 81.5°, probably a consequence of the ortho-interactions between methyl substituent and carbonyl oxygen on the pyrone ring and the carbomethoxy group on the benzene ring. The hydrogen of the hydroxyl group H(15) takes part in intramolecular hydrogen-bonding with the adjacent ester carbonyl oxygen H(15) ... O(17) 1.576Å .

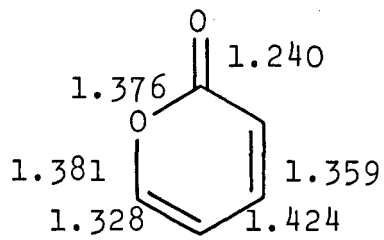
The crystal packing is of interest, exhibiting several short intermolecular contacts O(13)...C(22) 3.028Å. O(17)...C(6) 3.155Å and O(23)...C(21) 3.177Å .

Biosynthetically, it seems clear that alternarian acid is of heptaketide origin, derived from alternariol methyl ether via modification of the B ring. A biosynthetic pathway has been suggested and is shown in Figure 2.4. The suggested biogenetic process involves several steps, and the possibility of stable intermediates being produced also exists, although none was found in this investigation. Alternarian acid is the easiest pyrone structure that can be biosynthetically explained starting from alternariol methyl ether. The first step

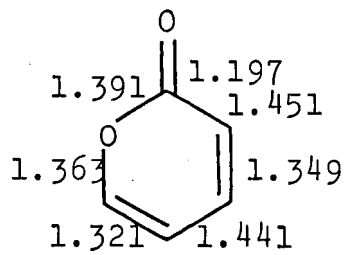
FIGURE 2.3

(a) Averaged bond lengths for the α-pyrone ring from three structures.

(b) Bond lengths found for the α-pyrone ring in alternarian acid dimethyl ester.



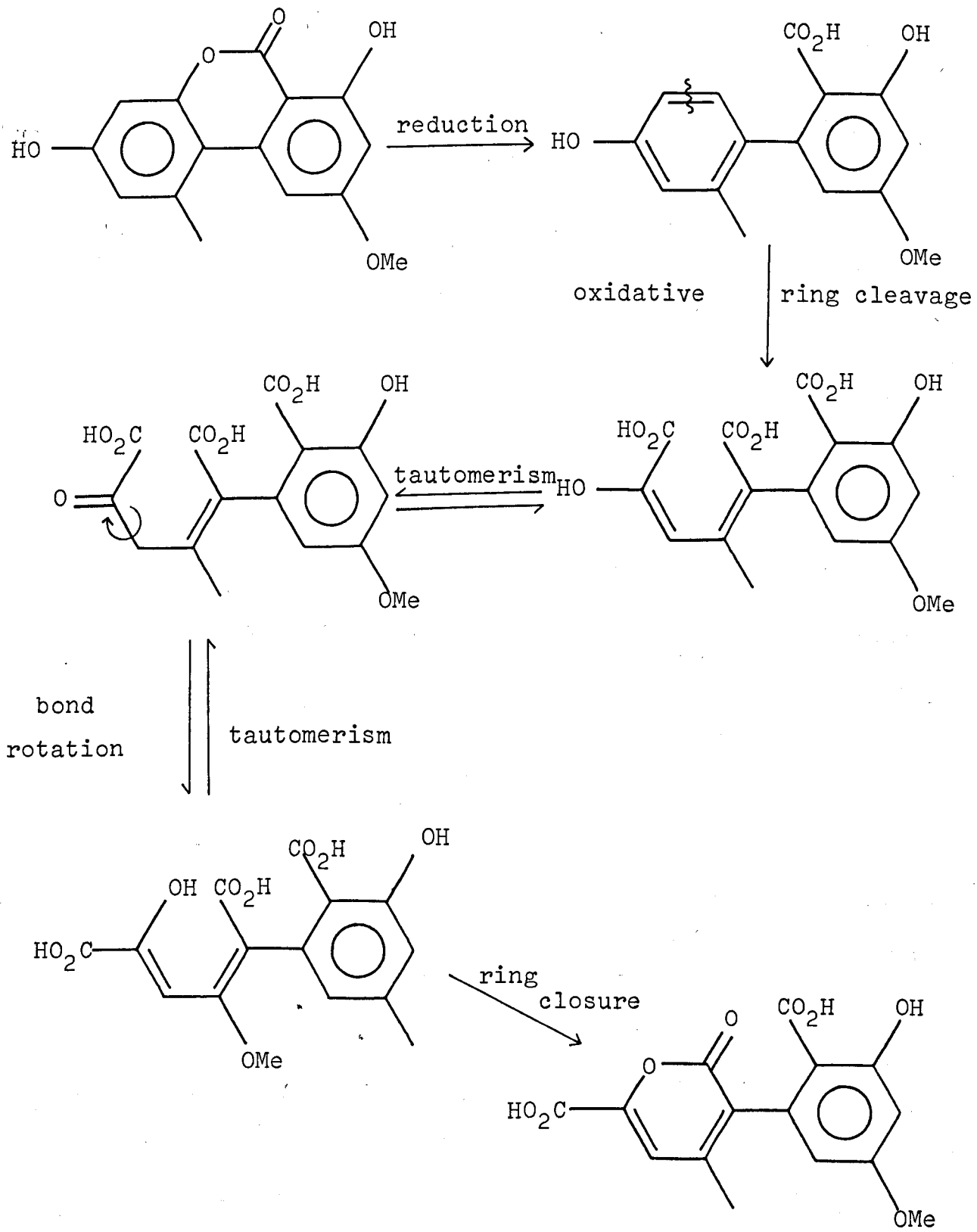
(a)



(b)

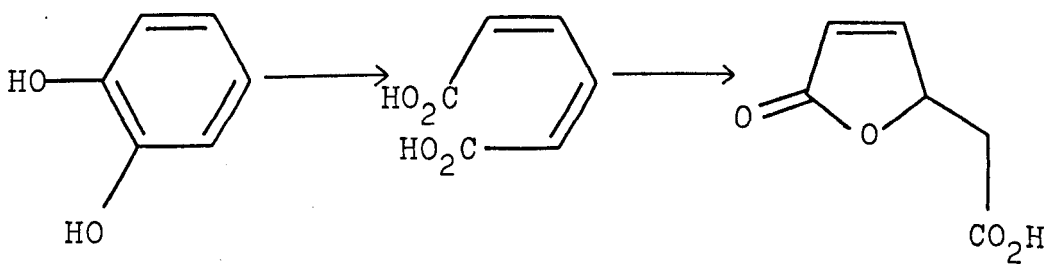
FIGURE 2.4

PROPOSED BIOSYNTHETIC PATHWAY FOR THE PRODUCTION OF ALTERNARIAN ACID FROM ALTERNARIOL METHYL ETHER.



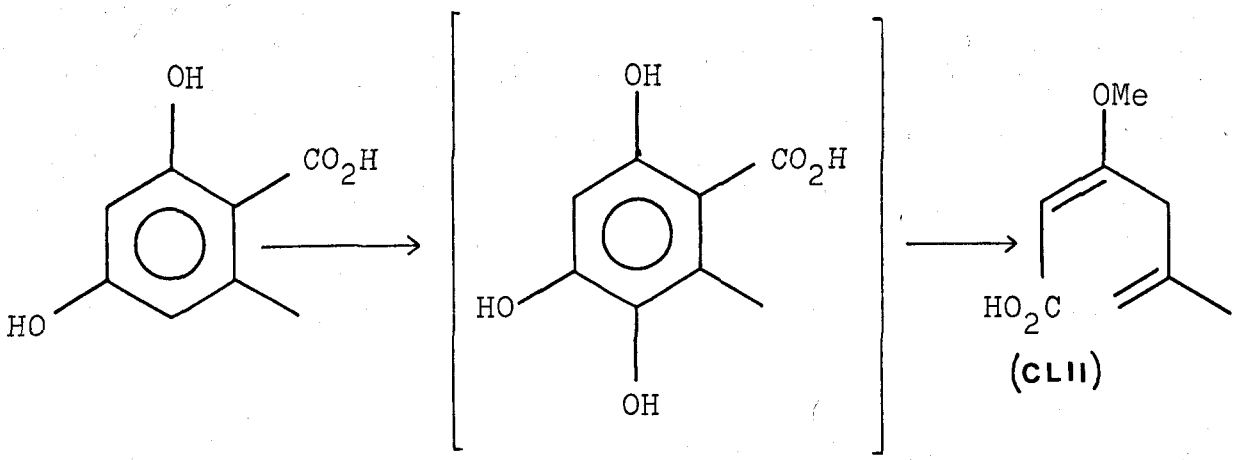
involves a reduction similar to that involved in the conversion of dehydroaltenusin to altenusin. The most important step is the oxidative ring cleavage of the enzyme-bound intermediate, which can then undergo keto-enol tautomerism followed by bond rotation. Ring closure of the intermediate will give the product, alternarian acid.

Alternarian acid is not the first example of cleavage of the B ring of an alternariol derivative. The altenuic acids are thought to be formed via oxidative ring fission of altenusin. This could yield the altenuic acids following ring cleavage of the catechol moiety similar to the metabolic degradation of catechol leading to cis, cis-muconic acid and muconolactone[234] (CL).

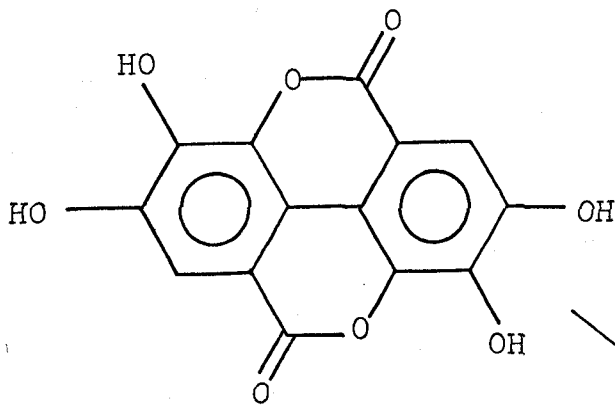


(CL)

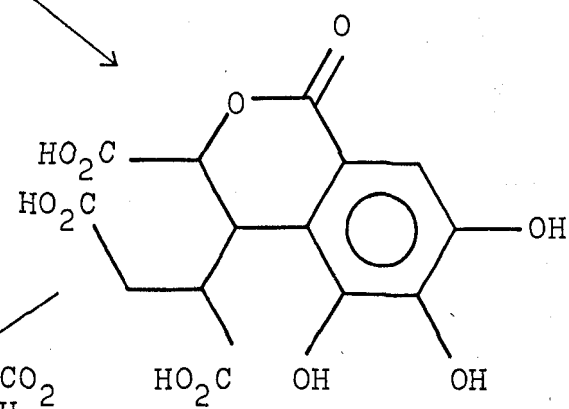
Similarly, orsellinic acid (CLI) can be converted by ring fission into penicillic acid (CLII).[239] Another example of a ring fission closely related to those involved in the formation of alternarian acid and the



(CLI) (CLII)

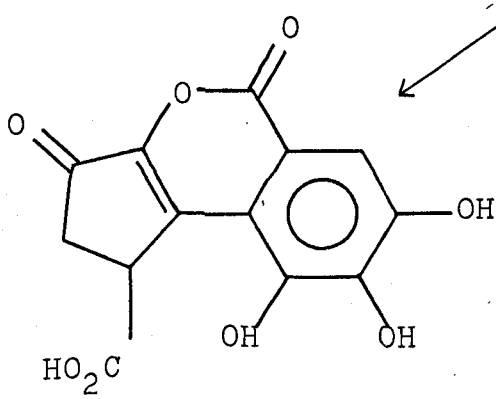


(CXLVI)



(CLIII)

$-CO_2$
$-H_2$



(CLIV)

altenuic acids is the conversion of ellagic acid (CXLVI) to chebulic[228] (CLIII) and brevifolincarboxylic acid (CLIX).[240]

To prove conclusively that the biosynthetic pathway postulated in Figure 2.4 was correct, it was decided to attempt a carbon labelling experiment using ^{14}C and ^{13}C-labelled sodium acetate as the precursor. Alternariol monomethyl ether is formed by head-to-tail condensations of seven acetate units (Figure 2.5a). However, during the step involving oxidative ring cleavage of the B ring of alternariol methyl ether, a bond between two carbons from the same acetate unit is broken (Figure 2.5b). If an enriched sample of 1,2 -^{13}C-labelled acetate was added to the growth medium and incorporated into the moledule, the lack of coupling between carbons 2' and 3' in the ^{13}C-n.m.r. spectrum of alternarian acid would show that the B ring had been broken as described.

To ascertain how much free acetate could be incorporated into alternarian acid from the growth medium, a labelling experiment using 1 ^{14}C -acetate was tried. This would also show if the organism was still producing alternarian acid, or if addition of acetate to the growth medium inhibited biosynthesis of alternarian acid. Far less ^{14}C -acetate is required for this experiment than for the ^{13}C -labelling experiment, which is expensive, and the quantity of ^{13}C -acetate that is required should be known approximately.

FIGURE 2.5a

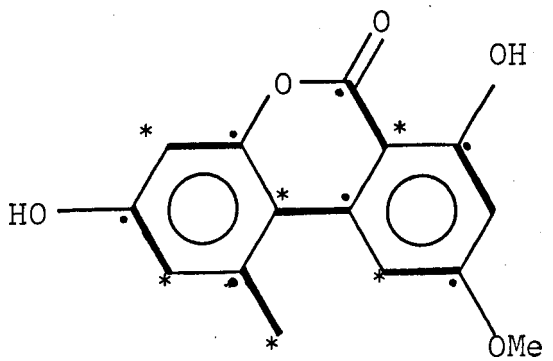
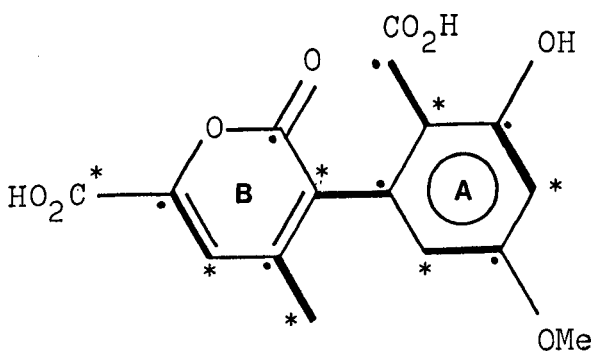


FIGURE 2.5b



▬ denotes carbon atoms originating from the same acetate unit.

* denotes carbon atoms originating from the methyl group of an acetate unit.

• denotes carbon atoms originating from the carboxy group of an acetate unit.

Eight pans containing 500 ml of growth medium each were inoculated with the same spore sample of Alternaria mali that was used for the production of alternarian acid.

Sterilized water (5 ml) was added to 250 μCi of 1-^{14}C -NaOAc; the mixture was shaken and 1 ml (50 μCi) removed and added to 800 mg of preweighed NaOAc. The mixture was made up to 8 ml with sterilized water.

Each pan was inoculated with 1 ml of the prepared labelled solution (two at a time) after 2, 4, 6 and 8 days. This was so that the ideal time of inoculation would be known for the incorporation of acetate into alternarian acid.

After twenty-eight days the cultures were harvested and the liquors were collected. The liquors from each of the two pans that were inoculated on the same day were combined, giving one litre of growth medium. 100 mg Of NaOAc had been inoculated into each pan giving a combined activity of 12.5 μCi for each sample.

The liquors were acidified with hydrochloric acid and extracted with 4 x 250 ml of ethyl acetate. The ethyl acetate was dried and evaporated, yielding a small quantity of oily solid which was washed with 2 x 25 ml diethyl ether. The solid residue was dissolved in methanol, and the solvent was left to evaporate. Methanol had previously been found to be an excellent solvent for obtaining crystals of alternarian acid but, in this case, no crystals were

obtained. H.P.L.C. analysis indicated the presence of alternarian acid, but this may have been a coincidental peak. Methylation of the solid with diazomethane gave a yellow oil, which was chromatographed on a preparative thin-layer plate, but none of the dimethyl ester of alternarian acid was isolated.

The labelling experiment was repeated using a fresher spore culture sample, but this was found to grow very slowly under the conditions described earlier, and no alternarian acid was detected on work up of the liquors. It was concluded that the A.mali strain no longer produced alternarian acid, and the experiment was abandoned.

When grown on an artificial medium, it is not unusual for many fungi to lose their ability to produce secondary metabolites. This phenomenon has previously been noticed in work done on Alternaria species. The ability of Alternaria tenuis auct., strain 94 to produce altenusin and dehydroaltenusin was lost after the wild type organism was grown for too long on an artificial growth medium. It was found that some strains remained active whereas others deteriorated rapidly. Since A.mali Roberts is morpholigically very similar to A.tenuis auct., it seems probable that the strain has deteriorated.

On examination of the structure of alternarian acid, it was suggested that hindered rotation of the bond linking the phenyl and α-pyrone ring systems existed. It is possible that the biosynthesis of alternarian acid

gives either the d- or l-isomer, which on work-up racemises to give optically inactive alternarian acid. However, as alternarian acid is not diasteriomeric, it must be modified in a way to make it diastereomeric. Several classical methods using n.m.r. spectroscopy have been used to determine optical purity, and these can also be used to determine whether hindered rotation exists in the molecule.

Pirkles method[241] of the use of a chiral solvent is simple, but suffers from the drawback that the induced shifts are often too small, sometimes being too small to be distinguished. Other approaches have included the chemical conversion of chiral alcohols and amines by treatment with optically active acid chlorides and subsequent analysis of their n.m.r. spectra.[242,243] For alternarian acid, an optically active alcohol could be used. This suffers from the disadvantage that care must be taken to isolate the derivatives of the compound without altering the enantiomeric ratio.

Chiral lanthanide shift reagents (LSR's) such as tris-(3-t-butyl-hydroxymethylene-d-camphorato)europium (III) have proved to be useful compounds.[244] Their major drawback, however, is their lack of specificity with multi-functional ligands. Also, for determining the free energy of rotation around a particular bond in a molecule, the bulk of the chiral shift reagent will also have a detrimental effect. For use with a ligand such as

alternarian acid dimethyl ester, the LSR is able to complex at several sites on the molecule, and its bulk may well interfere with free rotation around a bond. However, to get an approximate value for the free energy of rotation for alternarian acid, the LSR tris [3-(trifluoromethylhydroxymethylene)-d-camphorato] ,ytterbium (III) derivative was used with alternarian acid dimethyl ester (Alt). On addition of LSR to Alt, there are two possible equilibria:

$$dLSR + dAlt \underset{}{\overset{K^1}{\rightleftharpoons}} dLSR.dAlt$$

$$dLSR + lAlt \underset{}{\overset{K^2}{\rightleftharpoons}} dLSR.lAlt$$

Shifts may be different for two reasons:

(i) $K^1 \neq K^2$

(ii) Geometric differences of the two diastereoisomers.

N.M.R. spectra were obtained on a Bruker WM 250 spectrometer at a sweep width of 4,500 Hz using 8K data points for acquisition.

Using a 1:1 molar ratio of LSR to alternarian acid dimethyl ester, it was apparent from the n.m.r. spectrum that there was free rotation between the two ring systems. On cooling down the probe, splitting of the H8 and H9 methyl groups and H6 were observed. Splitting for the H6 hydrogen started at approximately 213K and the doublet

had shifted to δ 6.69. Without LSR added, the original position was δ 6.49, and LSR + Alt at 293K showed a peak at δ 6.63.

An approximate value for the free energy of bond rotation for the LSR alternarian acid dimethyl ester complex was calculated from the coalescence temperature (Tc), and separation of the signals ($\delta\nu$) at -80°C using equation (1).[245]

$$\frac{\Delta G}{RTc} = \log_e (\sqrt{2}R/\pi Nh) + \log_e (Tc/\delta\nu)$$

$$= 21.90 + \log_e (Tc/\delta\nu) \qquad (1)$$

$\delta\nu$ was taken as 19.65Hz

R = 2.075

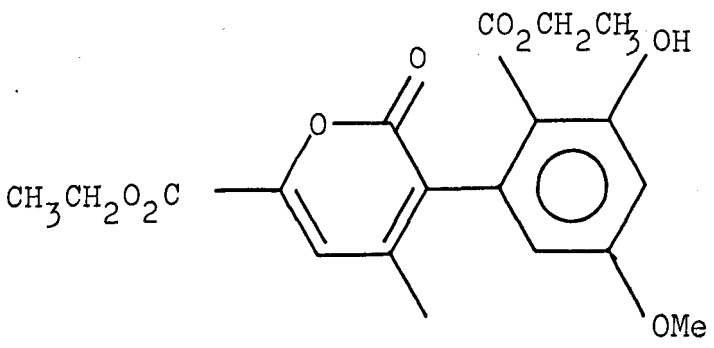
Using these values, ΔG was calculated as 9.685Kcal mol^{-1}.

It should be stressed that this figure is only approximate, and only of limited use due to the size of the LSR and the number of possible complexing sites of the molecule.

It was then decided to make the diethyl ester of alternarian acid. The methylene hydrogens on the ethyl

groups of the d- and l-forms of alternarian acid diethyl ester would be non-equivalent and should, therefore, on separation of the d- and l-forms on cooling, give differing δ values. The ethyl group, being considerably smaller than the LSR, should give a more accurate figure for the free energy of bond rotation between the phenyl and α-pyrone ring systems. Also, the question of where the LSR complexes with the alternarian acid derivative does not arise.

Alternarian acid diethyl ester (CLV) was synthesised by the action of boiling ethanol and concentrated H_2SO_4 on alternarian acid. Neutralisation followed by extraction with ethyl acetate and evaporation of solvent gave the



(CLV)

crude alternarian acid diethyl ester. Preparative t.l.c. using dichloromethane as eluate gave the pure product. The ^1H n.m.r. spectrum of alternarian acid diethyl ester is summarised in Table 2.10.

TABLE 2.10

P.M.R. SPECTRUM OF ALTERNARIAN ACID DIETHYL ESTER IN DICHLOROMETHANE

CHEMICAL SHIFT (δ)	INTEGRAL	MULTIPLICITY	SPLITTING (Hz)
1.26	3	triplet	6.5
1.39	3	triplet	7.5
2.00	3	singlet	
3.83	3	singlet	
4.15	2	quartet	6.5
4.41	2	quartet	7.5
6.22	1	doublet	2.5
6.53	1	doublet	2.5
7.09	1	singlet	
11.62	1	singlet	

Unfortunately on reduction of the probe temperature, no more splitting of any peaks was observed. At 193K, significant line broadening appeared, and the temperature was not decreased further. There are two possible reasons why peak splitting was not observed:

(i) At 293K there is free rotation between the phenyl and α-pyrone rings, resulting in a time averaged spectrum for the methylene hydrogens of the ethyl groups. However, when the temperature is lowered, there is no free rotation, and the d- and l-isomers are no longer in equilibrium. But for alternarian acid diethyl ester, there may be very little or no

difference between the δ values for the d- and l-isomers.

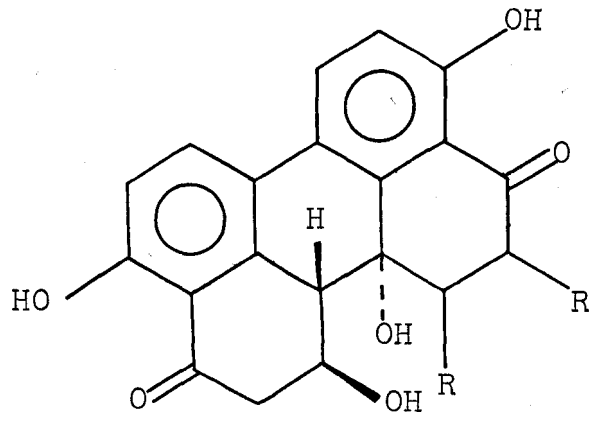
(ii) There is still free rotation between the two ring systems at 193K, so the d- and l-forms are not distinguishable.

CONCLUSIONS

Some phytotoxins have been isolated from both the *Alternaria* strains that have been investigated; kojic acid from *A.citri*, and alternariol monomethyl ether and altenuene from *A.mali*. These compounds can account for the general phytotoxicity of both species, but not for host-specificity. Although the phytotoxicity of several of the metabolites, notably alternarian acid, is unknown, it is unlikely that any of them show marked host-specificity. Alternarian acid, in common with other alternariol derivatives may exhibit biocidal activity but the quantities of the metabolites that were obtained were too small for extensive testing.

Other phytotoxic compounds have been isolated recently from *Alternaria* species. Alterperylenol (CLVI) and dihydroalterperylenol (CLVII),[246] from an unidentified *Alternaria* strain show phytotoxic and antifungal activity.

As techniques for the isolation and purification of natural products have become faster and more efficient, the number of known metabolites has greatly increased in recent years, and it seems likely that this increase will continue in the future.



(CLVI) R = H

(CLVII) R = H_2

EXPERIMENTAL

FUNGAL CULTURE

Alternaria mali Roberts, strain 106.24 obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands, was grown for twenty-eight days at 25°C in static culture on potato dextrose medium consisting of soluble potato extract (250 g), dextrose (750 g) and tap water (25 l), 500 ml of growth medium per pan.

EXTRACTION OF MYCELIUM FROM ALTERNARIA MALI.

The mycelium of *A.mali* was filtered from the growth medium, dried in a vacuum oven for twenty-four hours, and ground to a fine powder. The powder was placed in a Soxhlet extractor, and extracted with petroleum ether (b.p. 40 - 60°) for forty-eight hours, followed by extraction with ethyl acetate for forty-eight hours.

The petrol extract was dried and the solvent removed to leave a pale orange oil. Flash chromatography of the oil on silica-gel using petroleum ether (b.p. 40 - 60°) as eluent yielded a yellow oil. Leaching of the column with dichloromethane gave an oily yellow solid (G).

The ethyl acetate extract of the mycelium of *A.mali* was dried and the solvent evaporated to yield a dark brown oil. The oil was redissolved in ethyl acetate (250 ml) and washed with 4 x 250 ml aliquots of Na_2CO_3 solution (8% w/v), followed by 4 x 250 ml of 0.5M NaOH solution. The NaOH solution was acidified to pH 4 - 5 with dilute HCl, and back-extracted into ethyl acetate (4 x 250 ml).

The ethyl acetate was dried and evaporated to give a mixture of oil and solid. Preparative t.l.c. of the mixture on silica-gel in chloroform:methanol (9:1) showed two major bands which fluoresced in ultraviolet light. The two bands were extracted from the silica-gel using acetone in a Soxhlet extractor to give two solids, (J) with the lowest r.f. value, and (K) with the highest r.f. value.

IDENTIFICATION OF COMPOUND (G)

Compound (G) was crystallized from ethanol to give flaky crystals (524 mg). Compound (G) was identified as ergosterol (CXXXVIII), m.p. 159 - 161° (lit. 168 - 170°)[236] by comparison of its physical and spectroscopic data with an authentic sample.

IDENTIFICATION OF COMPOUND (J)

Compound (J) was an off-white solid which, on crystallization from methanol, gave colourless plates (34 mg). Compound (J) was identified as altenuene (XCII), m.p. 188 - 189° (lit. 190 - 191°).[143] R.f. ($CHCl_3$:MeOH, 95:5), 0.2. $\bar{\nu}_{max.}$ (KBr); 3500 - 3300 (br.), 1635, 1590, 1050 cm^{-1}. $\lambda_{max.}^{EtOH}$ nm (log ε); 320 (3.80), 2.80 (3.95), 242 (3.32). δ (d4-methanol); 1.50 (3H, s, CH_3), 1.98 (1H, m, CH), 2.40(1H, m, CH), 3.80 (1H, m, CH), 3.88 (3H, s, CH_3), 4.08 (1H, m, CH), 6.23 (1H, d, CH), 6.48 (1H, d, CH), 6.66 (1H, d, CH). M^+: 292.09618;

$C_{15}H_{16}O_6$ requires 292.09468. M/z (% R.A.); 292 (55), 277 (17), 274 (14), 248 (52), 228 (28), 220 (76), 177 (100).

IDENTIFICATION OF COMPOUND (K)

The pale yellow solid, compound (K), was crystallized from ethanol to give colourless needles, (433 mg). Compound (K) was identified as alternariol monomethyl ether (LXXXV), m.p. 263 - 264° (lit. 267°).[138] R.f. ($CHCl_2$: MeOH, 95:5); 0.54. $\bar{\nu}_{max.}$ (KBr); 3300 (br.), 1650, 1610, 1585 cm^{-1}. $\lambda_{max.}^{EtOH}$ nm (log ε); 217 (4.34), 259 (4.68), 297 (4.02), 303 (4.02), 336 (4.04). δ (d6-acetone), 2.80 (3H, s, CH_3), 3.99 (3H, s, CH_3), 6.62 (1H, d, CH), 6.77 (1H, d, CH), 6.86 (1H, d, CH), 7.34 (1H, d, CH), 11.97 (1H, s, OH). M^+ 272.06736; $C_{15}H_{12}O_5$ requires 272.06847. M/z (% R.A.), 272 (100), 243 (34), 229 (16), 201 (42).

EXTRACTION OF METABOLITES FROM THE LIQUORS OF ALTERNARIA MALI.

The mycelium of A.mali was filtered from the aqueous growth medium. The filtrate was concentrated to approximately four litres and extracted with light petroleum (b.p. 40 - 60°) followed by extraction with diethyl ether (4 x 1 l). The ether extract was dried and the solvent evaporated to yield a brown oily solid (L).

After being extracted with diethyl ether, the liquors were acidified to approximately pH 2 with dilute hydrochloric acid and extracted with 4 x 500 ml aliquots of

ethyl acetate. The organic layer was separated and dried, and the ethyl acetate solution concentrated to approximately 20 ml. After leaving at $0^{o}C$ for twenty-four hours, a crystalline solid and a brown oil settled on the bottom of the flask. The solvent was decanted off and the residual solid was washed with 4 x 25 ml aliquots of cold ethyl acetate, leaving a pale orange solid (M).

IDENTIFICATION OF COMPOUND (L).

Compound (L) was crystallized from ethanol to give small yellow prisms (226 mg). Recrystallization from ethanol did not remove the yellow coloration. Compound (L) was identified as dehydroaltenusin (XC), m.p. 190 - 191° (lit. 189 - 190°).[102a] Found: C, 60.99; H, 4.60%; $C_{15}H_{12}O_6$ requires C, 62.50; H, 4.20%. $\bar{\nu}_{max.}$ (KBr), 3370, 1665, 1630, 1615, 1070 cm^{-1}. $\lambda_{max.}^{EtOH}$ nm (log ε), 219 (4.50), 249 (3.88), 294 (3.84). δ ($CDCl_3$), 1.71 (3H, s, CH_3), 3.89 (3H, s, CH_3), 6.28 (1H, s, CH), 6.62 (1H, d, CH), 6.69 (1H, s, CH), 6.74 (1H, d, CH), 11.26 (1H, s, OH). ^{13}C p.p.m. 29.41, 55.91, 79.01, 99.72, 103.58, 104.20, 116.06, 120.69, 146.04, 152.89, 164.51, 166.18, 167.15, 180.61, 235.20. M^+ 288.0643; $C_{15}H_{12}O_6$ requires 288.0638. M/z (% R.A.), 288 (100), 273 (10.8), 260 (40.0), 259 (20.3), 243 (12.2), 232 (26.6), 217 (25.4), 177 (18.0).

ALTENUSIN (LXXXIX).

Dehydroaltenusin (100 mg) was dissolved in boiling ethanol (2 ml) and saturated sodium dithionite solution ($Na_2S_2O_4$, 2 ml) was added; the yellow colour was immediately discharged. The mixture was cooled and water (2 ml) was added. A colourless solid separated and was filtered, dried and recrystallized from CHCl. Colourless crystals of altenusin, m.p. 196 - 198° (lit. 201°)[102a] were obtained (43 mg, 42%). $\bar{\nu}_{max.}$ (nujol), 3400 - 3300 (br.), 1630, 1605, 1520, 1040 cm^{-1}. $\lambda_{max.}^{EtOH}$ nm (log ε), 219 (3.92), 247 (2.73), 294 (2.44). δ (d6-DMSO), 1.88 (3H, s, CH_3), 3.77 (3H, s, CH_3), 6.15 (1H, d, CH), 6.46 (1H, s, CH), 6.48 (1H, d, CH), 6.56 (1H, s, CH). M^+ 290.0788; $C_{15}H_{14}O_6$ requires 290.0790. M/z (% R.A.), 290 (62.7), 272 (100.0), 262 (31.3), 246 (22.1), 206 (43.5).

IDENTIFICATION OF COMPOUND (M)

Compound (M) was crystallized from methanol to give large, colourless cubes (908 mg). Compound (M) was identified as alternarian acid (CXLIX), m.p. 144 - 149°. Found: C, 52.46; H, 4.34%. $\bar{\nu}_{max.}$ (KBr), 3270 (br.), 2650 (br.), 1750, 1710, 1620, 1585, 1215, 965 cm^{-1}. $\lambda_{max.}^{EtOH}$ nm (log ε), 220 (4.53), 260 (4.11), 305 (4.16). δ (d4-methanol), 2.06 (3H, s, CH_3), 3.86 (3H, s, CH_3), 6.35 (1H, d, CH), 6.52 (1H, d, CH), 7.15 (1H, s, CH). ^{13}C (p.p.m.), 19.82 (q), 56.15 (q), 101.76 (d), 105.32 (s), 110.87 (d), 115.05 (d), 132.99 (s), 139.41 (s), 147.09 (s),

160.61 (s), 160.96 (s), 165.65 (s), 166.84 (s), 172.74 (s). M^+ 320.0486. M/z (% R.A.), 320 (41.4), 303 (15.9), 302 (100.0), 276 (68.3), 275 (66.0), 274 (21.6), 248 (64.6), 231 (36.2), 219 (24.5), 203 (51.5), 175 (35.5). H.p.l.c. retention time 140 seconds, H_2O:MeOH (80:20).

ALTERNARIAN ACID DIMETHYL ESTER (CXLVIII)

Alternarian acid (M; 74.4 mg) was added to a four molar excess of diazomethane (39 mg) in diethyl ether (25 ml) in a 50 ml conical flask. The mixture was stirred and as the alternarian acid dissolved, bubbles of gas were given off. After fifteen minutes acetic acid (5 ml) was added to the yellow solution to destroy excess diazomethane, and the solvent was evaporated in vacuo to leave a yellow oil. The oil was chromatographed on a preparative t.l.c. plate (silica-gel, CH_2Cl_2). Soxhlet extraction of the silica-gel using chloroform, followed by evaporation of the solvent yielded a colourless solid. Crystallization of the solid from ethyl acetate yielded small plates of alternarian acid dimethyl ester (58 mg, 72%), m.p. 153 - 155°C. Found: C, 57.18; H, 4.75%. $\bar{\nu}_{max.}$ (KBr), 1727, 1710, 1655, 1608, 1573, 969 cm^{-1}. $\lambda_{max.}^{EtOH}$ nm (log ε), 214 (4.48), 258 (4.16), 306 (4.18). δ ($CDCl_3$), 1.98 (3H, s, CH_3), 3.66 (3H, s, CH_3), 3.79 (3H, s, CH_3), 3.93 (3H, s, CH_3), 6.18 (1H, d, CH), 6.49 (1H, d, CH), 7.06 (1H, s, CH), 11.53 (1H, s, OH).

M^+, 348.08410. M/z (% R.A.), 348 (75.5), 316 (91.3), 289 (100.0), 261 (46.2), 245 (48.7), 233 (50.7), 201 (83.3), r.f. (silica-gel, CH_2Cl_2), 0.48.

ALTERNARIAN ACID DIETHYL ESTER (CLV)

Alternarian acid (M; 108.5 mg) was refluxed for ten hours in absolute ethanol (15 ml) and concentrated sulphuric acid (5 drops). After cooling, ethyl acetate (25 ml) was added and the mixture was washed with sodium bicarbonate solution (2 x 25 ml, 8% w/v). The organic layer was washed with water (20 ml) and dried. The solvent was evaporated to leave a yellow solid. The solid was chromatographed on a preparative t.l.c. plate (silica-gel, CH_2Cl_2). Soxhlet extraction of the silica-gel using chloroform, followed by evaporation of the solvent yielded a colourless solid, alternarian acid diethyl ester (66 mg, 52%), m.p. 158 - 160°C. $\bar{\nu}_{max.}$ (nujol), 3500 (br.), 1735, 1715, 1660, 1615, 1585, 1340, 1270 cm^{-1}. $\lambda_{max.}^{EtOH}$ nm (log ε), 214 (4.43), 260 (3.92), 309 (3.95). δ ($CDCl_2$), 1.26 (3H, t, CH_3), 1.39 (3H, t, CH_3), 2.00 (3H, s, CH_3), 3.83 (3H, s, CH_3), 4.15 (2H, q, CH_2), 4.41 (2H, q, CH_2), 6.22 (1H, d, CH), 6.53 (1H, d, CH), 7.09 (1H, s, CH), 11.62 (1H, s, OH). M^+, 376.09482, r.f. (silica-gel, CH_2Cl_2), 0.53.

REFERENCES

1. Y.Suhara, F.Sasaki, G.Foyama, K.Moeda, M.Ohno, *J.Am.Chem.Soc., 94,* 6501 (1972).
2. A.S.Tahori, "Pesticide Chemistry", Gordon and Breach, (1972), p.119.
3a. G.A.Strobel, *Ann.Rev.Plant Physiol., 25,* 541 (1974).
3b. R.K.S. Wood, A.Ballio, A.Graniti, "Phytotoxins in plant diseases", Academic Press (1972).
3c. S.Kadis, A.Ciegler, S.J.Ajl, "Microbial Toxins", Academic Press (1972), Vol. 8, pp. 131 - 247.
4. The Pesticide Manual : A world compendium; 6th edition, ed., C.R.Worthing, Pub. Glasshouse Crops Research Institute, p.490 (1979).
5. A. De Bary, *Botan.Z., 49,* 337 (1886).
6. C.M.Hutchinson, *India Dept.Agr.Mem.Bact.Ser., 67,* 1 (1913).
7. D.Gottlieb, *Phytopathology, 33,* 126 (1943).
8. A.E.Dimond, P.E.Waggoner, *Phytopathology, 43,* 229 (1953).
9. I.A.M. Cruikshank, *Ann.Rev.Phytopathol., 1,* 351, (1962).
10. F.Meehan, H.C.Murphy, *Science, 106,* 270 (1970).
11. R.P.Scheffer, R.B.Pringle, *Phytopathology, 52,* 750 (1962).
12. R.B.Pringle, A.C.Braun, *Nature, 181,* 1205 (1958).
13. D.Arrigoni, *J.C.S. Chem.Commun.,* 1342, (1972).
14. R.B.Pringle, R.P.Scheffer, *Phytopathology, 53,* 785 (1963).
15. T.J.Wolpert, L.D.Dunkle, *Phytopathology, 70,* 872, (1980).

16. R.B.Pringle, *Plant Physiol., 48,* 756, (1971).

17. R.P.Scheffer, A.J.Ullstrup, *Phytopathology, 55,* 1037, (1965).

18a. M.L.Gross, F.McCreery, F.Crow, K.B.Tomer, M.R.Pope, L.M.Cuiffetti, H.W.Knocke, J.M.Daly, L.D.Dunkle, *Tetrahedron Lett., 23,* 5381, (1982).

18b. J.M.Liesch, C.C.Sweeley, G.D.Stafford, M.S.Anderson, D.J.Weber, R.P.Scheffer, *Tetrahedron, 38,* 45, (1982).

19. J.D.Walton, E.D. Earle, B.W.Gibson, *Biochem.Biophys., Res.Commun., 107,* 785, (1982).

20. A.Hirohita, A.Suzuki, H.Susuki, S.Tamura, *Agr.Biol. Chem., 37,* 643, (1973).

21. T.Ueno, T.Nakashima, Y.Hayashi, H.Fukami, *ibid., 39,* 1115, (1975).

22. A.Closse, R.Huguenin, *Helv.Chim.Acta, 57,* 533, (1974).

23. T.Ueno, T.Nakashima, Y.Hayashi, H.Fukami, *Agric. Biol.Chem., 39,* 2081, (1975).

24. S.Tanaka, *Mem.Coll.Agric.Kyoto Univ., 28,* 1, (1933).

25. S.Nishimura, K.Kohmoto, H.Otani, *Recognition of Specific Plant Host-Parasite Interactions,* (U.S. - Japan Seminar), 133 - 146, (1979).

26. T.Nakashima, T.Ueno, H.Fukami, *Tetrahedron Lett., 23,* 4469, (1982).

27. K.Kohmoto, I.D.Khan, Y.Renbutsu, T.Taniguchi, S.Nishimura, *Physiol.Plant Pathol., 8,* 141, (1976).

28. T.Ueno, T.Nakashima, Y.Hayashi, H.Fukami, *Agric. Biol.Chem., 39,* 1115, (1975).

29. T.Okuno, Y.Ishita, A.Sugawara, Y.Mori, K.Sawai, T.Matsumoto, *Tetrahedron Lett., 16,* 335, (1975).

30a. K.Noda, Y.Shibata, Y.Shimohigashi, N.Izumiya, E. Gorss, *ibid., 21,* 763, (1980).

30b. T.Kanmera, H.Aoyagi, M.Waki, T.Kato, N.Izumiya, K.Noda, T.Ueno, *ibid., 22,* 3625, (1981).

31. R.G.Grogan, K.A.Kimble, I.Misaghi, *Phytopathology, 65,* 880, (1975).

32a. A.T.Bottini, D.G.Gilchrist, *Tetrahedron Lett., 22,* 2719, (1981).

32b. A.T.Bottini, J.R.Bowen, D.G.Gilchrist, *Tetrahedron Lett., 22,* 2723, (1981).

33. K.Kohmoto, R.P.Scheffer, J.O.Whiteside, *Phytopathology, 69,* 667, (1979).

34. Y.Kono, J.M.Daly, *Bioorganic Chemistry, 8,* 391, (1971).

35. G.W.Steiner, G.A.Strobel, *J.Biol.Chem., 246,* 4350, (1971).

36. W.L.Meyer, G.E.Templeton, C.I.Grable, R.Jones, L.F.Kuyper, *J.Am.Chem.Soc., 97,* 3802, (1975).

37. S.Takeuchi, M.Senn, R.W.Curtis, F.W.McLafferty, *Phytochemistry, 6,* 287, (1967).

38. M.Bodanszky, G.L.Stahl, *Proc.Natl.Acad.Sci., U.S.A., 71,* 2791, (1974).

39. R.J.Anderegg, K.Biemann, G.Buchi, M.Cushman, *J.Am. Chem.Soc., 98,* 3365, (1976).

40. S.Marumo, *Bull.Agric.Chem.Soc.Jpn., 23,* 428, (1959).

41. I.Ishikawa, Y.Ueno, H.Tsunoda, *J.Biochem., 67,* 753, (1970).

42. P.S.steyn, A.A.Tuinman, F.R.Van Heerden, P.H. Van Rooyen, P.L.Wessels, C.J.Rabie, *J.C.S., Chem. Commun., 47,* (1983).

43. B.F.Burrows, S.D.Mills, W.B.Turner, *J.C.S., Chem. Commun., 75,* (1965).

44. J.P.Ferezou, C.Riche, A.Quesneau-Thierry, C.Pascard-Billy, M.Barbier, J.F.Bousquet, G.Boudart, *Nouv. J.Chim., 1,* 327, (1977).

45. M.Devys, M.Berbier, A.Kollmann, J-F. Bousquet, *C.R.Hebd.Seances Acad.Sci.Ser.C, 286,* 457, (1978).

46. M.L.Bouillant, J.L.Pittet, J.Bernillon, J.Favre-Bonvin, N.Arpin, *Phytochemistry, 20,* 2705, (1981).

47. K.A.Frantzen, L.B.Johnson, D.L.Stuteville, *Phytopathology, 72,* 568, (1982).

48. A.J.Anderson, *Can.J.Bot., 58,* 2343, (1980).

49. G.Strobel, N.Van Alfen, K.D.Happner, W.McNeil, P.Albersheim, *Biochim.Biophys., Acta, 538,* 60, (1978).

50. K.J.Stevenson, J.A.Slater, S.Takai, *Phytochemistry, 18,* 235, (1979).

51. N.T.Keen, M.Long, *Physiol.Plant Pathol., 2,* 307, (1972).

52. N.T.Keen, M.Long, D.C.Erwin, *ibid., 2,* 317, (1972).

53. D.H.R.Barton, K.Barrow, Sir E.Chain, U.Ohnsorge, R.Thomas, *J.Chem.Soc., (C),* 1265, (1971).

54. G.Randazzo, A.Evidente, R.Capaso , S.Chiosi, C.G.Cassinovi, A.Ballio, *Phytopathol.Mediterr, 19,* 181, (1980).

55. T.Sassa, *Agric.Biol.Chem., 34,* 1588, (1970).

56. T.Sassa, M.Togashi, T.Kitaguchi, *Agric.Biol.Chem., 39,* 1735, (1975).

57. S.Nozoe, M.Morisaki, K.Tsuda, Y.Iitaka, N.Takahashi, S.Tamura, *J.Am.Chem.Soc., 87,* 4968, (1965).

58. L.Canonica, A.Fiecchi, M.Gallikenle, B.Ranzi, A. Scala, *Tetrahedron Lett.,* 1329, (1966).

59. S.Nozoe, K.Hirai, K.Tsuda, *ibid.,* 2211, (1966).

60. D.A.Hoisington, *Diss.Abstr.Int.B., 41,* 754, (1980).

61. A.T.McPhail, J.Kepler, M.Wall, J.Mason, C.Bassett, G.Sim, *J.Am.Chem.Soc., 89,* 1260, (1967).

62. M.Hirotani, J.O'Reilly, D.M.X.Donnelly, J.Polonsky, *Tetrahedron Lett., 18,* 651, (1977).

63. D.M.X. Donnelly, J.O'Reilly, J.Polonsky, G.W.Van Eijk, *Ibid., 23,* 5451, (1982).

64. P.DeMayo, E.Spencer, R.White, *Can.J.Chem., 39,* 1608, (1961).

65. A.W.Dawkins, *J.Chem.Soc., (C),* 116, (1966).

66. C.Riche, C.Pascard-Billy, M.Devys, A.Gaudener, M.Barbier, *Tetrahedron Lett.,* 2765, (1974).

67. T.K.Dahr, K.A.I.Siddiqui, E.Ali, *ibid., 23,* 5459, (1982).

68. J.F.Grove, R.Speake, G.Ward, *J.Chem.Soc., (C),* 230, (1966).

69. R.Goddard, I.K.Hatton, J.A.K.Howard, J.MacMillan, C.J.Gilmore, *J.C.S. Chem.Commun., 11,* 408, (1976).

70. Y.Kimura, T.Hamasaki, A.Suzuki, *Koen Yoshishu, 23,* 288, (1979).

71. M.Okuchi, M.Itoh, Y.Kaneko, S.Doi, *Agric.Biol.Chem., 32,* 394, (1968).

72. C.Wei, C.S.McLaughlin, *Biochem.Biophys.Res.Commun., 57,* 838, (1974).

73. B.Bohner, E.Fetz, E.Harri, H.P.Sigg, C.Stohl, C. Tamm, *Helv.Chim.Acta, 48,* 1079, (1965).

74. J.F.Grove, *Quart.Revs., 15,* 56, (1961).

75. W.Rademacher, J.E.Graebe, *Biochem.Biophys.Res. Commun., 91,* 35, (1980).

76. N.Murdofushi, M.Sugimoto, K.Itoh, N.Takahashi, *Agric.Biol.Chem., 43,* 2179, (1979).

77. J.MacMillan, Gibberellins-Chemistry, Physiology and Use, Monograph 5, Ed., J.R.Lenton, page 1, (1980).

78. P.S.Steyn, *Pure and Appl.Chem., 52,* 189, (1979).

79. N.Claydon, J.F.Grove, M.Hosken, *Chem.Ind., 20,* 344, (1974).

80. Idem., *Phytochemistry, 13,* 2567, (1974).

81. R.J. Ch.Lousberg, Y.Tirilly, *Experientia, 32,* 1394, (1976).

82. K.Kobayashi, T.Ui, *Physiol.Plant Pathol., 11,* 55, (1977).

83. Idem, *ibid., 14,* 129, (1979).

84. J.A.Ballantine, V.Ferrito, C.H.Hassal, V.I.P.Jones, *J.Chem.Soc., (C),* 56, (1969).

85. L.Trifinou, J.H.Bieri, R.Prewo, A.S.Dreiding, D.M. Rast, L.Hoesch, *Tetrahedron, 38,* 397, (1982).

86. H.Oku, T.Nankanishi, *Phytopathology, 53,* 1321, (1963).

87. I.Iwai, H.Mishima, *Chem.Ind.*, 186, (1965).

88. E.Hardegger, W.Reider, A.Walser, F.Kugler, *Helv. Chim.Acta, 49,* 1283, (1966).

89. A.A.Chalmers, C.P.Gorst-Allman, P.S.Steyn, R.Vleggar, W.F.Marasas, S.F.Kriek, *S.Afr.J.Chem., 31,* 111, (1978).

90. R.A.Baker, J.H.Tatum, S.Namec, *Phytopathology, 71,* 951, (1981).

91. H.R.Arnstein, A.H.Cook, *J.Chem.Soc.,* 1021, (1947).

92. P.S.Steyn, C.W.Holzapfel, *J.S.Afr.Chem.Inst., 20,* 186, (1967).

93. C.J.Kruger, P.S.Steyn, R.Vleggar, C.J.Rabie, *J.C.S.Chem.Commun.,* 441, (1979).

94. S.Kuyama, *J.Org.Chem., 27,* 939, (1962).

95. R.J.Ch.Lousberg, U.Weiss, C.A.Salemink, A.Arnone, L.Merlini, G.Nasini, *J.C.S.Chem.Commun.,* 1463, (1971).

96. M.E.Daub, *Phytopathology, 72,* 370, (1982).

97a. S.Sakamura, H.Niki, Y.Obata, R.Sakai, T.Matsumoto, *Agric.Biol.Chem., 33,* 690, (1969).

97b. S.Sakamura, J.Ito, R.Sakai, *ibid., 35,* 105, (1971).

98. K.Tamari, J.Kaji, *Nippon Nougei Kagaku Kaishi, 28,* 254. (1954).

99. S.Iwasaki, S.Nozoe, S.Okuda, Z.Sata, T.Kozaka, *Tetrahedron Lett.,* 3977, (1969).

100. T.Sassa, M.Nukina, M.Ikeda, T.Umezawa, H.Tasaki, *Agric.Biol.Chem., 45,* 2161, (1981).

101. N.Umetsu, J.Kaji, K.Tamari, *ibid., 37,* 451, (1973).

102a T.Rosett, R.H.Sankhala, C.E.Stickings, M.E.Taylor, R.Thomas, *Biochem.J.*, *67*, 390, (1957).

102b C.E.Stickings, *Biochem.J.*, *72*, 332, (1959).

103. T.Kinoshita, M.Ohkawa, Y.Tanaka, *Ann.Phytopathol. Soc.Jap.*, *38*, 397, (1972).

104. Y.Mikami, Y.Nishijima, H.Iimura, A.Suzuki, S.Tamara, *Agric.Biol.Chem.*, *35*, 611, (1971).

105. E.Gaumann, *Phytopathology*, *47*, 342, (1957).

106. H.M.Ward, *Ann.Bot.*, *19*, 1, (1905).

107. D.R.Perrin, W.Bottomley, *J.Amer.Chem.Soc.*, *84*, 1919, (1962).

108. D.R.Perrin, *Tetrahedron Lett.*, *29*, (1964).

109. K.Kubota, *Tetrahedron*, *4*, 68, (1958).

110. N.Suzuki, *Bull.Natl.Inst.Agr.Sci.(Japan),Ser.C*, *8*, 69, (1957).

111. K.C.Chexal, J.P.Springer, J.Clardy, R.J.Cole, J.W.Kirksey, J.W.Durner, H.G.Cutler, B.J.Strawter, *J.Am.Chem.Soc.*, *98*, 6748, (1976).

112. J.L.Aubagnac, F.M.Devienne, R.Combarieu, *Tetrahedron Lett.*, *23*, 5263, (1982).

113. G.B.Lucas, R.W.Pero, J.P.Snow, D.Harvan, *J.Agric. Food Chem.*, *19*, 1274, (1971).

114. G.B.Lucas, *Tob.Sci.*, *15*, 37, (1971).

115 P.W.Brian, P.J.Curtis, H.G.Hemming, J.M.Wright, *Nature*, *164*, 534, (1949).

116. J.R.Bartels-Keith, *J.Chem.Soc.*, 1662, (1960).

117. A.Stoessl, *J.C.S. Chem.Commun.*, 307, (1967).

118. A.Stoessl, *Can.J.Chem.*, *47*, 767, (1969).

119. R.Suemitsu, J.Iwai, K.Kawaguchi, N.Haitani, *Bull. Agric.Chem.Soc.Jap., 23,* 547, (1959).

120. R.H.Thompson, "Naturally occurring quinones", p.196, Butterworth, London, (1957).

121. R.Suemitsu, J.Iwai, K.Kawaguchi, *Agric.Biol.Chem., 39,* 2249, (1975).

122. J.N.Ashley, H.Raistrick, T.Richards, *Biochem.J., 33,* 1291, (1939).

123. R.Suemitsu, J.Iwai, K.Kawaguchi, N.Haitani, N.Kitagawa, *Agric.Biol.Chem., 41,* 2289, (1977).

124. A.Stoessl, C.H.Unwin, J.B.Stothers, *Tetrahedron Lett., 27,* 2481, (1979).

125. M.M.Wheeler, D.M.Wheeler, G.W.Peterson, *Phytochemistry, 14,* 288, (1975).

126. A.M.Becker, R.W.Rickards, K.J.Schmalzl, H.C.Yick, *J.Antibiot., 31,* 324, (1978).

127. W.V.Lavatte, R.Bentley, *Arch.Biochem.Biophys., 108,* 287, (1964).

128. A.N.Starrat, *Can.J.Chem., 46,* 767, (1968).

129. J.H.Birkinshaw, P.Chaplen, R.Lahoz-Oliver, *Biochem.J., 67,* 155, (1957).

130. A.N.Starrat, G.A.White, *Phytochemistry, 7,* 1883, (1968).

131. O.C.Musgrave, *J.Chem.Soc.,* 4301, (1956).

132. H.D.Munro, O.C.Musgrave, R.Templeton, *J.Chem.Soc., (C),* 947, (1967).

133. N.Sugiyama, C.Kashima, M.Yamamoto, T.Sugaya, R.Mohri, *Bull.Chem.Soc.Jap., 39,* 1573, (1966).

134. S-B. Hyeon, A.Ozaki, A.Suzuki, S.Tamura, *Agric. Biol.Chem., 40,* 1663, (1976).

135. K.Kameda, H.Aoki, H.Tanaka, M.Namiki, *ibid., 37,* 2137, (1973).

136. K.Kameda, M.Namiki, *Chem.Lett., 12,* 1491, (1974).

137. H.Raistrick, C.E.Stickings, R.Thomas, *Biochem.J., 55,* 421, (1953).

138. R.Thomas, *ibid., 80,* 234, (1961).

139. R.W.Pero, D.Harvan, M.C.Blois, *Tetrahedron Lett., 12,* 945, (1973).

140. R.G.Coombe, J.J.Jacobs, T.R.Watson, *Aust.J.Chem., 23,* 2343, (1970).

141. D.Rogers, D.Williams, R.Thomas, *J.Chem.Soc.Chem. Commun.,* 393, (1971).

142. J.D.Williams, R.Thomas, *Tetrahedron Lett.,* 639, (1973).

143. R.Pero, R.Owens, S.Dale, D.Harvan, *Biochim.Biophys. Acta, 230,* 170, (1971).

144. A.T.McPhail, R.W.Miller, D.Harvan, R.W.Pero, *J.Chem.Soc., Chem.Commun., 18,* 632, (1973).

145. R.Thomas, *Proc.Chem.Soc., 88,*(1959).

146. S.Gatenbeck, S.Hermodsson, *Acta Chem.Scand., 19,* 65, (1965).

147. S.Sjoland, S.Gatenbeck, *ibid., 20,* 1053, (1966).

148. D.Harvan, R.W.Pero, "Mycotoxins and other fungal related food problems", *Adv.Chem.Ser., 149,* 351, (1976), ed., J.V.Rodricks.

149. R.W. Pero, H.Posner, M.Blois, D.Harvan, J.W. Spalding, *Environ.Health Perspect., 4,* 87, (1973).

150. D.Harvan, R.W.Pero, *Adv.Chem.Ser., 149,* 344, (1976).

151. I.Hiroe, S.Aoe, *J.Fac.Agric.,Tottori Univ., 2,* 1, (1954).

152. A.Ciegler, L.A.Lindenfelser, *Sep.Exp., 25,* 719, (1969).

153. C.S.Kumar, A.S.Rao, *Can.J.Bot., 57,* 1255, (1979).

154. C.O.Gatterman, *J.Med.Chem., 8,* 483, (1965).

155. F.A.Miller, W.A.Wrightsel, B.J.Sloan, J.Ehrlich, J.C.French, *Nature, 200,* 1338, (1963).

156. Ch.Stoll, J.Renz, E.Gaumann, *Phytopathol.Z., 29,* 388, (1957).

157. E.Hardegger, P.Liechti, L.M.Jackmann, A.Boller, Pl.A.Plattner, *Helv.Chim.Acta, 46,* 60, (1963).

158. K.Tamari, N.Ogasawara, J.Kaji, "The rice blast disease", pp. 35 - 68, Johns Hopkins Press, Baltimore, (1963).

159. Pl.A.Plattner, U.Nager, A.Boller, *Helv.Chim.Acta, 31,* 594, (1948).

160. C.Lippe, *Nature, 218,* 196, (1968).

161. O.Nowosielski, B-D.Knezek, B.G.Ellis, *Amer.Chem. Soc., Div.Water, Air Waste Chem., 11,* 32, (1971).

162. H.Haque, A.D.Russell, *Antimicrob.Agents Chemother., 5,* 447, (1974).

163. G.Schwarzenback, "Complexometric titrations", p. 6 Methuen, London, (1957).

164. Personal communication, Dr. J.S.E. Holker.

165. K.Saito, *Botan.Mag., Tokyo, 21,* 7, (1907).

166. T.Yabuta, *J.Chem.Soc.,* 575, (1924).

167. H.E.Morton, W.Kocholaty, R.Junowics-Kocholaty, A.Kelner, *J.Bacteriol., 50,* 579, (1945).

168. E.Gaumann, A.von Arx, *Ber.Schweiz.Botan.Ges., 57,* 174, (1947).

169. H.R.V.Arnstein, R.Bentley, *Biochem.J., 54,* 493, (1953).

170. Idem, *ibid., 54,* 508, (1953).

171. Idem, *ibid., 54,* 517, (1953).

172. Idem, *ibid., 62,* 403, (1956).

173. G.H.Stout, L.H.Jensen, "X-ray structure determination : A practical guide", N.Y.MacMillan Company (1968).

174. P.Maine, S.J.Fiske, S.E.Hull, L.Lessinger, G.Germain, J.P.Declercq, M.M.Woolfson, (1980). Multan 80. A system of computer programs for the automatic solution of crystal structures from X-ray diffraction data. University of York, England and Louvain, Belgium.

175. J.M.Stewart, G.J.Kruger, H.L.Ammon, C.Dickinson, S.R.Hall, (1972). The X-ray system - version of June 1972. Tech.Ref., TR-192, Computer Science Centre, University of Maryland, College Park, Maryland.

176. A.Holm, C.Cristophersen, T.Ottersen, H.Hope. A.Christensen, *Acta Chem.Scand., Ser.B., 31,* 687, (1977).

177. L.P.Battaglia, A.P.Corradi, M.Nardelli, C.Pelizzi, M.E.V.Tani, *J.Chem.Soc., Dalton Trans.,* 1076, (1976).

178. T.Liljefors, N.L.Allinger, *J.Amer.Chem.Soc., 98,* 2745, (1976).

179. M.M.Blight, J.F.Grove, *J.Chem.Soc., Perkin Trans. I,* 1691, (1974).

180. H.Hikino, S.Nabetani, T.Takemoto, *J.Pharm.Soc., Japan, 93,* 619, (1973).

181. P.J.Suter, W.B.Turner, *J.Chem.Soc., (C),* 2240, (1967).

182. Y.Kimura, T.Inoue, S.Tamura, *Agric.Biol.Chem., 37,* 2213, (1973).

183a E.Honkanen, R.Moisio, P.Karvonen, *Acta Chem.Scand., 19,* 370, (1965).

183b W.H.McFadden, E.A.Day, M.J.Diamond, *Analyt.Chem., 37,* 89, (1965).

184. N.J.McCorkindale, J.L.C.Wright, P.W.Brian, S.M.Clarke, S.A.Hutchinson, *Tetrahedron Lett.,* 727, (1968).

185. A.J.Birch, A.A.Qureshi, R.W.Richards, *Aust.J.Chem., 21,* 2775, (1968).

186. A.Yoshikoshi, *Japan Kokai,* 73, 40, 796 and 797.

187. D.C.Aldridge, A.Borrow, E.E.L.Gerring, *Ger.Offen., 2,* 415, 102.

188. K.Sakata, H.Masago, A.Sakurai, *Tetrahedron Lett., 23,* 2095, (1982).

189. N.J. McCorkindale, personal communication, Fungal Metabolites (W.B.Turner), p.289. Academic Press, New York, (1971).

190. D.C.Aldridge, R.Bowling, J.C.Swait, *Ger.Offen.,* 2, 801, 399.

191. D.Brookes, B.K.Tidd, W.B.Turner, *J.Chem.Soc.*, 5385, (1963).

192. D.C.Aldridge, W.B.Turner, *J.Chem.Soc., (C)*, 2431, (1971).

193. M.Kato, R.Tanaka, A.Yoshikoshi, *J.Chem.Soc., Chem. Commun.*, 1561, (1971).

194. M.Kato, M.Kageyama, R.Tanaka, K.Kuwahara, A.Yoshikoshi, *J.Org.Chem., 40*, 1932, (1975).

195. R.M.Carlsen, A.R.Oyler, *ibid., 41*, 4065, (1976).

196. R.C.Anderson, B.Fraser-Reid, *Tetrahedron Lett., 19*, 3233, (1978).

197. T.Sakai, M.Yoshida, S.Kohmoto, M.Utaka, A.Takeda, *Tetrahedron Lett., 23*, 5185, (1982).

198. H.Ohrui, N.Sueda, H.Kuzuhara, *Nippon Kagaku Kaishi, 5*, 769, (1981).

199. J.W.Emsley, J.Feeney, L.H.Sutcliffe, "High resolution nuclear magnetic resonance spectroscopy", Vol. 1, p.166, Vol. 2, p. 678, Pergamon, Oxford, (1965).

200. N.J.McCorkindale, W.P.Blackstock, G.A.Johnstone, T.P.Roy, J.A.Troke, *Iupac Int.Symp.Chem.Nat.Prod.*, 151, (1978).

201. S.Tamura, *Adv.Pestic.Sci., Plenary Lect.Symp.Pap. Int.Congr.Pestic.Chem., 4*, 356, (1978) (Pub. 1979).

202. T.Hamasaki, H.Kuwano, K.Isono, Y.Hatsuda, K.Fukuyama, T.Tsukihara, Y.Katsube, *Agric.Biol.Chem., 39*, 749, (1975).

203. K.Fukuyama, H.Kawai, T.Tsukihara, K.Tsukihara, Y.Katsube, T.Hamasaki, Y.Hatsuda, H.Kuwano, *Bull. Chem.Soc.Japn., 48*, 2949, (1975).

204. R.O.Gould, T.J.Simpson, M.D.Walkinshaw, *Tetrahedron Lett.*, *22*, 1047, (1981).

205. C.H.Calzadilla, C.Ferguson, S.A.Hutchinson, N.J.McCorkindale, unpublished observations cited in W.B.Turner, "Fungal Metabolites", p. 230, Academic Press, London, (1971).

206. N.J.McCorkindale, C.H.Calzadilla, S.A.Hutchinson, D.H.Kitson, G.Ferguson, I.M.Campbell, *Tetrahedron*, *37*, 649, (1981).

207. D.M.X. Donnelly, J.O'Reilly, A.Chiaroni, J.Polonsky, *J.Chem.Soc., Perkin Trans. I*, *10*, 2196, (1980).

208. M. DeBernardi, G.Mellerio, G.Vidari, P.Vita-Finzi, G.Fronza, *ibid.*, 221, (1980).

209. N.J.McCorkindale, C.H.Calzadilla, R.L.Baxter, *Tetrahedron*, *37*, 1991, (1981).

210. L.M.Seitz, D.B. Sauer, R.Burroughs, H.E.Mohr, J.D.Hubbard, *Phytopathology*, *69*, 1202, (1979).

211. W.V. Lavatte, R.Bentley, *Arch.Biochem.Biophys.*, *108*, 287, (1964).

212. L.M.Seitz, H.E.Mohr, R.Burroughs, D.B.Sauer, *Cereal Chem.*, *54*, 1207, (1977).

213. L.C.Brown, J.J.Jacobs, *Aust.J.Chem.*, *28*, 2317, (1975).

214. L.M.Seitz, J.V.Paukstelis, *J.Agric.Food Chem.*, *25*, 838, (1977).

215. P.Wieland, V.Prelog, *Helv.Chim.Acta*, *30*, 1028, (1947).

216. K.E.Schulte, G.Rucker, H.Fachmann, *Tetrahedron Lett.*, 4763, (1968).

217. M.W.Miller, "The Pfizer Handbook of Microbial Metabolites", p.408, McGraw-Hill, New York, (1961).

218. Thanks to Dr. D.J.Chadwick for his practical skills and computing.

219. The S.E.R.C. Microdensitometer Service, Daresbury Laboratory.

220. The Cambridge Crystallographic Database.

221. J.A.Thoma, *Anal.Chem., 35,* 214, (1963).

222. A.Jeanes, C.S.Wise, R.J.Dimler, *ibid., 23,* 415, (1951).

223. M.Brenner, A.Niederweiser, *Experientia, 15,* 237, (1961).

224. N.Zollner, G.Wolfram, *Klin.Wochschr., 40,* 1098, (1962).

225. R.D.Bennett, E.Heftmann, *J.Chromatog., 12,* 245, (1963).

226. J.C.Overeem, A.van Dijkman, *Rec.Trav.Chim.Pays-Bas, 87,* 940, (1965).

227. K.Kameda, H.Aoki, M.Namiki, J.C.Overeem, *Tetrahedron Lett.,* 103, (1974).

228. T.O.Schmidt, W.Meyer, *Liebigs Ann., 571,* 1, (1951).

229. R.Thomas, *Biochem.J., 78,* 748, (1961).

230. J.B.D.MacKenzie, A.Robertson, W.B.Whalley, *J.Chem. Soc.,* 2965, (1950).

231. A.J.Birch, P.Fitton, E.Pride, A.J.Ryan, H.Smith, W.B.Whalley, *ibid.,* 4576, (1958).

232. Thanks to Dr. I.D.Entwistle and P.D.Regan, Shell Research, Sittingbourne,

233. D.C.Rohrer, D.W.Fullerton, E.Kitatsuji, T.Nambara, E.Yoshii, *Acta.Crystallogr., Sect.B, 38,* 1865, (1982);

L.R.Nasimbeni, M.L.Niven, G.R.Pettit, Y.Kamano, M.Inoue, J.J.Einck, *ibid.*, *38*, 2163, (1982). T.Debaedemaeker, U.Thewalt, W.Kreiser, H.A.F. Heineman, *Chem.Ber.*, *112*, 423, (1979).

234. J.A.Elvidge, R.P.Linstead, B.A.Orkin, P.Sims, H.Baer, D.B.Pattison, *J.Chem.Soc.*, 2228, (1950).

235. L.G.Sillen, A.E.Martell, "Stability constants of metal - ion complexes", special publication, 17, (1964), Chemical Society, London.

236. The Merck Index, 8th Edition, p.416, ed., P.G. Stecher, Publ. Merck and Company Incorporated (1968).

237. The Merck Index, 8th Edition, p.644, ed. P.G. Stecher, Pub., Merck and Company Incorporated (1968).

238. P.S.O'Colla, E.E.Lee, D.McGrath, *Sci.Proc.Roy.Dublin Soc.Ser.A.*, *1*, 337, (1963).

239. K.Mosbach, *Acta Chem.Scand.*, *14*, 457, (1960).

240. R.D.Haworth, J.Grimshaw, *Chem. and Ind.*, 199, (1950). T.O.Schmidt, *Angew.Chem.*, *68*, 103, (1956). T.O.Schmidt, R.Eckert, *Z.Naturf*, *116*, 757, (1956).

241. W.H.Pirkle, *J.Amer.Chem.Soc.*, *88*, 1837, (1966).

242. M.Raban, K.Mislow, *Tetrahedron Lett.*, 4249, (1965).

243. J.A.Dale, H.S.Mosher, *J.Amer.Chem.Soc.*, *90*, 3732, (1968) and *95*, 512, (1973).

244. G.M.Whitesides, D.Lewis, *ibid.*, *92*, 6979, (1970).

245. R.J.Abraham, P.Loftus, Proton and Carbon-^{13}NMR Spectroscopy, p.167, Heyden, London, (1978).

246. T.Okuno, I.Natsume, K.Sawai, K.Sawamura, A.Furusaki, T. Matsumoto, *Tetrahedron Lett.*, *24*, 5653, (1983).