FUNGAL METABOLITES IN FORESTRY AND AGRICULTURE: ISOLATION, CHARACTERIZATION AND APPLICATIONS.

A Thesis

by

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Submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ISBN 0-315-72109-X

To George, Nikki and Zacharie

for they are the future...

May the love for science that their grandparents,

George and Zafiria,

inspired in their father and me, grow stronger in them!

To my parents George and Zafiria,
for they have always been the greatest driving force for my achievements
and the primary contributors to my education.

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ABSTRACT

The fungus *Pisolithus tinctorius* was found to produce the antifungal metabolites *p*-hydroxybenzoylformic acid and (R)-(-)-*p*-hydroxymandelic acid. The absolute stereochemistry of the latter compound was established *via* stereospecific synthesis of both (R) and (S) enantiomers and NMR techniques. The structure-activity relationship was examined through the use of related compounds. Both of the natural products and a few of their analogues were found to inhibit spore germination and cause cell-specific disintegration (hyphal lysis) to a number of phytopathogenic and dermatogenic fungi. It was concluded that *P. tinctorius*, which exists symbiotically on the roots of most forest trees, provides protection to its host trees against disease causing fungi.

The fungus *Suillus cavipes* was shown to promote dichotomous branching and root development in hypocotyl cuttings of coniferous seedlings by inducing the biosynthesis of the phytohormone ethylene.

Finally, the metabolites of the fungus *Phomopsis convolvulus*, a host-specific pathogen of field bindweed (*Convolvulus arvensis*) were investigated. The two known steroids, ergosta-5,7,22-trien-3-ol (ergosterol) and ergosta-6,22-dien-5α,8α-epidioxy-3-ol (ergosterol peroxide) were shown to be metabolites of *P. convolvulus*. In addition, four novel metabolites were isolated and structurally identified. Two of these metabolites, 4-carboxy-3-hydroxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone and 4-carboxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone, were shown to exhibit strong phytotoxicity against field bindweed. Weak phytotoxicity was also observed with the other two metabolites, 4-(hydroxymethyl)-7-methoxy-6-methyl-1(3H)-isobenzofuranone and 3-(4-methoxy-3-methyl-α-pyron-6-yl)-2-methyl-2-butenoic acid, as well as with ergosterol peroxide. Attempts toward the synthesis of the disulfide analogue of ergosterol peroxide were initiated.

PRODUITS NATURELS DE CHAMPIGNONS EN FORESTERIE ET EN AGRICULTURE: PURIFICATION, CARACTÉRISATION ET APPLICATIONS

par Youla S. Tsantrizos

RÉSUMÉ

Il a été découvert que les métabolites fongicides, l'acide *p*-hydroxybenzoylformique et l'acide (R)-(-)-*p*-hydroxymandélique, sont produits par le champignon *Pisolithus tinctorius*. La configuration absolue de l'acide *p*-hydroxymandélique a été établie par la synthèse stéréospécifique des énantiomères et par la résonance magnétique nucléaire du proton (RMN) de dérivés. La relation entre la structure et l'activité biologique des métabolites et de composés similaires a été examinée. Les produits naturels et quelques analogues préviennent la germination des spores et causent la désintégration cellulaire spécifique de certains champignons phytopathogènes et dermatogènes. Il a été conclu que le *P. tinctorius*, qui existe en symbiose sur les racines des arbres forestiers, protège son hôte contre les effects d'organismes pathogènes.

Il a été démontré que le champignon *Suillus cavipes* stimule la division dichotomisée et la rhizogénèse des boutures hypocotyles des jeunes conifères. Le *S. cavipes* induit la biosynthèse d'une phytohormone, l'éthylène.

Les métabolites du champignon *Phomopsis convolvulus*, qui s'attaque spécifiquement à la mauvaise herbe *Convolvulus arvensis*, ont été étudiés. Deux stéroides connus, l'ergostatriène-5,7,22-ol-3 (ergosterol) et l'ergostadiène-6,22-épidioxy-5α,8α-ol-3 (peroxide d'ergosterol), ont été isolés des cultures de *P. convolvulus*. De plus, quatre nouveaux métabolites ont été isolés et leur structure déterminée. De ceux-ci, le carboxy-4-hydroxy-3-méthyl-6-isobenzofuranone-1(3H) et le carboxy-4-méthoxy-7-méthyl-6-isobenzofuranone-1(3H) sont de puissants agents phytotoxiques pour le *C. arvensis*. Les deux autres métabolites, le (hydroxyméthyl)-4-méthoxy-7-méthyl-6-isobenzofuranone-1(3H) et l'acide (méthoxy-4-méthyl-3-pyronne-α-yl-6)-5-méthyl-2-butène-2-oïque, ainsi que le peroxide d'ergostérol ont démontré une faible toxicité contre le *C. arvensis*. La synthèse d'un analogue disulfide du peroxide d'ergostérol a été ébauchée.

ACKNOWLEDGEMENTS

I feel fortunate and privileged to have pursued my doctoral studies in the Chemistry Department of McGill University. The support, advice and friendship of so many members of the department have made this experience intellectually and personally very enjoyable.

I would like to express my deepest appreciation and gratitude to Professor Kelvin Ogilvie who, in spite of the geographic distance between us, never failed to provide his support and guidance. His leadership qualities, attitude towards his students and his dedication to science will be an inspiration to me for the years to come.

I am particularly indebted to Professor David Harpp, not only for being the first of my educators to kindle my interests towards organic chemistry, but also for his advice and moral support which were indispensable to the successful completion of my studies.

A special thanks to Dr. Françoise Sauriol for her expert advice on NMR and Dr. Orval Mamer for helpful discussions on mass spectrometry.

From my fellow researchers, I would like to especially thank Patricia Folkins for her friendship, support and invaluable assistance with the preparation of this manuscript.

I would also like to thank Normand Hébert and Yves St-Denis for translating the abstract into French and Chantal Marotte for typing parts of the manuscript.

I would like to thank my collaborators, Professor Alan Watson (Plant Pathology Department, McDonald College, McGill University), Professor André Fortin and Harry Koppe (Forestry Department, Laval University) for their contributions to the biological aspects of this thesis. I also would like to thank again Professor David Harpp and Patricia Folkins for their advice on sulfur chemistry.

I would like to thank Professors George Just and Jik Chin for their advice on various topics, and the use of equipment in their laboratories.

I would like to thank, in a very special way, my friends John Gutpell, Susan Solymoss and Sandy Glashan not only for their assistance with the preparation of this manuscript but for their love and encouragement when it was most needed.

I would like to thank my uncle and aunt, John and Stamatia Manoliadis, for their continuous love, support and help in so many ways.

I would like to express my gratitude to Dr. Paul Nguyen-ba, Dr. Masad J. Damha, Dr. Sharon Bennett, Jana Pika, Charles Williams, Warren Chew, Stephen Kawai and Vrej Jubian, for their friendship and helpful discussions.

Finally, I would like to thank Vanier College for its support in providing me with a professional leave of absence in order to pursue my doctoral studies, and the Quebec Government (FCAR and Ministère de l'Enseignement supérieur et de la Science, Direction générale de l'enseignement collégial) for financial support.

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ABBREVIATIONS

Ac	acetyl
ACC	1-aminocyclopropane-1-carboxylic acid
APT	attached proton test
AVG	aminoethoxyvinylglycine
BINAL-H	2,2'-dihydroxy-1,1'-binaphthyl lithium aluminum hydride
br	broad
bu	butyl
° C	degree Celsius
cm	centimetre
COLOC	heteronuclear shift correlation spectroscopy via long range coupling
COSY	correlation spectroscopy
d	doublet
D	deuterium
dd	doublet of doublets
DEPT	distortionless enhancement by polarization transfer
dt	doublet of triplets
DMSO	dimethylsulfoxide
ee	enantiomeric excess
EI	electron impact
Et	ethyl
EtOAc	ethyl acetate
EtOH	ethanol
FAB	fast atom bombardment
g	gram
GIC	growth inhibition factor
h	hour
HETCOR	heteronuclear correlation spectroscopy
HPLC	high pressure liquid chromatography
i	iso
IR	infrared

L	litre
m	multiplet
m	meta
M	molar
μ	micron
Me	methyl
MeOH	methanol
μL	microlitre
μM	micromolar
mg	milligram
min	minute
MMN	Melin Norkans medium
mmol	millimole
mol	mole
m.p.	melting point
ms	mass spectrum
MTBA	4-methylthio-2-oxobutanoic acid
N	normality
n	normal
nm	nanometer
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
o	ortho
p	para
PDA	potato dextrose agar
PDB	potato dextrose broth
Pet. ether	petroleum ether
Ph	phenyl
ppm	parts per million
Pr	propyl
Rf	relative mobility
rpm	revolutions per minute
r.t.	room temperature
s	singlet

sec	second
sh	sharp
shd	shoulder
t	triplet
t	tertiary
TDMA	tridodecylmethylammonium
TDMAC	tridodecylmethylammonium chloride
THF	tetrahydrofuran
TLC	thin layer chromatography
UV	ultraviolet
v	volume

"There are no such things as pure and applied science,
there are only science and the applications of science."

Louis Pasteur (1822-1895)

CHAPTER 1

INTRODUCTION

1. 1 The Morphology and History of Fungi

Fungi are a diverse group of microorganisms whose beneficial and detrimental effects have a major impact on the world economy. They are used in the production of food, beverages, pharmaceutical and agricultural products. At the same time, pathogenic fungi are being increasingly recognized as important in human diseases and cause billions of dollars of agricultural crop losses annually.

Fungi grow as either unicellular **yeasts** or multicellular filaments known as **molds.** Some of them are dimorphic, capable of growing as a yeast or as a mold depending on the environmental conditions. The basic structural units of a mold are tubular filaments called **hyphae.** A mass of intertwined hyphae constitutes a vegetative structure known as the **mycelium.** In addition, fungi which reproduce sexually can form fruiting bodies such as mushrooms or puffballs. The vegetative body has a strong cell wall made of variously-linked biopolymers of glucan (glucose), chitosan (glucosamines) and chitin (N-acetylglucosamine), but in some cases it can consist entirely of chitin.

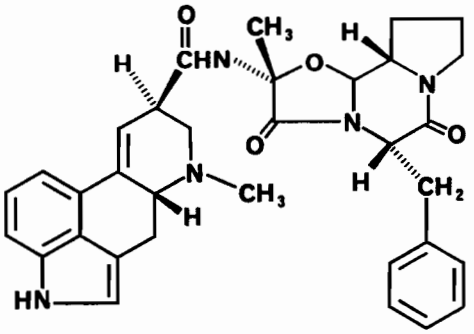
Fungi can reproduce asexually or sexually. The asexual fungi generate various kinds of unicellular spores by division of their cell nucleus. The spores can be **sporangiospores** (enclosed in a sac-like structure) or **conidia** (not enclosed) and they develop on specialized structures called **sporophores.** The sexually reproducing fungi form spores each carrying only one set of the homologous chromosomes. The diploid condition is reestablished when two spores fuse and germinate.

Yeasts and especially molds are well adapted to compete in nature with other microorganisms. They colonize large areas of plant tissues and soils, and extend their hyphae through many barriers, including the complex tissue of wood and poor soils, in search of nutrient resources. As such, some fungi are the most important class of plant pathogens causing significant losses to agriculture and forestry. At the same time, others affect plants in a positive way. In the latter group of fungi are the symbiotic **mycorrhizae** which colonize the roots of trees, increasing the host's ability to obtain nutrients that are far beyond the reach of its roots.

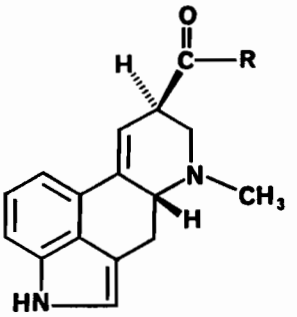
Chemically, fungi produce metabolites which also have a wide spectrum of biological activities. These natural products can be of benefit or detriment to both plants and animals and have

played an important role in history. For example, ergotism, an illness associated with convulsions, hallucinations and gangrene, was common in France between 950 and 1600 A.D.[1] This lethal form of food poisoning was caused by the consumption of grains infected with the mold *Claviceps purpurea*. However, in 1582 the Frankfurt physician Adam Lonitzer reported the use of this fungus as a medication for quickening childbirth.[2] The use of this medicine was discontinued in the late 1800s when physicians became aware of its dangers.

The first active, alkaloidal preparation from *Claviceps purpurea* was isolated in 1907, and in 1945 Stoll[3] isolated ergotamine (**1a**), the first pure active metabolite. Lysergic acid (**1b**), the nucleus common to all ergot alkaloids was subsequently characterized, and both the natural products and their synthetic analogues, became a major development for chemistry and medicine. The uterotonic (oxytotic) principle ergonovine (or ergobasine, **1c**) was soon discovered and its maleate salt is still used in obstetrics to induce labor. The strong vasoconstricting effect of ergotamine[4] (**1a**) has found therapeutic use in the treatment of severe migraine headaches.



(1a) Ergotamine



(1b) R= -OH

(1c) R= -NHCH(CH$_3$)CH$_2$OH

(1d) R= -N(CH$_2$CH$_3$)$_2$

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1. It is even believed to have been responsible for the creation of the alleged witches of Salem Massachusetts in 1692.

2. A. Hofmann, in *My Problem Child*, translated by J. Ott, McGraw-Hill Book Company, New York, N. Y., (1979).

3. Stoll, *Helv. Chim. Acta* **28**, 1283 (1945).

4. B. Kreilgard, in *Analytical Profiles of Drug Substances*, Ed. K. Florey, Academic Press, New York, vol. **6**, 113 (1977).

Several other compound, including the potent psychomimetic drug lysergic acid diethylamide (LSD, **1d**) are based on the chemical structure of ergot alkaloids and a great deal of scientific research has been devoted to the understanding of their chemistry. The biological effects and possible uses of fungal metabolites, like the ergot alkaloids, has been of interest to researchers in other field of science as well. In the last few years, the establishment of the interdisciplinary field of biotechnology has intensified these investigations and has led to many promising new developments.

Placing, however, the anticipated benefits of the growing field of biotechnology in the appropriate historical, economic and social perspective, it is important to realize that the ergot alkaloids are only a few of the many interesting examples of the impact of fungi to the history of science.

The use of fungi in the production of foods and beverages has its roots deep in antiquity.[5] The ability of yeast to produce beer from grains was discovered by the Sumerians and Babylonians before 6,000 B.C. The Egyptians discovered the process of leavening bread 2,000 years later. Thousands of years before the existence of microorganisms was even recognized, moldy cheese, bread, and soybean curds were used by practitioners of folk medicine to treat infected wounds.

In the 17th century, the pioneering Dutch microscopist Anton van Leeuwenhoek[6] observed the presence of "animalcules" in water and decaying matter. Almost two centuries later in the 1870s, the French chemist Louis Pasteur, the English physicist John Tyndall, and an English physician William Roberts, directly observed the antagonistic effects of one microorganism on another. Pasteur, with his characteristic foresight, suggested that the phenomenon might have therapeutic potentials. For the next 50 years, various microbial preparations were tried as medicines, but they were either too toxic or not effective in *vivo*. Finally in 1928, the discovery by Sir Alexander Fleming that the fungus *Penicillium notatum* inhibits the growth of the bacteria *Staphylococcus aureus* marked the onset of the era of antibiotics.[7]

The tremendous amount of effort that followed Fleming's discovery has led to the development of many new drugs from microbial metabolites. Fungi of the *Penicillium* and *Cephalosporium* species are now used commercially for the production of broad spectrum β-lactam antibiotics. In the manufacturing of steroids, several stereospecific conversions are carried out using

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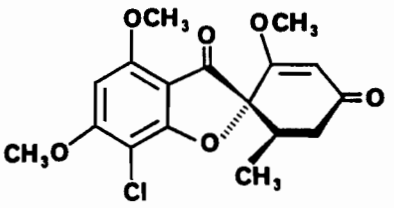
5. T.D. Brock, *Milestones in Microbiology*, Prentice-Hall, Englewood Cliffs, N.J. (1961).

6. C. Dobell, *Antony van Leeuwenhoek and His "Little Animals"*, Dover, New York (1960).

7. R. Reid, *Microbes and Men*, Saturday Review Press, New York (1975).

the fungi *Rhizopus nigricans* and *Culvularia lunata*. The natural product griseofulvin[8] (2), was first isolated in 1939 from *Penicillium griseofulvin* and its structure was characterized by Grove[9] in 1951. It is now produced commercially from the fermentation broth of the same microorganism and it serves as one of the most important antifungal agents for the treatment of human dermatophyte infections.



Griseofulvin (2)

Perhaps the most significant contribution of fungi to the field of medicine in recent years is the immunosuppressive drug cyclosporin A.[10] This metabolite of *Tolypocladium influtum Gams* is a cyclic oligopeptide of eleven amino acids which has greatly enhanced the success rate of organ transplants.

The industrial production of such pharmaceutical products involves large scale, strictly-controlled fermentation conditions. In the case of penicillin, for instance, the strain *Penicillium chrysogenum* is grown in several 100,000 litre fermentation tanks, all linked on a staggered production schedule in order to provide continuous output of the antibiotic. In addition, the strain of fungus used (*P. chrysogenum*) produces 10,000 times more penicillin per unit volume of broth than did Fleming's original culture of *P. notatum*.

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8. W. Huber, in *Antibiotics*, Eds. J.W. Corcoran and F.E. Hahn, Springer-Verlag, New York, vol. 3, 606 (1975).

9. J.F. Grove, J. MacMillan, T.P.C. Mulholland and M.A. Thorold Rogers, *J. Chem. Soc.*, 3977 (1952).

10. a) A. Ruegger , *Helv. Chim. Acta* **59**, 1075 (1976).
    b) M. Dreyfuss , *J. Appl. Microbiol.* **3**, 125 (1976).
    c) M.Y. Gordon and J.W. Singer, *Nature* **279**, 433 (1979).
    d) P.J. Tutschka , *Blood* **61**, 318 (1983).

1. 2 Potential Applications of Fungi in Agriculture

In nature, unlike the precisely controlled environment of the industrial fermentation tank, a handful of soil is the locus of an unruly turmoil of competing microorganisms. Crop losses due to fungal diseases and post-harvest food spoilage amount to billions of dollars annually. The severe outbreak of potato blight in Ireland in 1845-1846 was caused by the fungus *Phytophthora infestans* and led to the death of one million people and the emigration of a million and a quarter more. More recently, the infection of cereals with the fungus *Helminthosporium sativum* led to an estimated loss of $50 million in Western Canada[11] alone over a period of 25 years.

However, as in the case of the ergot fungus, the investigation of a damaging microorganism can sometimes lead to the development of new, useful products. For instance, upon investigation of *Helminthosporium maydis* (also called *Drechslera maydis*), the fungus which devastated corn crops in the United States and Canada in the summer of 1970,[12] it was found that *H. maydis* is genetically similar to another member of the species, *H. sorghicola* (also called *Drechslera sorghicola*), which is a host-specific pathogen of Johnson grass (*Sorghum halapense* L.). Johnson grass is a serious and hard to control weed in all tropical and semitropical areas of the world. The active metabolites of the fungus are now well known and have the potential of being developed into new herbicides. Such compounds can also serve as models for the synthesis of more commercially suitable analogues of the natural products, and provide practical means for the selection of seedlings and tissues of crop plants which are genetically disease resistant.

In considering then, the potential application of fungi and their metabolites to agriculture, the subtle question of the interaction of microorganisms with one another and the biosphere as a whole must be explored. Knowledge of the chemistry of the molecules involved in these interactions would certainly play an important role in the understanding of pathogenicity. The organisms themselves must be examined, in terms of their biological effects in nature, their host specificity and their growth needs in order to develop optimum fermentation conditions for the production of their interesting secondary metabolites. Finally, reliable bioassays must be developed in order to facilitate the isolation of biologically active compounds and the investigation of their effects on plants and animals.

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11. B.J. Sallans, *Can. Plant Disease Survey* **38**, 11 (1958).

12. L.A. Tatum, *Science* **171**, 1113 (1971).

1. 2. 1 Mycoherbicides

In some cases, a microorganism's beneficial or detrimental effects cannot be attributed to a specific metabolite. However, the fungus itself may still be of value and possibly marketed in one of its living forms. Mycoherbicides represent such an example; they are simple plant pathogenic fungi which can be used in an inundative strategy to control weeds. In the last few years, two such fungi have been developed and registered as agricultural products.[13] In 1981, the pathogen *Phytophthora palmivora* (Butler) Butler[14] was registered as a mycoherbicide for the control of strangler (milkweed) vine (*Morrenia odorata* Lindl.) in Florida's citrus groves. This product was marketed by Abbott Laboratories under the name "DeVine^{Tm}". A year later, Upjohn company marketed "Collego^{Tm}", a dry powder of *Colletotrichum gloeosporioides* (Penz.)[15] spores. Collego is now used for the selective control of northern jointvetch (*Aeschynomene virginica*), an important weed in rice and soybean fields.

1. 2. 2 Fungal Metabolites

Ideally, a commercial product must be inexpensive to produce and store, and have a predictable level of biological activity. These criteria can more easily be met by pure natural compounds or their synthetic analogues, than by the living microorganisms themselves. Hence, in addition to purely scientific interest, there is practical justification for the research efforts undertaken in order to successfully apply the potential benefit of fungal metabolites to agriculture.

Fungal metabolites are as diverse chemically as they are in biological activity. However, the examples which are most relevant to agriculture and forestry are those of metabolites which can promote the growth of crop plants or those which are toxic to common pests such as weeds, insects and microbial pathogens. Some interesting examples of such compounds are given below in order to illustrate their chemical diversity and their potential benefits.

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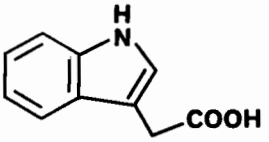
13. D.O. TeBeest and G.E. Templeton, *Plant Disease* **69**, 6 (1985).

14. S. Woodhed, *Phytopathology* **71**, 913 (1981).

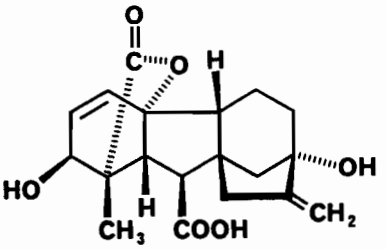
15. D.O. TeBeest, *Plant. Dis.* **66**, 469 (1982).

1. 2. 2a Phytohormones

Fungi which promote growth and induce morphological changes to plants have been extensively studied. These effects are attributed largely to the ectomycorrhizal fungi which live symbiotically on the roots of trees. As previously mentioned, their large mycelium network serves as a transport system for nutrients between the soil and the roots of their hosts. In addition, these fungi produce a number of plant hormones which stimulate growth and control the development of different plant tissues. Several reports have appeared in the literature on the production of auxins[16] (indoleacetic acid, 3), gibberellins (4), ethylene (5) and cytokinin-like metabolites from ectomycorrhizal fungi.[17] The gibberellins are the only ones of these plant hormones which are produced commercially through the fermentation of fungi.



Indoleacetic acid (3)



Gibberellic acid (4)

The gibberellins[18] comprise a family of over 60 phytohormones having a diterpenoid acid structure. These types of compounds were originally isolated in 1938 from *Fusarium moniliforme*

16. a) C.M. Baser, H.E. Garrett, R.J. Mitchell, G.S. Cox and C.J. Starbuck, *Can. J. For. Res.* 17, 36 (1987).
    b) W.T. Frankenberger and M. Poth, *Appl. Environ. Microbiol.* 53, 2908 (1987).

17. a) I. Ho, *Can. J. For. Res.* 17, 31 (1987).
    b) K. M. Hanley and D.W. Greene, *HortScience* 22, 591 (1987).

18 J. MacMillan, *Recent Aspects of the Chemistry and Biosynthesis of the Gibberellins* in "The Chemistry and Biochemistry of Plant Hormones", Eds. V.C. Runeckles, E. Sondheimer and D.C. Walton, Academic Press, New York, vol.7 (1974).

(*Gibberella fujikuroi*), the causative fungus of Bakanae disease in rice.[19] Gibberellic acid (GA3, 4), the most important member of the family, and a mixture of gibberellins GA4/GA7 are commercially available from the fermentation of *F. moniliforme* and are used extensively in agriculture.[20] Their applications include the induction of male flowers in cucumbers for seed production, stimulation of grape vines to produce larger berries, promotion of artichoke bud formation and acceleration of the germination of barley in order to reduce malting time and increase the amount of fermentable carbohydrates.

Structurally, ethylene (5) is the simplest member of the fungal metabolites which has phytohormonal activity. Observations on the effects of ethylene can be found in the literature dating back to 1858.[21] In 1910, Cousins[22] reported that gases from oranges caused premature ripening of bananas in mixed commercial shipments. It is now almost certain that the ethylene produced could not have come from the oranges, since oranges produce very little ethylene, but from fungi growing on the fruits. In 1940 Biale[23] and Miller[24] independently discovered that the common mold of citrus fruits, *Penicillium digitatum*, produces ethylene at high rates. Today the involvement of ethylene in such plant phenomena as ripening, senescence, root formation and flowering is well established,[25] and several fungi have been shown to be involved in its production.[26,27] Consequently, ethylene has found numerous applications in the food and agriculture industries where its power has been harnessed *via* the ethylene-releasing synthetic compound 2-chloroethylphosphonic acid[28] (ethephon,

19. S. Yabuta and K. Sumiki, *J. Agr. Chem. Soc. Japan* **14**, 1526 (1938).

20. J.N. Turner, *Outlook Agr.* 7, 14 (1972).

21. G.W. Fahnstock, *Proc. Acad. Nat. Sci. Phila.* **1**, (1858).

22. H.H. Cousins, *J. Am. Dep. Agr. Annu. Rep.* **7**, 15 (1910).

23. J.B. Biale, *Science* **91**, 458 (1940).

24. E.V. Miller, J.R. Winston and D.F. Fisher, *J. Agr. Res.* **60**, 269 (1940).

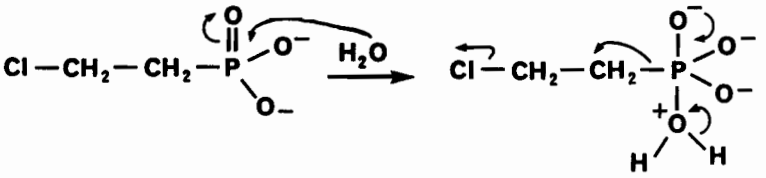
25. a) F.B. Abeles, *Ethylene in Plant Biology*, Academic, New York (1973).
b) S.F. Yang, in *Recent Advances in Phytochemistry*, , Eds. V.C. Runeckles, E. Sondheimer and D.C. Walton, Academic Press, New York, vol. 7, 131 (1974).

26. L. Ilac and R.W. Curtis, *Science* **159**, 1357 (1968).

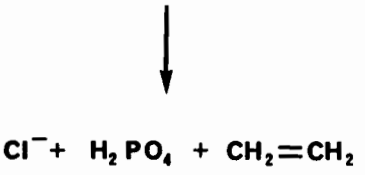
27. J.H. Graham and R.G. Linderman, *Can. J. Microbiol.* **26**, 1340 (1980).

28. a) A.R. Cooke and D.I. Randall, *Nature* **218**, 974 (1968).
b) J.A. Maynard and J.M. Swan, *Aust. J. Chem.* **16**, 596 (1963).

6). Ethephon is an agrochemical of significant commercial value and it is presently used as a herbicide in the late fall where it breaks the dormancy of weeds causing suicidal germination. It is also used to control fruit and vegetable ripening.



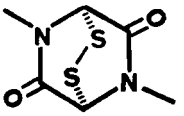
Ethephon (6)



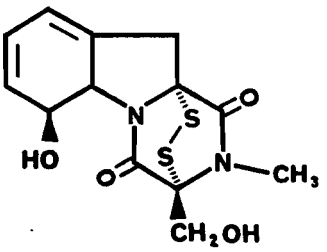
Ethylene (5)

1. 2. 2b Antifungal toxins:

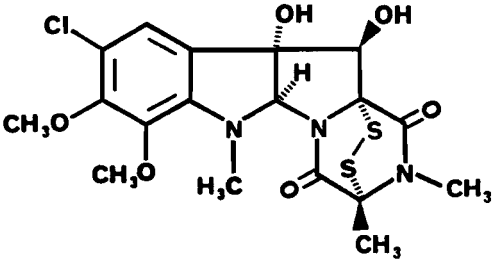
Several fungal metabolites have been reported in the recent literature possessing antifungal activity. Some interesting examples of these metabolites belong to the family of natural products which are characterized by an epidithiodioxopiperazine ring (7), like the compounds gliotoxin (8) and sporidesmin (9).



(7)



Gliotoxin (8)



Sporidesmin (9)

Gliotoxin was originally discovered in the late 1930's by Weindling and Emerson.[29] Since then, many members of this family have been isolated and shown to exhibit antibiotic and antiviral activity, in addition to their antifungal properties. Both the natural products and a number of

29. a) R. Weindling and O.H. Emerson, *Phytopathology* **26**, 1068 (1936).
b) R. Weindling and O.H. Emerson, *Phytopathology* **27**, 1175 (1937).

synthetic analogues[30] have been shown to inhibit the activity of viral reverse transcriptase[31] in Rauscher leukemia, Rauscher sarcoma and Coxsackies viruses.[30,31,32,33] Recently, gliotoxins have been reported in the literature to have immunosuppressive activity. Immunologically compromised patients[34] often suffer from aspergillosis, a lung infection disease caused by the *Aspergillus* fungi which are believed to contribute to the over-all failing immune system of the patients by producing gliotoxins.

Gliotoxins are metabolites of a number of soil fungi including *Aspergillus fumigatus*, *Penicillium terlikowskii*, *Gliocladium fimbiatum* and *Trichoderma viride*. They are structurally related to sporidesmin (9), aranotin[35] (10) and the antifungal macrocyclic emestin[36] (11). All of these metabolites are characterized by the epidithiodioxopiperazine ring which is believed to be responsible for their biological activity. In fact, Taylor[37] and Cavallito[38] have independently shown that reduction of the disulfide bridge leads to total loss of the antibiotic activity of gliotoxins against *Staphylococcus aureus*. Further, oxidation of the reduced dithiol with iodine, to reform the disulfide bridge, leads to complete regeneration of the biological activity.

Although the pharmacological use of these natural products is at the moment hindered by their high toxicity to mammalian cells, their possible use in agriculture as antifungal agents and in the

30. H.C.J. Ottenheijm, J.D.M. Herscheid, G.P.C. Kerkhoff and T.F. Spande, *J. Org. Chem.* **40**, 2147 (1975).

31. H.C.J. Ottenheijm, J.D.M. Herscheid, M.W. Tijhuis and M. Oosterbaan, *J. Med. Chem.* **21**, 796 (1978).

32. A. Taylor, in *Microbial Toxins*, Eds. S. Kadis, A. Ciegler and S. J. Ajl, Academic Press, New York, vol. 7, 337 (1971).

33. K.C. Murdock, *J. Med. Chem.* **17**, 827 (1974).

34. a) A. Mullbacher and R.D. Eichner, *Proc. Natl. Acad. Sci. USA* **81**, 3835 (1984).
b) R.D. Eichner and A. Mullbacher, *Aust. J. Exp. Biol. Med. Sci.* **62** (Pt. 4), 479 (1984).

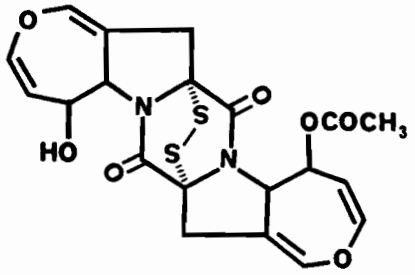
35. R. Nagarajan, L.L. Huckstep, D.H. Lively, D.C. DeLong, M.M. Marsh and N. Neuss, *J. Am. Chem Soc.* **90**, 2980 (1968).

36. H. Seya, K. Nozawa, S. Nakajima, K. Kawai ans S. Udagawa, *J. Chem. Soc. Perkin Trans. I*, 109 (1986).

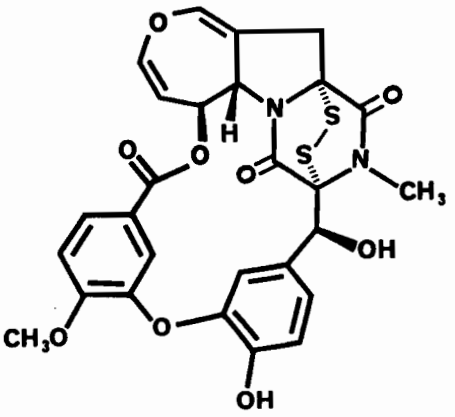
37. a) W.D. Jamieson, R. Rahman and A. Taylor, *J. Chem. Soc. (c)*, 1564 (1969).
b) D. Brewer, D. Hannah and A. Taylor, *Can. J. Microbiol.* **12**, 1187 (1966).

38. C.J. Cavallito, J.H. Bailey and W.F. Warner, *J. Am. Chem. Soc.* **68**, 715 (1946).

protection of wood against wood-rotting fungi is presently under investigation.[39]



Aranotin (10)



Emestin (11)

1. 2. 2c Phytotoxins

The search for microbial metabolites having phytotoxic activity was first undertaken in order to clarify the biochemical basis of fungal pathogenicity to crop plants. However, fungi that produce phytotoxins do not necessarily infect only crop plants. In fact, a systematic search for fungal metabolites which are toxic to agriculturally important weeds has led to the isolation of natural products which could potentially be used as herbicides.

Phytotoxicity is a host-parasite interaction which can be mediated by a number of different interfering mechanisms. The host plant can be deprived of nutrients and essential metabolites which are utilized by the pathogen for its own development. In response, the plant itself may initiate the production of metabolites which are called **phytoalexins.**[40] These are stress compounds which are involved in the defence mechanisms of most plants. They usually possess antifungal or insecticidal activity, but they may also be partly toxic to the host itself.[41] Finally, the more aggressive of plant

39. J.C. Li, Centre de Recherches Industrielles du Quebec, Production of Biological Fungicides, unpublished work.

40. a) A.R. Putnam, *Chem. and Eng. News*, April 4, 34 (1983).
b) J.A. Schneider and K. Nakanishi, *J. Chem. Soc. Chem. Commun.*, 353 (1983).

41. H.P. Fisher and D. Bellus, *Pestic. Sci.* **14**, 334 (1983).

pathogens will produce phytotoxic metabolites in order to break down the defence barriers of their hosts. The release of such compounds into plant tissues can often be determined by examining the symptoms developing in the diseased plant. Yellowing, wilting, brightly-colored lesions and necrosis are commonly caused by phytotoxins. If these symptoms develop at areas distant from the site of infection, then the involvement of a phytotoxic compound can be strongly suspected.

The chemical and biological aspects of fungal phytotoxins have been extensively discussed in several review articles.[42] Although it is not intended to list all known examples here, a few of the most important families of plant pathogens which produce phytotoxins will be described. These pathogens belong to the species *of Alternaria*, *Helminthosporium* and *Ascochyta* and they are well known for their ability to produce metabolites having diverse molecular structures, biological activities and host selectivity.

Ascochyta:

The genus *Ascochyta* includes fungi which are pathogenic to a variety of crop plants and weeds. Although the chemistry involved in its pathogenecity is not always known, a number of interesting metabolites have been identified and shown to possess antibiotic, antiviral and phytotoxic activities.

Ascochitine (12) is a principle phytotoxic compound of *Ascochyta fabae*, a pathogen responsible for brown spot disease in broad beans and chickpea plants. This pathogen was first identified in North America at the University of Saskatchewan, Saskatoon, in 1973, where seeds from a large variety of chickpea plants were imported from different countries in order to assess the potential of this legume as a crop plant in Western Canada.[43] The active metabolite, ascochitine, was isolated from the culture filtrates of *A. fabae* by Oku and Nakanishi[44] who also observed its antibiotic

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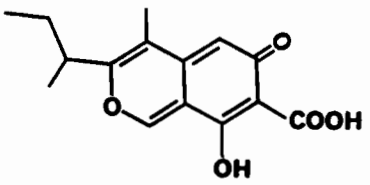
42. a) G.A. Strobel, *Ann. Rev. Biochem.* **51**, 309 (1982).
b) S.O. Duke, *Weed Sciece* **2**, 15 (1986).
c) R.N. Goodman, Z. Kirtly and K.R. Wood, in *The Biochemistry and Physiology of Plant Disease*, University of Missouri Press, Columbus, chapter 9 (1986).
e) H.G. Cutler, in *The Science of Allelopathy*, , Eds. A.R. Putman and C.S. Tong, John Wiley and Son, New York, chapter 9 (1986).
f) S.O. Duke and J. Lydon, *Weed Technology* **1**, 122 (1987).

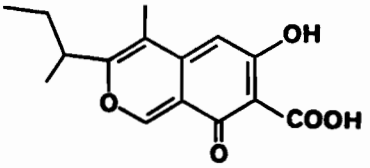
43. R.A.A. Morrall and D.L. McKenzie, *Plant Disease Reporter* **58**, 342 (1974).

44. H. Oku and T. Nakanishi, *Phytopathology* **53**, 1321 (1963).

activity against a number of bacteria, yeasts and other fungi. The same compound was previously isolated from *Ascochyta pisi* by Bertini[45] and for nearly 15 years ascochitine was believed to have the *para*-quinone[46] structure 12. Recently, however, biosynthetic studies and ^{13}C-^{1}H coupled NMR studies[47] indicated that the *ortho*-quinone 13 is the correct structure of ascochitine.



(12)



(13)

The macrocyclic lactone ascotoxin (14) is an other phytotoxic metabolite from this species. It was isolated from the mycelium of *Ascochyta imperfecta*,[48] a mold responsible for rotting disease in clover, and has been shown to be the principle phytotoxin of this fungus. Ascotoxin is identical in structure to decumbin which was previously isolated from the culture filtrate of *Penicillium decumbens*[49] and other *Penicillium* fungi.

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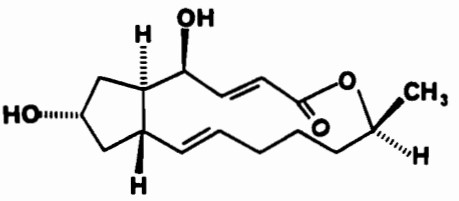
45. S. Bertini, *Ann. Stazione chim.-agrar. sper. Roma* **11**, 545 (1956).

46. a) I. Iwai and H. Mishima, *Chem. and Ind.*, 186 (1965).
b) M.N. Galbraith and W.B. Whalley, *J. Chem. Soc. (C)*, 3557 (1971).

47. L. Colombo, C. Gennari, G. Ricca, C. Scolastico and F. Aragozzini, *J. Chem Soc. Perkin I*, 675 (1980).

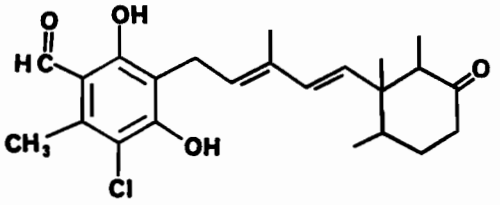
48. Y. Suzuki, H. Tanaka, H. Aoki and T. Tamura, *Agr. Biol. Chem.* 34, 395 (1970).

49. V.L. Singleton, N. Bohonos and A.J. Ullstrup, *Nature* **181**, 1072 (1958).

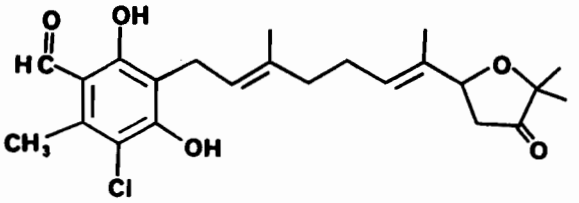


Ascotoxin / Decumbin (14)

Metabolites exhibiting antiviral properties have also been isolated from the *Ascochyta* species. The compounds ascochlorin[50] **(15)** and ascofuranone[51] **(16)** have both been isolated from the mycelium of *Ascochyta viciae*, a microorganism which causes black spots on infected plants. They have been shown to inhibit the growth of several RNA and DNA viruses.



Ascochlorin (15)



Ascofuranone (16)

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50. a) G. Tamura, S. Suzuki, A. Takatsuki, K. Ando and K. Arima, *J. Antibiot.* **21**, 539 (1968).
b) Y. Nawata, K. Ando, G. Tamura, K. Arima and Y. Iitaka, *J. Antibiot.* **22**, 511 (1969).

51. H. Sasaki, T. Okutomi, T. Hosokawa, Y. Nawata and K. Ando, *Tetrahedron Lett.*, 2541 (1972).

Helminthosporium:

The species *Helminthosporium* includes the fungi *H. sacchari* (pathogen of sugarcane), *H. victoriae* (pathogen of oats), *H. carbonum* (pathogen of corn), *H. oryzae* (pathogen of rice), *H. sativum* (pathogen of several crop cereals) and *H. maydis* (pathogen of Southern corn). All of these fungi are highly pathogenic, host-specific and pose a serious threat to agriculture world wide. For example, *H. oryzae* (now reclassified as *Drechslera oryzae*) caused a massive epidemic of brown spot disease in rice during the great Bengal famine of 1943,[52] during which an estimated two million people died of starvation in India. Other areas of the world were also severely affected. Structurally, most of the toxic metabolites which have thus far been isolated from the *Helminthosporium* species, seem to have a terpenoid moiety as part of their structure.

The phytotoxin of *H. sacchari* was first isolated by Steiner and Strobel[53] in 1971 and was proposed to be an α-galactoside of cyclopropanediol. The purified toxin was shown to cause the same type of red runners, arising from eye-shaped lesions on the leaves of sugarcane, as the fungus itself. Further biological studies on the phytotoxicity and host selectivity of the toxin were published in the same year by Steiner and Byther.[54] Several reports[55] on the general structure of this metabolite have appeared since. A β-1-5-linked oligogalactofuranoside linked to a sesquiterpenoid moiety has been proposed. However, the precise structure is still not known.

Similarly, a number of publications on the toxic effects of *H. victoriae*[56] appeared in the 1960s, but the isolation of the main active metabolite, victorin, proved to be exceedingly difficult since upon purification the toxin was found to be unstable.[57] Finally in 1972, Dorn and Arigoni[58] reported

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52. S.Y. Padmanabhan, *Ann. Rev. Phytopath.* **11**, 11 (1973).

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

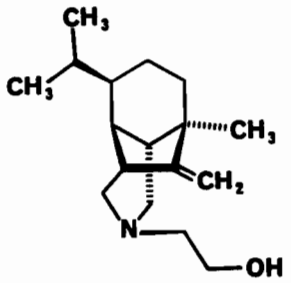
54. G.W. Steiner and R.S. Byther, *Phytopathology* **61**, 691 (1971).

55. a) R.C. Beier, B.P. Mundy and G. Strobel, *Can. J. Chem.* **58**, 2800 (1980).
b) R.S. Livingston and R.P. Scheffer, *J. Biol. Chem.* **256**, 1705 (1981).
c) U. Macko, K. Goodfriend, T. Wachs, J.A.A. Renwick, W. Acklin and D. Arigoni, *Experientia* **37**, 923 (1981).

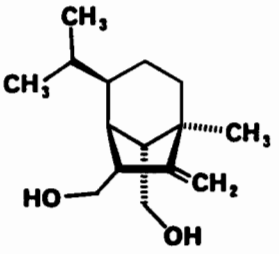
56. a) J.M. Gardner and R.P. Scheffer, *Physiological Plant Pathology* **3**, 147 (1973), and references therein.
b) K.E. Damann, J.M. Gardner and R.P. Scheffer, *Phytopathology* **64**, 652 (1974).

57. R.B. Pringle and A.C. Braun, *Phytopathology* **47**, 369 (1957).

the isolation and structure determination of the compound victoxinine (17) which is a component of victorin. The biological precursor of victoxinine, dihydroprehelminthosporol (18), was also isolated from the culture media of *H. victoriae* and found to have considerable phytotoxicity. The same terpenoid was isolated last year from a *Bipolaris* fungus[59] which is pathogenic to Johnson grass. Biological testing showed that dihydroprehelminthosporol induces lesions on the leaves of Johnson grass which are very similar in appearance to those produced during infection of the plant by the fungus.



Victoxinine (17)



Dihydroprehelminthosporol (18)

The closely related phytotoxic compounds prehelminthosporol (19) and prehelminthosporal (20), are produced by *H. sativum* (now reclassified as *Bipolaris sorokiniana*)[60] a disease-causing fungus of crop cereals (mentioned previously). Interestingly, the presence of an unstable hemiacetal in these compounds led to their initial isolation as the aldehydes helminthosporol[61] (21) and helminthosporal[62] (22) respectively.

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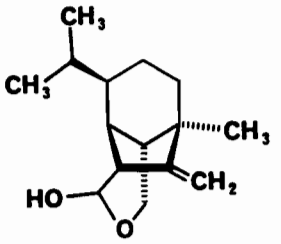
58. F.Dorn and D. Arigoni, *J. Chem. Soc. Chem. Commun.*, 1342 (1972).

59. L.M. Pena-Rodriguez, N.A. Armingeon and W.S. Chilton, *J. Nat. Prod.* **51**, 821 (1988)

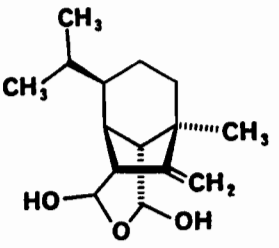
60. a) P. De Mayo, R.E. Williams and E.Y. Spencer, *Can. J. Chem.* **43**, 1357 (1965).
    b) M. Nukina, H. Hattori and S. Marumo, *J. Am. Chem. Soc.* **97**, 2542 (1975).

61. S. Tamura, A. Sakurai, K. Kainuma and M. Takai, *Agr. Biol. Chem. Tokyo* **27**, 738 (1963).

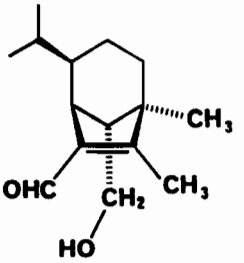
62. a) P. De Mayo, E.Y. Spencer and R.W. White, *Can. J. Chem.* **39**, 1608 (1961).
    b) P. De Mayo, E.Y. Spencer and R.W. White, *Can. J. Chem.* **41**, 2996 (1963).



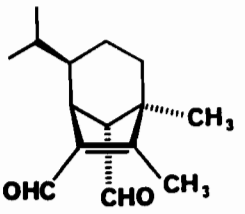
Prehelminthosporol (19)



Prehelminthosporal (20)



Helminthosporol (21)



Helminthosporal (22)

Finally, a C-25 terpenoid structure is characteristic of the phytotoxins called ophiobolins. These compounds are produced by three members of the *Helminthosporium* species, *H. maydis*, *H. sorghicola* and *H. oryzae*. Such types of carbotricyclic ring systems have also been found in the phytotoxic metabolites produced by other species of fungi. Two such examples are the non-host-specific plant toxins fusicoccin[63,64] and the family of cotylenins.[65]

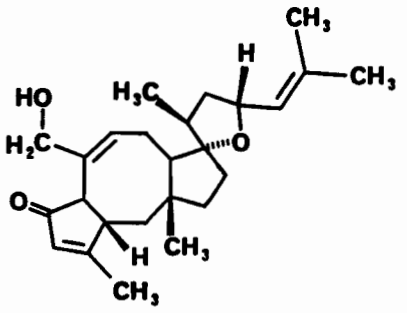
Host specific phytotoxins from *H. maydis* (also called *Drechslera maydis*) were first isolated

63. K.D. Barrow, D.H.R. Barton, E.B. Chain, U.F.W. Ohnsorge and R. Thomas, *J. Chem. Soc. Chem. Commun.*, 1197 (1968).

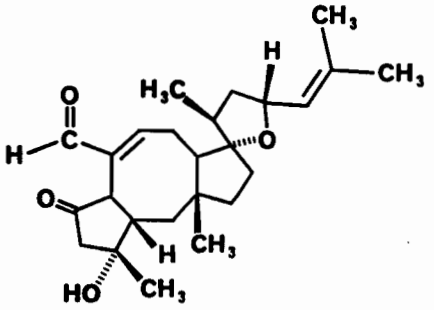
64. Fusicoccin is the major phytotoxin of the fungus *Fusicoccum amygdali* which is the causal agent of leaf wilting and necrosis in almond and peach trees.

65. T. Sassa and A. Takahama, *Agric. Biol. Chem.* 39, 2213 (1975).

by Strobel[66] in 1974, although, their structural identity was unclear at the time. In 1980, a polyketo-polyalcohol structure was proposed,[67] but it was not until 1987 that X-ray crystallography of one of these metabolites, ophiobolin I (23), confirmed that they belonged to the family of ophiobolins.[68] Although all of the ophiobolins isolated from *H. maydis* are phytotoxic, only the 6-epiophiobolin A (24) shows the same host-selectivity as the microorganism.



Ophiobolin I (23)



6-Epiophiobolin A (24)

Ophiobolins had been isolated previously from *H. oryzae*[69] (now called *Drechslera oryzae*) and other fungi.[70] Extensive review[71] articles describing the chemistry and biological activity of more than ten already-known compounds have been written, and new members[72] of this class of plant toxins continue to appear in the literature.

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66. A.L. Karr, D.B. Karr and G.A. Strobel, *Plant. Physiol.* 53, 250 (1974).

67. Y. Kono, S. Takeuchi, A Kawarada, J.M. Daly and H.W. Knoche, *Tetrahedron Lett.* 21, 1537 (1980).

68. F. Sugawara, G. Strobel, R.N. Strange, J.N. Siedow, G.D. Van Duyne and J. Clardy, *Proc. Natl. Acad. Sci. USA* 84, 3081 (1987).

69. L. Canonical, A. Fiecchi, M. Galli Kienle and A. Scala, *Tetrahedron Lett.*, 1211 (1966).

70. S. Nozoe, K. Hirai and K. Tsuda, *Tetrahedron Lett.*, 2211 (1966), and references therein.

71. G. Strobel, D. Kenfield and F. Sugawara, *Phytoparasitica* 16, 145 (1988).

72. F. Sugawara and N. Takahashi, G. Strobel, C.-H. Yum, G. Gray, Y. Fu and J. Clardy, *J. Org. Chem.* 53, 2170 (1988).

Alternaria:

The *Alternaria* species is a large family of biologically important microorganisms which are all very similar in morphology. They are known to be aggressive saprophytes and pathogens of several crop plants and weeds. A number of phytotoxins have been isolated from them and shown to be non-host-selective. However, there are at least six *Alternaria* parasites which are known to produce phytotoxic metabolites which are very host-specific and just as host-specific as the pathogens themselves. In spite of their similarities they are therefore considered to be distinct pathotypes of the *Alternaria alternata* family and a number of excellent review articles[73,74] have been written about them.

Although the focus of this review is to provide some examples of metabolites which could be of benefit to agriculture, it should be mentioned that the *Alternaria* fungi produce compounds which have diverse biological activities. Several *Alternaria* metabolites have been found to possess antibiotic, insecticidal, antiviral and mycotoxic properties, in addition to being phytotoxins. A number of review articles[75,76] have been written describing the chemistry and biology of the *Alternaria* mycotoxins.

An interesting example of a broad toxicity natural product is the compound tenuazonic acid[77] (25). Originally isolated from *Alternaria tenuis*,[78] it was later found to be a ubiquitous fungal metabolite produced by a few *Alternaria* fungi and members of the *Aspergillus*, *Phoma* and *Pyricularia* species. Its Mg^{+} and Ca^{2+} complexes[79] have been implicated in acute haematologic disorders

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73. S. Nishimura and K. Kohmoto, *Ann. Rev. Phytopathol.* **21**, 87 (1983).

74. G.E. Templeton, in *Microbial Toxins*, Ed. S. Kadis and A. Ciegler, New York, Academic, vol. **8**, 169 (1972).

75. D.J. Harvan and R.W. Pero, in *Mycotoxins and Other Fungal Related Food Problems*, Ed. J.V. Rodricks, Washington, DC, Am. Chem. Soc., 344 (1976).

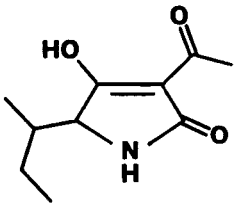
76. P.S. Steyn, in *Mycotoxins in Human and Animal Health*, Eds. J.V. Rodrick, C.W. Hesseltine and M.A. Mehlman, Illinois, Pathotox Publ., 419 (1977).

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

78. R. Rosett, R.H. Sankhala, C.E. Stickings, M.E.U. Taylor and R. Thomas, *Biochem. J.* **67**, 390 (1957).

79. P.S. Steyn and C.J. Rabie, *Phytochemistry* **15**, 1977 (1976).

among African populations consuming grains infected with *Phoma sorghina*. This relatively simple compound has been found to inhibit the growth of tumor cells,[80] to have antiviral activity,[81] antibacterial activity[77] and insecticidal activity.[82] In mammals, it has been shown to inhibit peptide bond formation during protein synthesis[83] at the ribosome level. Its phytotoxic properties have also been extensively studied and, very recently, the relationship between its structure and toxicity to plants has been investigated.[84]



Tenuazonic acid (25)

The *Alternaria* fungi infect such crop plants as tomatoes (*A. lycopersici*), strawberries, and apple, pear (*A. mali*) and citrus trees (*A. citri*) among others. Unlike the *Helminthosporium* species which produces phytotoxic metabolites of mostly two general structures, the ophiobolines and the helminthosporides, the *Alternaria* fungi produce phytotoxic metabolites with tremendous variations in

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80. a) C. Gitterman, E. Dulaney, E. Kaczka, G. Campbell, D. Hendlin and B. Woodruff, *Cancer Res.* 24, 440 (1964).
b) C. Gitterman, *J. Med. Chem.* 8, 483 (1964).

81. F. Miller, W. Rightsel, B. Sloan, J. Ehrlich, J. French, Q. Bartz and G. Dixon, *Nature* 200, 1338 (1963).

82. M. Cole and G. Rolinson, *Appl. Microb.* 24, 660 (1972).

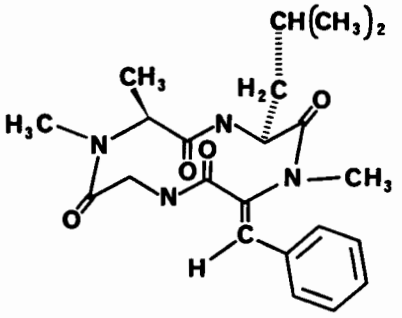
83. a) L. Carrasco and D. Vazquez, *Biochem. Biophys. Acta* 319, 209 (1973).
b) M. Barbacid and D. Vazquez, *J. Mol. Biol.* 84, 603 (1974).

84. M.H. Lebrun, L. Nicolas, M. Boutar, F. Gaudemer, S. Ranomenjanahary and A. Gaudemer, *Phytochemistry* 27, 77 (1988), and references therein.

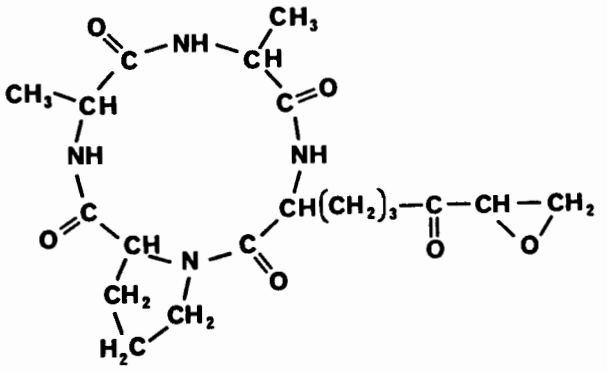
their chemical composition.

Toxins with a peptide-like structure are produced by several members the species, *A. tenuis*, *A. carbonum*, *A. brassicae*, *A. kikuchiana* and *A. mali*.

A. tenuis is a pathogen which causes chlorosis on the cotyledons of germinating cotton and many other dicotyledonous plants. The chemical structure of its active metabolite was elucidated by Meyer[85] to be that of the cyclic tetrapeptide, tentoxin (**26**). A few years later Rich[86] proposed a new conformation for the structure of this peptide and demonstrated a strong correlation between the conformational structure of the molecule and its biological activity. Similar cyclic peptides are produced by the corn pathogen *A. carbonum* (compound **27**)[87a,b] and the canola pathogen *A. brassicae*.[87c]



Tentoxin (26)



HC-Toxin (27)

The fungus *A. kikuchiana*, a distinct pathotype which causes black spot disease in Japanese pears, produces metabolites which are partly peptide and partly unsaturated acid/ester in nature. Involvement of a toxic metabolite in the pathogenicity of *A. kikuchiana* was first suggested by

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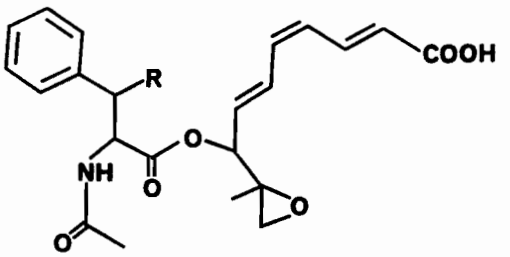
85. W.L. Meyer, G.E. Templeton, C.I. Grable, R. Jones, L.F. Kuyper, R.B. Lewis, C.W. Sigel and S.H. Woodhead, *J. Am. Chem. Soc.* **97**, 3802 (1975), and references therein.

86. a) D.H. Rich and P.K. Bhatnagar, *J. Am. Chem. Soc.* **100**, 2218 (1978).
b) D.H. Rich, P.K. Bhatnagar, R.D. Jasensky, J.A. Steele, T.F. Uchytil and R.D. Durbin, *Bioorg. Chem.* **7**, 207 (1978).

87. a) R.B. Pringle and R.P. Scheffer, *Phytopathology* **57**, 1169 (1967).
b) S.O. Duke, in *The Science of Allelopathy*, Eds. A.R. Putnam and C.S. Tang, John Wiley and Sons, New York, chap. 17 (1986).
c) W.A. Ayer and L.M. Pena-Rodriguez, *J. Nat. Prod.* **50**, 400 (1987).

Tanaka[88] in 1933. However, the complete identity of this toxin did not become known until 50 years later when Nakashima[89] isolated and identified the host-specific toxins AK-toxin I and II (28), from its culture broth. The AK-toxin I is extremely potent causing veinal necrosis and rapid K^+ loss on leaves of Japanese pears at a concentration of 10^{-8} to 10^{-9} M.



(I) R=CH_3

(II) R=H

AK-Toxins (28)

The last active metabolites which have a peptide structure are the AM-toxins (29), produced by the apple pathotype of *A. alternata* (*A. mali*). The main phytotoxin, AM-toxin I, was isolated independently by two groups and shown to be the cyclic depsipeptide 29 (I).[90] Two other, minor toxins, AM-toxins (II, III) were also isolated and found to differ from AM-toxin I only in the *para*-substitution of the aromatic ring. Final proof of the structural assignment for the AM-toxins, along with some indication as to their structure-toxicity[42a,91] relationship, was obtained through total

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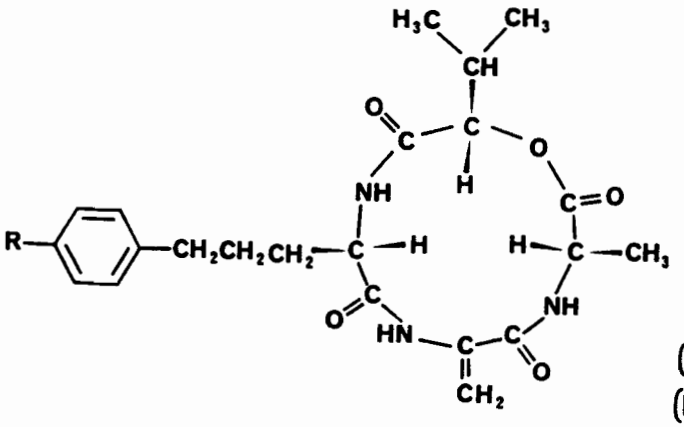
88. S. Tanaka, *Mem. Coll. Agric. Kyoto Imp. Univ.* **28**, 1 (1933).

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

90. a) T. Okuno, Y. Ishita, K. Sawai and T. Matsumoto, *Chem. Lett.*, 635 (1974).
b) T. Ueno, T. Nakashima, Y. Hayashi and H. Fukami, *Agric. Biol. Chem.* **39**, 1115 (1975).

91. K. Noda, Y. Shibata, Y. Shimohigashi, N. Izumiya and E. Gross, *Tertahedron Lett.* **21**, 763 (1980), and references therein.

synthesis[92] of the toxins themselves and some of their analogues.



(I) R=OCH_3
(II) R=H
(III) R=OH

AM-Toxins (29)

α-Pyrones are yet another class of phytotoxic compounds produced by *Alternaria* fungi. Two distinct pathogens of *Alternaria citri* (host-specific pathogens of citrus trees) have been shown to produce five different metabolites[93] having an α-pyrone (30) or dihydropyrone (31) structure. Although all of these metabolites are toxins which display the same host-selectivity as the microorganisms which produce them, the highest amount of activity was observed with the dihydropyrone compound 32.

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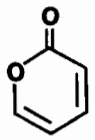
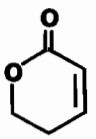
92. a) T. Kanmera, H. Aoyagi, M. Waki, T. Kato, N. Izumiya, K. Noda and T. Ueno, *Tetrahedron Lett.* 22, 3625 (1981).
b) S. Lee, H. Aoyagi, Y. Shimohigashi, N. Izumiya, T. Ueno and H. Fukami, *Tetrahedron Lett.*, 843 (1976).
c) Y. Shimohigashi, S. Lee, T. Kato, N. Izumiya, T. Ueno and H. Fukami, *Chem. Lett.*, 1411 (1977).

93. a) J.M. Gardner, Y. Kono, J.H. Tatum, Y. Suzuki and S. Takeuchi, *Phytochemistry* 24, 2861 (1985).
b) Y. Kono, J.M. Gardner, Y. Suzuki and S. Takeuchi, *Phytochemistry* 24, 2869 (1985).
c) Y. Kono, J.M. Gardner, K. Kobayashi, Y. Suzuki, S. Takeuchi and T. Sakurai, *Phytochemistry* 25, 69 (1986).



(30)



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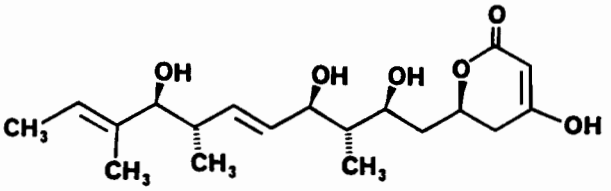
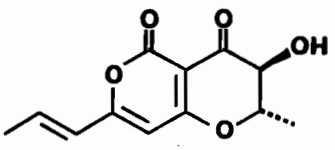


(32)

The structurally related compounds radicinin[94] (33), radicinol[94] (34), deoxyradicinin[95] (35) and 3-epideoxyradicinol[96] (36) have been isolated from the *Alternaria helianthi* and *Alternaria chrysanthemi* fungi. They all possess phytotoxic activity and, in addition, radicinin displays antibacterial,[94] insecticidal, antifungal and plant growth regulatory activity.[97]



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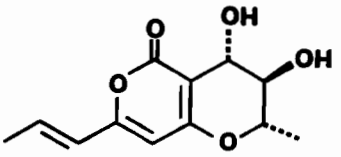


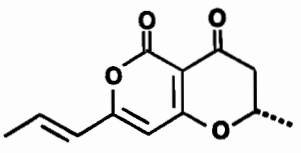
(34)

94. D.J. Robeson, G.R. Gray and G.A. Strobel, *Phytochemistry* **21**, 2359 (1982), and references therein.

95. D.J. Robeson and G.A. Strobel, *Phytochemistry* **21**, 1821 (1982).

96. D.J. Robeson and G.A. Strobel, *Phytochemistry* **23**, 767 (1984).

97. T. Yokota, T. Ishikura and A. Ozaki, Japanese Patent No. 11 997, cited in *Chem. Abstr.* 67, 898 18b (1967).



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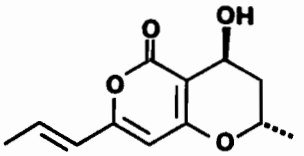

(36)

The above list of active compounds which have been isolated from the *Alternaria* species is far from complete. However, these selected examples illustrate the complexity and diversity of metabolites found even within a single species of very closely related fungi.

1.3 Project Objectives

The main objective of this thesis was to investigate three agriculturally important fungi, *Pisolithus tinctorius*, *Scuillus cavipes*, and *Phomopsis convolvulus*, each one of which was associated with unique biological phenomena. Initially, it was postulated that these phenomena were caused by biologically active fungal metabolites , hence, their isolation, identification and some understanding of their biological activity was attempted.

Pisolithus tinctorius and *Scuillus cavipes* are two ectomycorrhizal fungi which stimulate the growth of forest trees and other host plants.

Recently, Kope and Fortin[98] have shown that the cell-free culture medium of *Pisolithus tinctorius* inhibits spore germination and causes hyphal lysis to a number of plant pathogenic fungi. The biologically active metabolites responsible for these effects were isolated and structurally

98. H.H. Kope and J.A. Fortin, *New Phytologist* 113, 000 (1989) in press.

characterized. They were found to be the aromatic compounds *p*-hydroxybenzoylformic acid (**37**) and (R)-(-)-*p*-hydroxymandelic acid (**38**). In order to gain some insight into the mode of action of these antibiotics and into the relationship between chemical structure and biological activity, both enantiomers of metabolite **38** were synthesized. Their biological effects, along with a number of other structurally related compounds, were examined.

The second microorganism, the fungus *Scuillus cavipes* was originally observed to strongly promote the formation of roots and root primordial[99] on hypocotyl cuttings of coniferous trees. The possible production of a novel metabolite having phytohormonal activity was implicated. Hence, an investigation of this fungus was initiated, although it was quickly discovered that the compound involved was ethylene gas. It was shown that the morphological changes observed when hypocotyl cutting were exposed to small amounts of ethephon (**6**) were very similar to those produced by *S. cavipes*. The production of ethylene gas directly by the fungus was ruled out; the isolation of a metabolite which can act as its precursor was unsuccessful.

Lastly, the chemical structure and biological activity of some interesting metabolites of *Phomopsis convolvulus* were investigated. *Phomopsis convolvulus* is a host-specific pathogen of field bindweed which, upon infection, displays all of the characteristics associated with the production of phytotoxins. Since field bindweed is an important agricultural pest, which at the moment is difficult to control, *P. convolvulus* represented a good candidate for the development of new agricultural products.

From the growth media of *P. convolvulus* the steroid compounds ergosterol (**89**) and ergosterol peroxide (**90**), the phthalides 4-carboxy-3-hydroxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (**91**), 4-(hydroxymethyl)-7-methoxy-6-methyl-1(3H)-isobenzofuranone (**92**) and 4-carboxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (**93**), and the α-pyrone 3-(4-methoxy-3-methyl-α-pyron-6-yl)-2-methyl-2-butenoic acid (**94**) were isolated. Bioassays were developed and used to investigate the overall phytotoxicity of the above metabolites and their possible role in the pathogenicity of *Phomopsis convolvulus* towards field bindweed.

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99. Primordial consist of very actively dividing cells which have not yet begun to show morphological differentiation.

CHAPTER 2

ANTIFUNGAL ANTIBIOTICS FROM PISOLITHUS TINCTORIUS

2.1 Introduction

The mycorrhizal fungus *Pisolithus tinctorius*, isolated from the soil and roots of a broad range of host plants,[100] is well known for its ability to colonize the roots of trees, thereby, becoming their extension. This symbiotic existence has been shown to be extremely advantageous to the host plants,[101] especially in habitats characterized by poor environmental conditions such as high temperature and very low humidity, as well as soils of low fertility and high concentrations of pollutants. *P. tinctorius* has adapted effectively to mine spoils, sand dunes, and areas of eroded, rocky and shallow soil. The potential of this microorganism in aiding reforestation and reclamation efforts has been examined by several researchers. It was found, for instance, that pine seedlings transplanted onto a pyritic coal mine site and infected with *P. tinctorius* achieve twice the height and stem diameter of uninfected trees, after a period of five years.[102] The hypothesis that the fungus could promote growth by interfering with *Thiobacillus* bacteria, which catalyze the production of H_2SO_4 from pyrite, has also been examined,[102] however, this turned out not to be the reason for its beneficial affects. Similarly, *P. tinctorius*-infected loblolly (*Pinus taeda*) and shortleaf (*Pinus echinata*) pine seedlings, transplanted onto eroded forest sites, have been found to have substantially high growth rates especially upon the addition of sewage sludge.[103] Protection against air pollutants (O_3 and SO_2) of the feeder-roots of pine trees has also been observed[104] in the presence of this fungus.

100. D.H. Marx, *Can. J. Microbiol.* **23**, 217 (1977).

101. a) W.J. Brill, *Scientific American* **245**, 199 (1981).
b) G. Gay, R. Rouillon and G. Bruchet, *Colloq. INRA 13(Mycorhizes, Partie Integr. Plante: Biol. Perspect. Util.)*, 163 (1982).
c) D.W. Greene, W.J. Manning and D.R. Cooley, *HortScience* **17**, 655 (1982).

102. J.W. Hendrix, C. Stevens Hunt and D.M. Maronek, *Can. J. Microbiol.* **31**, 878 (1985).

103. C.R. Berry and D.H. Marx, *For. Sci.* **22**, 351 (1976).

104. a) H.E. Garrett, J.L. Carney and H.G. Hedrick, *Can. J. For. Res.* **12**, 141 (1982).
b) J.L. Carney, H.E. Garrett and H.G. Hedrick, *Phytopathology* **68**, 1160 (1978).

It is clearly evident that *P. tinctorius* is of great value to agriculture and forestry. In general, it is believed that the microorganism aids the development of its host plants in three ways. The primary mechanism involves its large network of mycelia which it spreads far beyond the reach of the host's root system, seeking nutrients. The accumulation and transfer of Mg, P, S, K, Ca and other minerals from the fungus to host trees[105] has been proposed as the major factor in its ability to stimulate plant growth in low fertility soils. Secondly, accelerated plant development has been associated with the production of metabolites which are phytohormones. The *in vitro* production of the auxin indole acetic acid was observed in all eight isolates of *P. tinctorius* tested by Ho,[106] as well as in those tested by others.[107] In some cases, the addition of low levels (10^{-8}M) of the amino acid L-tryptophan was required to induce production of auxin.[108] Cytokinin,[106] a cell division-promoting hormone, and gibberellin[106] or gibberellin-like[109] growth promoting hormones are also produced by the fungus.

The third mechanism by which *P. tinctorius* aids in the growth of its hosts is by protecting them against disease. Marx[110] was first to report the antagonistic properties of mycorrhizal fungi against pathogenic fungi and soil bacteria. Recently, Kope and Fortin[98] have observed that the liquid culture of *P. tinctorius* exhibits strong antifungal activity against several pathogenic fungi including *R. praticola*, *S. brunnea* and *T. hartigii*. Hyphal lysis, characteristic of antifungal antibiotics which inhibit chitin synthesis (e.g. polyoxins[111]), was shown to occur within 12-24 hours of exposure of a test organism to the liquid culture medium of *P. tinctorius*.

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105. P. Sihanonth and R.L. Todd, *Ecol. Bull.* **25** (Soil Org. Components Ecosyst.), 392 (1977).

106. I. Ho, *Can. J. For. Res.* **17**, 31 (1987).

107. M. Ek, P.O. Ljungquist and E. Stenstroem, *New Phytol.* **94**, 401 (1983).

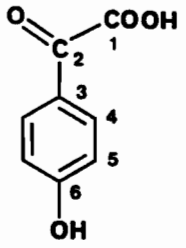
108. W.T. Frankenberger, Jr. and M. Poth, *Appl. Environ. Microbiol.* **53**, 2908 (1987).

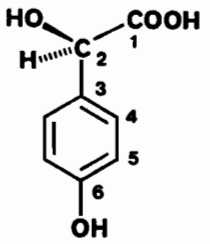
109. K.M. Hanley and D.W. Greene, *HortScience* **22**, 591 (1987).

110. a) D.H. Marx, *Phytopathology* **59**, 153 (1969).
b) D.H. Marx, *Phytopathology* **60**, 1472 (1970).

111. a) K. Isono, K. Asahi and S. Suzuki, *J.Am.Chem.Soc.* **91**, 7490 (1969).
b) K. Isono and S. Suzuki, *Heterocycles* **13**, 333 (1979).
c) A. Endo, K. Kakiki and T. Misato, *J.Bacteriol.* **104**, 189 (1970).
d) A. Endo and T. Misato, *Biochem. Biophys. Res. Comm.* **37**, 718 (1969).

Since the liquid cultures used for biological testing were completely free of spores and mycelium, it became evident that secondary metabolites released in the growth medium were responsible for the antifungal activity. The aim of this section of the thesis is to discuss the isolation and structure determination of two metabolites, *p*-hydroxybenzoylformic acid (37) and the (R)-(-)-enantiomer of *p*-hydroxymandelic acid (38), believed to be the compounds responsible for the observed antifungal activity of *Pisolithus tinctorius*. In addition, the enantiomer of metabolite 38, (S)-(+)-*p*-hydroxymandelic acid, was synthesized and used along with a number of other structural analogues of the natural products to study the structure-activity relationship.

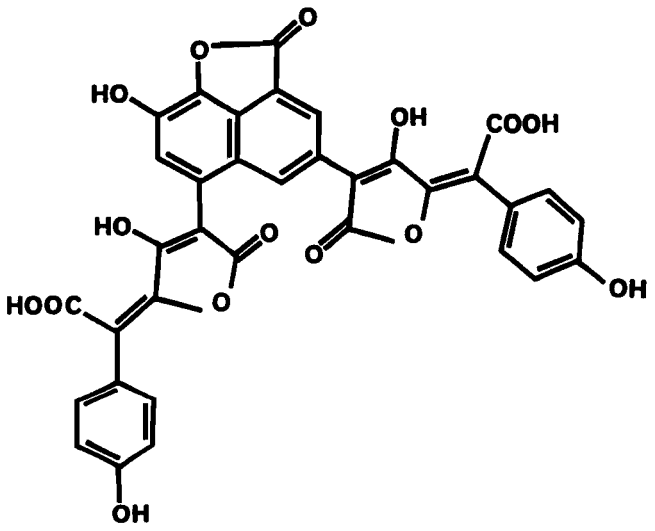




***para*-Hydroxybenzoylformic acid (37)** **(R)-(-)-*para*-Hydroxymandelic acid (38)**

2. 2 Isolation of metabolites *p*-hydroxybenzoylformic acid and *p*-hydroxymandelic acid

Pisolithus tinctorius inoculum incubated in Modified Melin-Norkrans liquid medium[110a] for 50-55 days was filtered to remove all vegetative growth. The dark brown liquid culture was evaporated to a very small volume and then freeze-dried to give a solid. The intensity of the brown color of the medium was often indicative of the concentration of biologically active metabolites present. The production of dark-colored, phenolic, *p*-hydroxybenzoic and humic acid-like metabolites have been noted previously[112] and in 1985 Gill[113] reported the isolation of the brightly red pigment norbadion A(39) from the sporophores of *P. tinctorius*. He also suggested at that time that the compounds responsible for the intense pigmentation of the fungus's fruiting bodies could play an important role in its physiology and ecology. The structural similarities between compounds 37 and 38 and the *p*-hydroxylated pulvinic acid(40) side-chains of norbadion A are quite evident.



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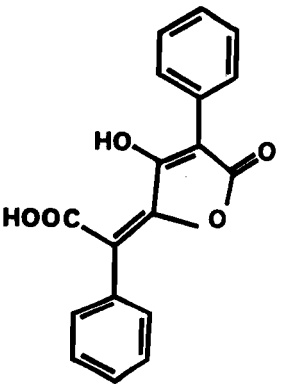


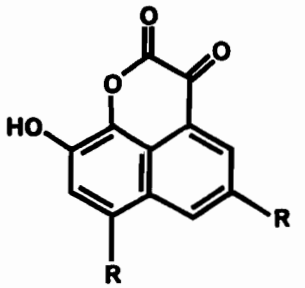
(40)

112. K.H. Tan, P. Sihanonth and R.L. Todd, *Soil Sci. Soc. Am. J.* 42, 906 (1978).

113. M. Gill and D.A. Lally, *Phytochemistry* 24, 1351 (1985).

In fact, a large number of pigments which are structurally related to pulvinic acid have been isolated from other fungi. Badion A (41), bisnorbadioquinone A (42), O-methylpulviquinone A (43) and their biogenetic precursor, xerocomic acid (44), have been isolated from the chocolate-brown pileus of the highly prized edible fungus bay boletus (*Xerocomus badius*).[114] It is rather interesting that the bright-red pigment 44 has been shown to be responsible for the blue coloration of damaged[115] flesh of bay boletus.

Recently, Gill and Watling[116] showed the presence of compounds 41 and 42 in *Pisolithus* as well, and proposed a common biosynthetic scheme for all these pigments from *p*-hydroxyphenylpyruvic acid. It was also suggested that the isolation of hydroxylated pulvinic acid compounds from a large number of fungi, including the *Boletus* and *Pisolithus* species, may be of taxonomic significance.



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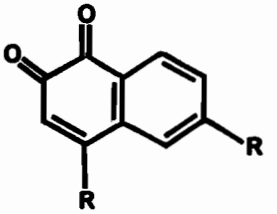

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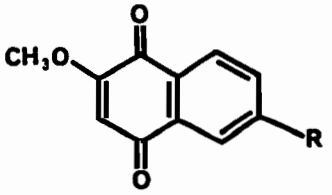

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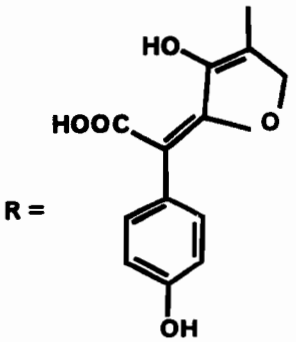

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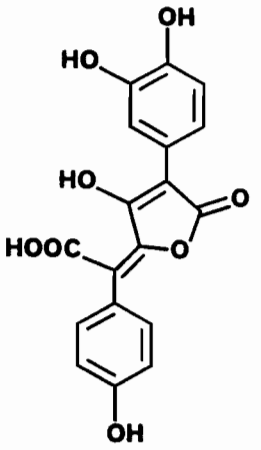
114. B. Steffan and W. Steglich, *Angew. Chem. Int. Ed. Engl.* **23**, 445 (1984).

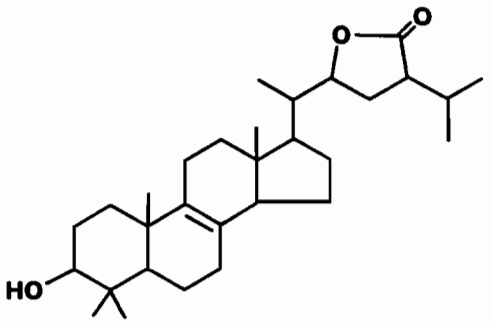
115. W. Steglich, W. Further and A. Prox, *Z. Naturforsch.* **B23**, 1044 (1968).

116. M. Gill and R. Watling, *Pl. Syst. Evol.* **154**, 225 (1986).

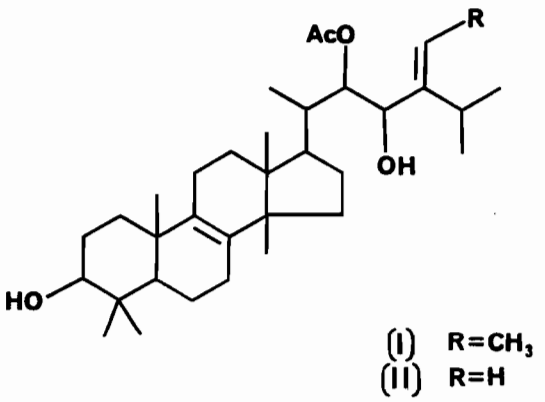


(44)

It is worth noting that amongst the various types of compounds which have been isolated from *P. tinctorius* and which are characteristic of this fungus are the tetracyclic triterpene carboxylic acids pisolactone[117] (45) and the steroids 46 (I, II).[118]



Pisolactone (45)



(46)

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117. A.M. Lobo, P.M. de Abreu, S. Prabhakar and L.S. Godinho, *Tetrahedron Lett.* **24**, 2205 (1983).

118. A.M. Lobo, P.M. de Abreu, S. Prabhakar and L.S. Godinho, *Tetrahedron Lett.* **26**, 2589 (1985).

Soxhlet extraction of the freeze-dried culture of *P. tinctorius* with diethyl ether for 2-7 days (500 mL/day/15g of solid) led to the isolation of an orange oil which inhibited the germination of conidia of the plant pathogen *Truncatella hartigii* at concentrations of 500 μg/mL. The yields obtained were quite variable, increasing proportionally with extraction time, at the expense of the purity of the biologically active constituents. Soxhlet extracts of the same sample, which were isolated and purified each day for seven consecutive days, indicated that the optimum extraction period was three days. Beyond the three day period, the amounts of active metabolites isolated were minimal, whereas the amounts of dark color pigments extracted increased proportionally with time. These results were further supported by the level of toxicity observed for each of these extracts.

Further purification using silica gel flash chromatography[119] was unsuccessful leading to very poor recovery of the overall mass. However, it was observed that all of the biological activity recovered was contained in the most polar fractions (collected after the column was eluted with 5% ethanol in ethyl acetate for a long period of time).

Cellulose column chromatography, eluted with *n*-butanol saturated with water/glacial acetic acid (95/5), allowed partial removal of the most polar and most non-polar components of the mixture. The active polar fractions were recombined, evaporated to near dryness and applied to a 0.33 mm Whatman paper. Overnight descending chromatography with a solvent mixture of 2-propanol/0.5 M NH_4HCO_3 (4/1) led to the separation of this crude material into five bands, visible under uv. In addition, several brightly colored pigments (brown, green, red, orange and yellow) were present at Rf=0.0-0.1. All bands were tested for biological activity but only the intensely uv-active band at Rf=0.22-0.32 was found to exhibit antifungal activity.

Further purification of this mixture proved to be difficult, even when high pressure liquid chromatography techniques were attempted using a variety of solvent systems and column packing materials. The conditions that showed the most promising results involved an aqueous buffer of 1.0 M NH_4OAc + $5.0x10^{-3}$ M *n*-$Bu_4NH_2PO_4$ at a flow rate of 2.0 mL/min, using a C_{18} reverse phase column and a uv detector set at 254nm. Under these conditions, at least six major peaks were recorded (Fig. 1) along with several minor ones. Isolation and desalting of the most strongly absorbing compound gave a biologically inactive gelatinous material. It became evident that the isolation of every peak of this crude would be a very tedious and time consuming process. In addition, repeated HPLC experiments with standard amounts of crude samples at various detector wavelengths (240-280nm) caused such dramatic changes in the intensity of the observed peaks (Fig. 1), that it became necessary to examine the uv characteristics of the active compounds before HPLC

119. W.C. Still. M. Kahn and A. Mitra, *J. Org. Chem.* **43**, 2923 (1978).

could be use effectively.

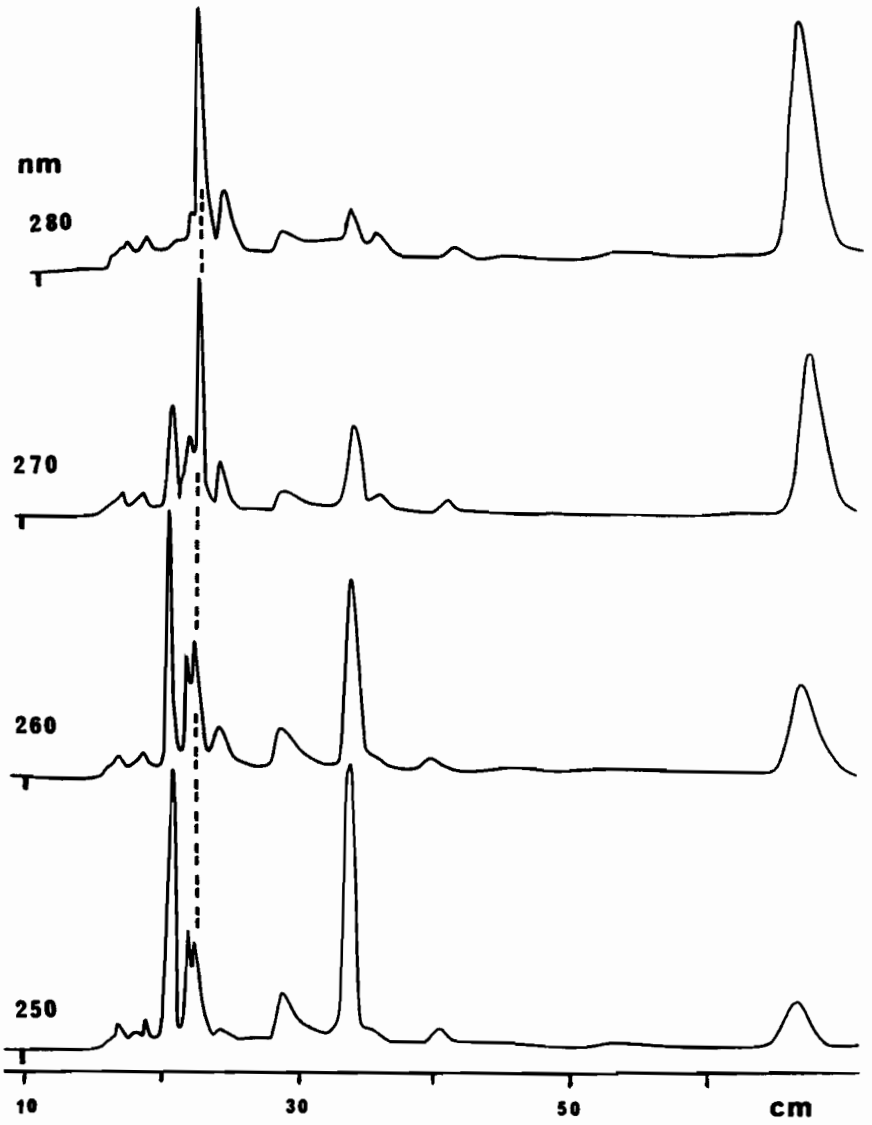

Fig. 1: HPLC of biologically active crude at variable wavelengths (nm); chart speed=2.5cm/min, flow rate=2.0mL/min (0-25cm), 3.0mL/min (26-60cm).

The above mixture was purified again using descending paper chromatography and eluted with a solvent mixture of *n*-butanol saturated with water/glacial acetic acid (95/5). Visualization of the bands was fairly difficult, due to the extensive band-diffusion and overlap; it could only be done in a completely dark room under a uv lamp. Compound **38** appeared as a very weak band at Rf= ~0.6-0.7, whereas the major metabolite(**37**) streaked over a large area having an Rf from ~0.2 to ~0.6. All other bands were found to be biologically inactive materials. The active compounds, **37** and **38**, were isolated, almost pure, as creamy-white solids which darkened in color upon exposure to light and possibly air. Both compounds arrested hyphal growth of *T. hartigii* and caused lysis of the tips within 24 hours, at concentrations of 50-150 μg/mL. At the same concentrations, a 0% germination of *T. hartigii* conidia was observed after 48 hours.

The uv spectra of both compounds was recorded at acidic, neutral and basic conditions and a bathochromic shift, characteristic of carboxylic acids and phenols, was observed (Fig. 2 and 3). At neutral and acidic solutions the uv_{max} absorptions were at 296 nm and 228 nm for compounds **37** and **38** respectively.

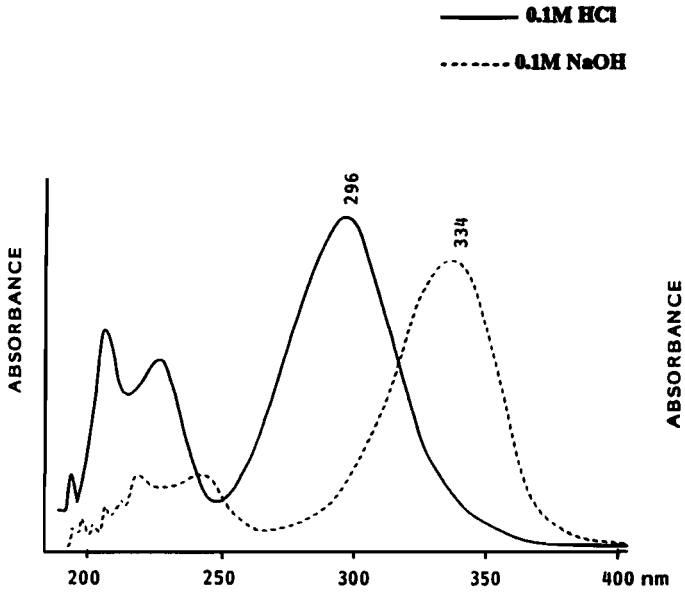

Fig. 2: UV of metabolite 37

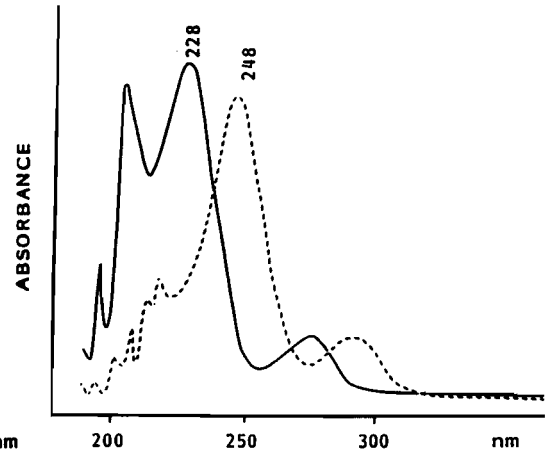

Fig. 3: UV of metabolite 38

All bands isolated from the second paper chromatography were analyzed by C_{18} reverse phase HPLC using a solvent system which did not require subsequent desalting of the pure compounds (91.7% H_2O, 6.4% CH_3OH, 1.8% CH_3COOH). The HPLC results were recorded at 290 and 254 nm and the isolated compounds were re-chromatographed on cellulose TLC in order to confirm the retention times of compounds **37** and **38** (Fig. 4) at 7-8 and 4-5 minutes respectively. Finally both active metabolites were purified using a C_{18} reverse phase column with the solvent mixture of 91.7% H_2O, 7.3% CH_3OH and 0.9% CH_3COOH, at a flow rate of 2.0 mL/min. Compounds **37** and **38** eluted at 5-6 minutes and 8-9 minutes respectively.[120]

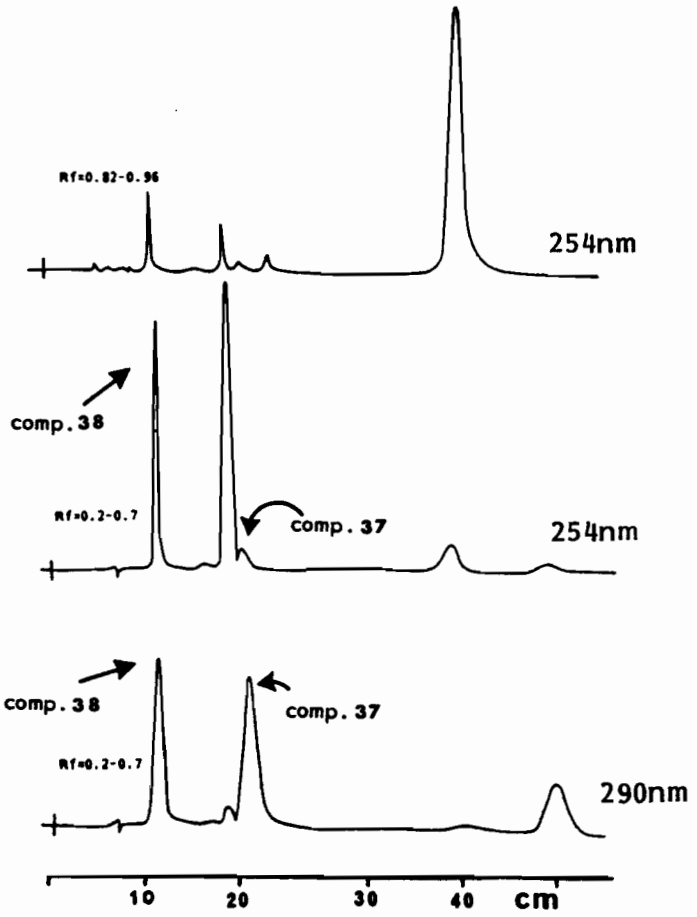

Fig. 4: HPLC results of metabolites 37 and 38.

120. The order at which compounds **37** and **38** elute from the column is reversed compared to previous experiments. However, it should be noted that the size, age and manufacturers of the two columns were different. In addition, the solvent system used here was much less acidic than before but much safer for the packing material of the column used.

2. 3 Structure determination of antifungal metabolites 37 and 38

2. 3. 1 *para*-Hydroxybenzoylformic acid (37)

The ^{1}H NMR of compound 37 in DMSO (Fig. 5), contained only two equal intensity doublets at 6.8 and 7.7 ppm (J=8 Hz), and a broad signal at 10.7 ppm of half the intensity of each doublet.[121]

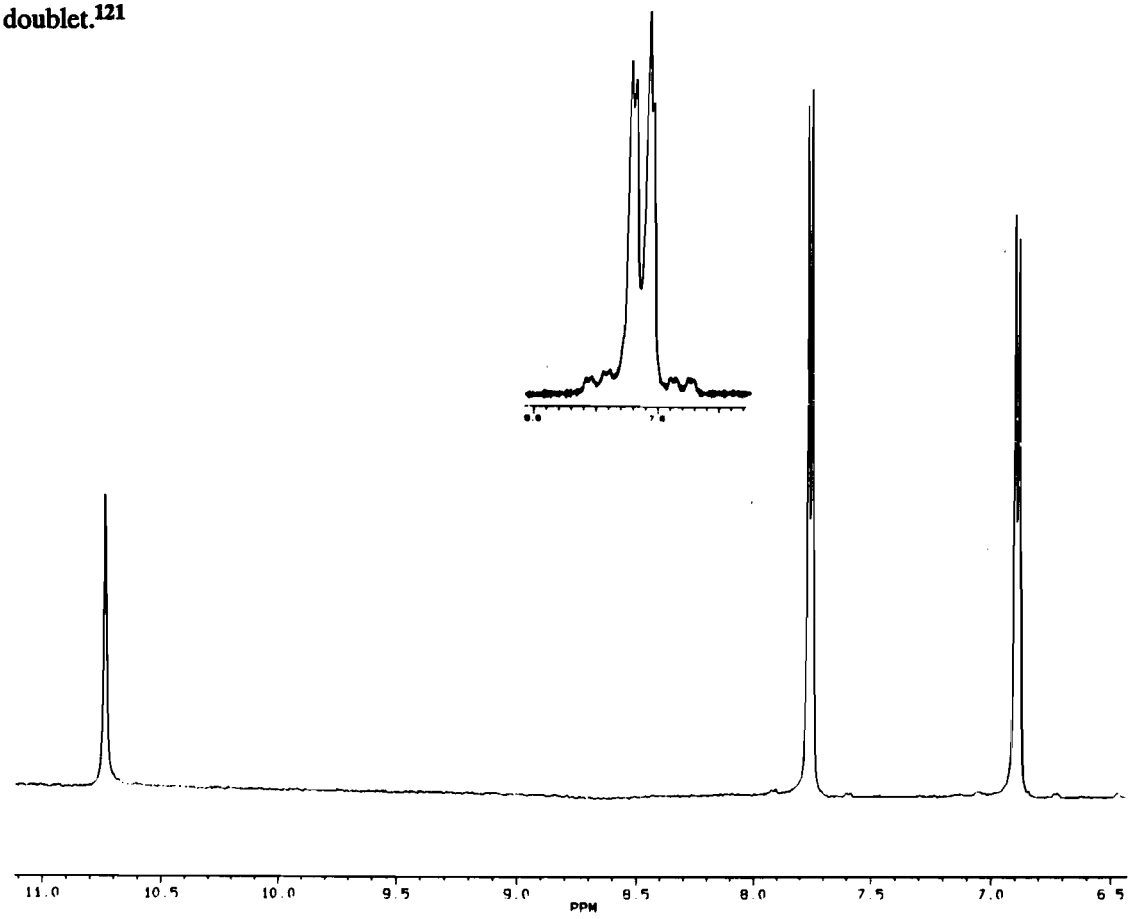

Fig. 5: ^{1}H NMR(400 MHz, DMSO) of metabolite 37.

121. Compound 37 is very hygroscopic; drying under high vacuum for several days often led to darkening of its color without complete removal of the water.

COSY homonuclear shift correlation NMR (Fig. 6) showed that the two doublets were coupled to each other, and a high resolution spectra revealed further splitting of each doublet by 0.85 Hz. Hence, the data was characteristic of a *para*-disubstituted benzene ring.

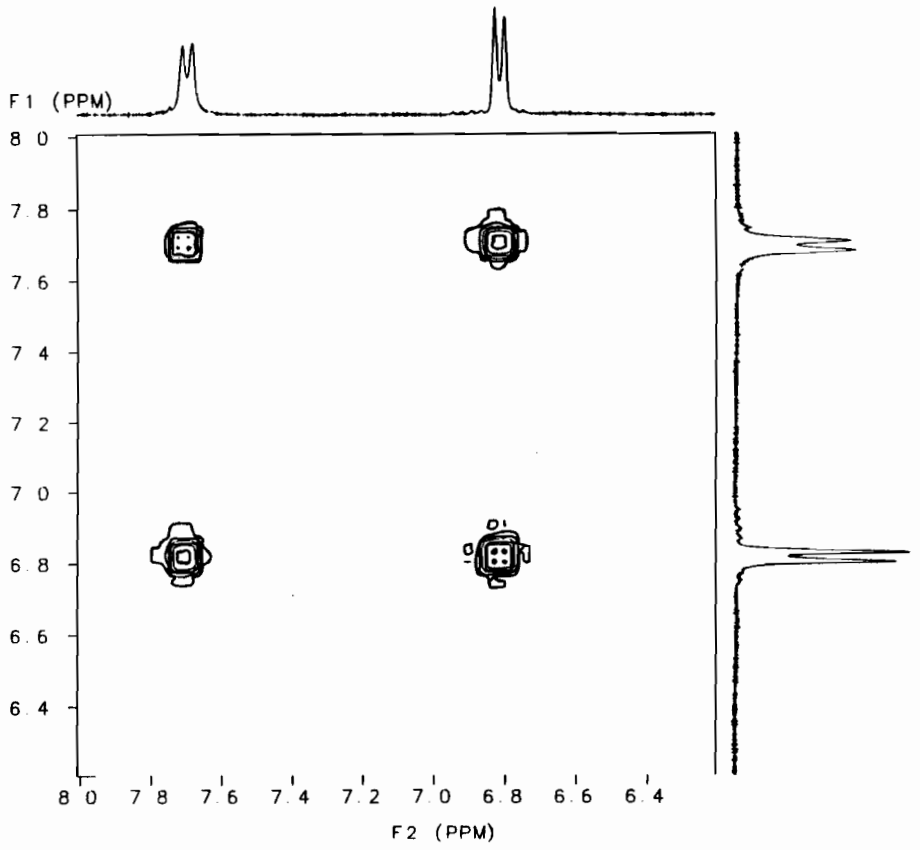

Fig. 6: COSY NMR (300 MHz, D_2O) of metabolite 37.

^{13}C NMR in DMSO showed six types of carbons; two very intense signals at 116.0 and 132.3 ppm, again supporting a *para*-disubstituted aromatic ring, and four weak signals at 125.6, 163.9, ~170 (broad) and ~194 ppm (broad) (Fig. 7a). Attached Proton Test (APT) NMR (Fig. 7b) indicated that all of the four latter signals were quaternary.

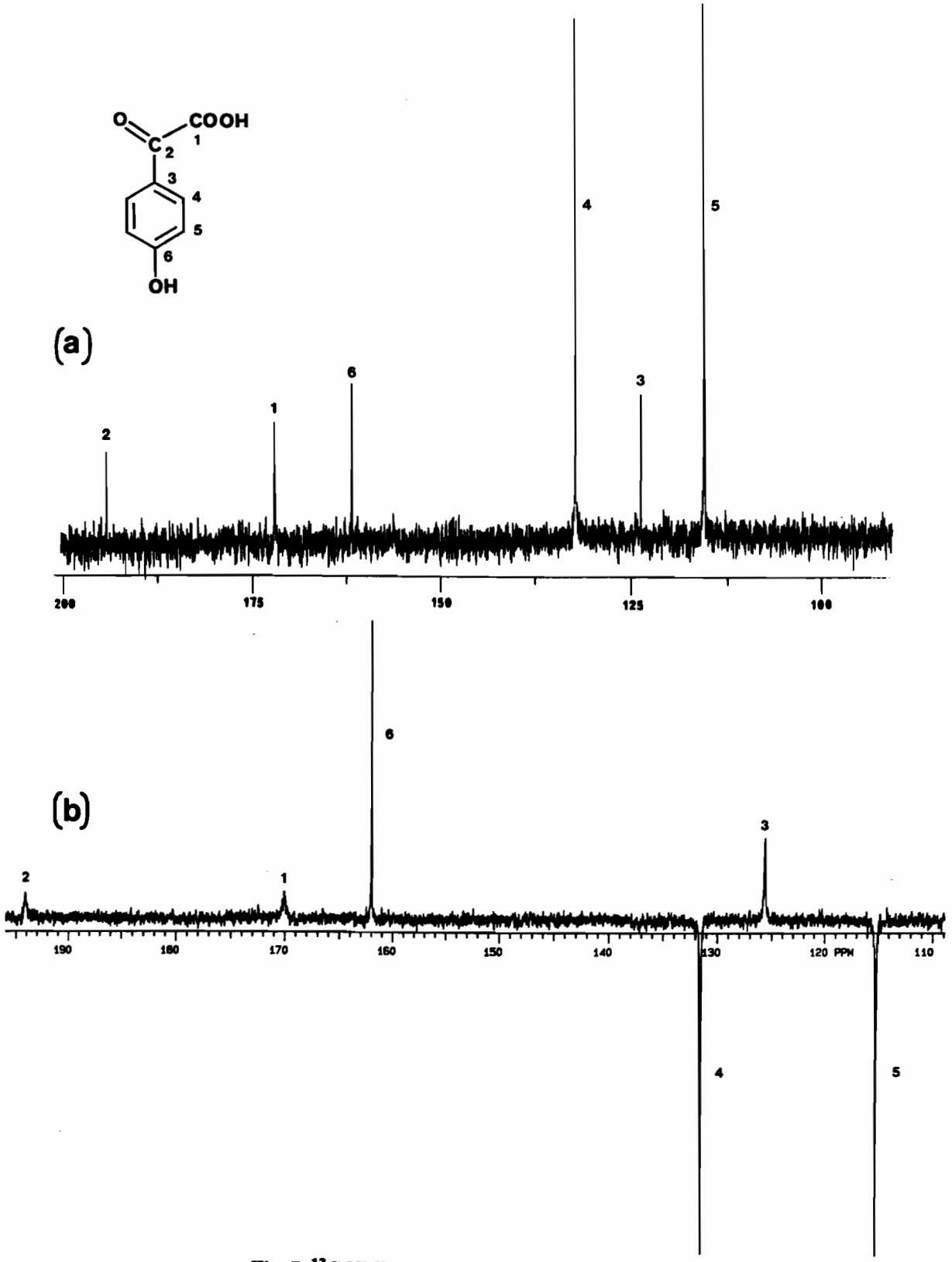

Fig. 7: ^{13}C NMR(300 MHz, DMSO) of metabolite 37;
a: ^{13}C (decoupled) spectra, b: APT spectra.

As it was mentioned previously, the bathochromic shift (from 296nm in acid to 334nm in base) exhibited by the uv spectra of metabolite **37** (Fig. 2) indicated the presence of an acidic and/or a phenolic compound. The IR (in KBr) showed absorptions at ~3380 and ~3150cm^{-1} (-OH), carbonyl absorptions at 1735 and 1654cm^{-1}, a set of peaks supporting a carboxylate ion at 1608 and 1400cm^{-1}, and a *para*-disubstituted benzene ring (1586, 855cm^{-1}). Apparently, carboxylic acids extracted from mineral-based microbial cultures are often isolated as mixtures of the protonated and salt form.[122d]

Chemical ionization and FAB (both positive and negative) mass spectra of **37** showed numerous low intensity species with no predominant molecular ion. ^{252}Cf-plasma desorption mass spectrometry,[122] however, suggested a molar mass of 166. The spectrum of an electrosprayed sample[122c] gave strong positive and negative ions at 211.2 $(M^{2-}+H^{+}+2Na^{+})^{+}$ and 165.1 $(M^{2-}+H^{+})^{-}$ respectively. These results were further supported by the ions obtained from the ^{252}Cf-plasma desorption mass spectra using tridodecylmethylammonium chloride (TDMAC) as a cationic surfactant;[122a,b] 1774.7 $[(TDMA^{+})_3(M^{2-})]^{+}$, 866.7$[(TDMA^{+})(M^{2-}+H^{+})_2]^{-}$, 165.1 $(M^{2-}+H^{+})^{-}$ and 121.1 $(M^{2-}+H^{+}-CO_2)^{-}$. In addition, it became evident that compound **37** can exist as an anionic species, it has two ionizable protons and it is most likely a carboxylic acid.

Although pure compound **37** was isolated as a cream-colored solid, crystallization efforts in H_2O, H_2O/EtOH and pure EtOH were unsuccessful. It was observed, however, that exposure of the sample to pure ethanol over a period of a couple of days, at room temperature, led to the partial esterification of the acid, as noted by ^{1}H and ^{13}C NMR. The ease with which this esterification takes place is quiet common for α-keto acids. In addition, the chemical shifts observed when the ^{1}H NMR was carried out in a solutions of pH 8.1, had values equal to the average between the chemical shifts at pH 1.5 and pH 12.5. Such shift changes are typical of phenols, *para*-substituted with an electron withdrawing group and they suggested a pKa value for this phenol of ~8.1. The structure of *p*-hydroxybenzoylformic acid (**37**) was finally proposed.

Ammonia CI mass spectrometry of the methyl ester derivative of **37** gave a strong molecular ion at 181 $(M^{+}+1)$, 198 $(M+NH4^{+})$ and a base peak at 121 $(HO\text{-}C_6H_4\text{-}CO^{+})$. Both the ^{1}H and ^{13}C NMRs were as expected (see experimental section for details). Hence, the proposed structure was well supported by all data including the FAB MS results which in retrospect could be interpreted (Tab. 1).

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122 a) C.J. McNeal and R.D. Macfarlane, *Biochem. Biophys. Res. Comm.* **139**, 18 (1986).
b) C.J. McNeal and R.D. Macfarlane, *J. Am. Chem. Soc.* **108**, 2132 (1986).
c) B. Sundqvist and R.D. Macfarlane, *Mass Spec. Reviews* **4**, 421 (1985).
d) C.J. McNeal, Texas A&M University, personal communication.

Table 1: FAB MS Fragments of Metabolite 37

FAB^{+ve}			FAB^{-ve}			
m/z	Fragment	+	Matrix	m/z	Fragment	+	Matri
x							
121	$(HOC_6H_4CO)^+$						
167	$(M+H)^+$			165	$(M-H)^-$		
				183[a]	$(M-H)^-+H_2O$		
259	$(M+H)^+$		92	257	$(M-H)^-$		92
277[a]	$(M+H)^+ + H_2O$		92	275[a]	$(M-H)^- + H_2O$		92
297[b]	$(M+K)^+$		92				
				331[c]	$(2M-H)^-$		
				349	$(M-H)^-$		92x2
				367[a]	$(M-H)^- + H_2O$		92x2
389[b]	$(M+K)^+$		92x2				
				423[c]	$(2M-H)^-$		92
443	$(M+H)^+$		92x3	441	$(M-H)^-$		92x3
				459[a]	$(M-H)^- + H_2O$		92x3
481[b]	$(M+K)^+$		92x3				
				515[c]	$(2M-H)^-$		92x2
535	$(M+H)^+$		92x4	533	$(M-H)^-$		92x4
				551[a]	$(M-H)^-$+H2O		92x4
573[b]	$(M+K)^+$		92x4				
627	$(M+H)^+$		92x5	625	$(M-H)^-$		92x5

FAB^{+ve} and FAB^{-ve} spectra were recorded on a ZAB-HS instrument using glycerol as the matrix.
[a] hydrate of comp. 37, [b] potassium salts, [c]dimers.

Since the sodium salt of this compound (37) turned out to be commercially available the ^{1}H and ^{13}C NMR spectra, of both the actual compounds (Tab. 2) and their methyl esters 47 (Tab. 3), were compared. All spectral data, including uv in acid and base, were found to be identical. It should be noted that the carbon chemical shifts varied with the pH of the solution most notably so for the two carbonyl carbons (Tab. 2). In both the natural product and the synthetic compound an expected downfield shift of ~4-5 ppm was observed for the formation of the carboxylate ion from the acid.

Table 2: NMR data of metabolite 37 vs p-hydroxybenzoylformic acid.

^{1}H NMR: (200 MHz, DMSO, δ-values, solvent as internal standard)

	Metabolite 37	**p-Hydroxybenzoylformic acid**
($2H_4$, d, J=8.0 Hz)	6.9	6.8
($2H_5$, d, J=8.0 Hz)	7.7	7.7
[-OH, s(br.)]	10.7	10.7

^{13}C NMR: (300 MHz, DMSO, δ-values, solvent as internal standard)

	Metabolite 37		**p-Hydroxybenzoylformic acid**	
	salt	**acid**	**salt**	**acid**
C-1	170.2	166.8	170.2	166.8
C-2	194.3	187.0	194.5	187.0
C-3	125.7	123.3	125.6	123.3
C-4	131.5	132.2	131.5	132.3
C-5	115.1	116.0	115.2	116.0
C-6	162.1	163.8	162.2	163.9

Table 3: NMR data of the "natural" methyl ester 47 vs the commercial samples.

^{1}H NMR: (200 MHz, $CDCl_3$, δ-values, solvent as internal standard)

	of Metabolite	of p-Hydroxybenzoylformic acid
($2H_4$, d, J=8.9 Hz)	6.9	6.9
($2H_5$, d, J=8.9 Hz)	7.9	7.9
[-OH, s(br)]	~6.7	~6.6
(CH_3, s)	3.9	3.9

^{13}C NMR: (300 MHz, $CDCl_3$, δ-values, solvent as internal standard)

	of Metabolite	of p-Hydroxybenzoylformic acid
CH_3	52.8	52.9
C-1	164.4	164.6
C-2	184.8	185.0
C-3	125.4	124.9
C-4	133.0	133.2
C-5	115.9	116.1
C-6	162.0	162.7

2. 3. 2 *para*-Hydroxymandelic acid (38)

The ^1H NMR of compound **38** in D_2O (Fig.8) showed three types of non-exchangeable protons; two doublets at 7.13 ppm and 6.72 ppm (J=8.2 Hz), characteristic of a para-substituted aromatic ring, and a broad singlet at ~5.0 ppm (integration of 2:2:1 respectively). Since it was nearly impossible to free the sample of water the ^1H NMR in DMSO failed to reveal the presence of any exchangeable protons in the metabolite.

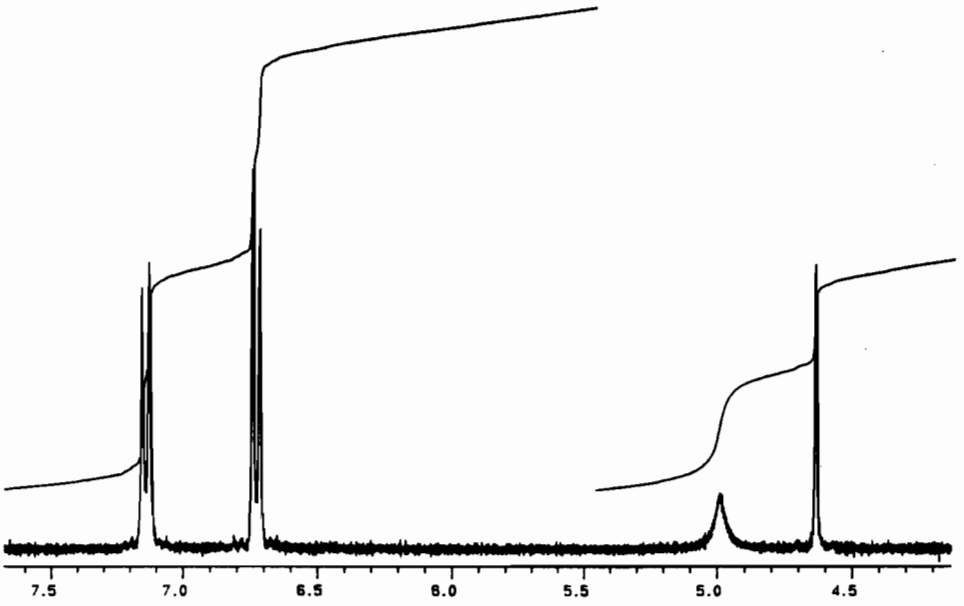

Fig. 8: ^1H NMR(300 MHz, D_2O) of metabolite 38.

^{13}C NMR (Fig. 9) in D_2O+DMSO revealed the presence of six types of carbons; at 74.4 ppm (probably a C-OH), two very intense signals at 117.3, 130.4 ppm, weak signals at 132.3 ppm, 157.5

ppm (phenolic carbon) and 177.5 ppm (most likely carboxylic acid). Distortionless Enhancement by Polarization Transfer (DEPT) NMR indicated that the carbon at 74.4 ppm had one proton attached to it.

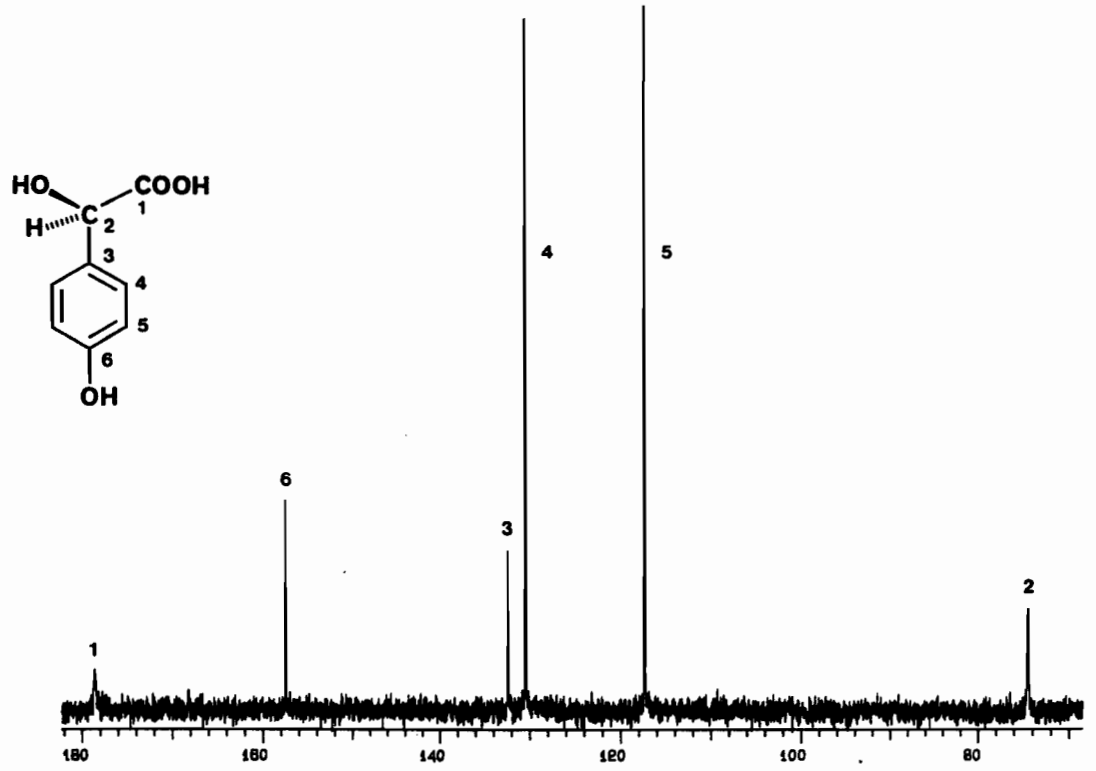

Fig. 9: ^{13}C NMR (300 MHz, D_2O+DMSO) of metabolite 38.

A satisfactory mass spectra could not be obtained either with CI (NH_3) or FAB mass spectrometry. ^{252}Cf-plasma desorption mass spectrometry,[122] however, gave a molecular ion of mass 168.1. The electrosprayed sample of compound **38** gave a positive ion at 213.2 $(M^{2-}+H^+ +2Na^+)^+$ and a negative ion at 167.1 $(M^{2-}+H^+)^-$. These results were further supported by the ions obtained from the ^{252}Cf-plasma desorption spectra of this sample adsorbed onto a tridodecylmethylammonium chloride (TDMAC) cationic surfactant; 167.1 $(M^{2-}+H^+)^-$, 869.9 $[(TDMA^+)(M^{2-}+H^+)^{2-}]^-$, 1776.6 $[(TDMA^+)_3M^{2-}]^+$ and 121.1 $(M^{2-}-HCO_2H)^-$.

Given the close similarities of spectral data between metabolites **37** and **38**, and the difference of only two mass units between their respective molecular ions, the structure of *p*-hydroxymandelic acid (**38**) was proposed. A commercial sample of D/L *p*-hydroxymandelic acid was used to compare spectral data. As expected all sets of data were identical. The natural product displayed a negative optical rotation, hence, its chirality remained to be determined.

2. 4 Determination of the absolute stereochemistry of natural product 38

2. 4. 1 Determination of optical purity using chiral solvating agents.

Metabolite 38, isolated from *P. tinctorius*, was found to exhibit a negative optical rotation ($[\alpha]_D$ = -2.7 °). To the best of our knowledge, literature information on the optical properties of *p*-hydroxymandelic acid is not available, hence, it was decided to attempt the determination of the absolute stereochemistry of this natural product using NMR techniques involving chiral shift reagents.

It was twenty years ago when Hinckley[123] first described the ability of a "shift reagent" to spread out the proton signals in the NMR of cholesterol. Since then, an abundant amount of work has been published on the applications of such compounds in facilitating structure determination. One of the most important contributions from this area of research is the development and utilization of chiral shift reagents which constitute probes for obtaining stereochemical information. Their application is based on their ability to induce shifts of different magnitudes on protons found in different stereochemical environments. Integration of the resolved signals provides a direct measure of enantiomeric ratios in a given sample.

Such a determination was first achieved for enantiomeric mixtures of chiral amines by Whitesides and Lewis[124] using tris[3-(*tert*-butylhydroxymethylene)-*d*-camphorato]europium(III). Numerous lanthanide metal shift reagents are presently available which can be used for the determination of enantiomeric purity.

Chiral solvating agents provide an alternate method for such measurements. Enantiomerically pure alcohols of the general structure 48, originally developed by Pirkle and coworkers, have been used for this purpose. They have been shown to permit not only the direct determination of the enantiomeric purity of a sample but often allow correlations to be made about absolute configuration.

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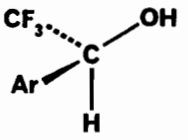
123. C.C. Hinckley, *J. Am. Chem. Soc.* 91, 5160 (1969).

124. G.M. Whitesides and D.M. Lewis, *J. Am. Chem. Soc.* 92, 6979 (1970).



(48) a) Ar= phenyl
b) Ar= 9-anthryl

(R)-(-)-2,2,2-trifluorophenylethanol (**48a**) was first used by Pirkle and Beare[125] for studying the optical purity of chiral sulfoxides. A year later a model was suggested which was capable of explaining the origin and sense of the NMR nonequivalence observed with methyl esters of amino acids.[126] Later, a more refined model was used to effectively determine the enantiomeric composition and absolute configuration of chiral oxaziridines. The formation of short-lived, chelate-like, diastereomeric complexes, of type **49** and **50**, was proposed which can cause nonequivalence of the substituents R_1 and R_2 due to the magnetic field contributions of the aromatic electron cloud of the shift reagent. The formation of such complexes requires the presence of two basic groups, B_1 and B_2, where B_1 is more basic than B_2. It is expected[127] that the more basic substituent will hydrogen bond preferentially to the hydroxyl proton of the solvating agent, where the less basic group will form a non-classical hydrogen bond with the acidic carbinyl hydrogen. The inductive effects of the adjacent electron withdrawing groups (trifluoromethyl, hydroxyl, 9-anthryl[128]) serve to render this hydrogen somewhat acidic so that it is able to participate in a dipole-dipole interaction.[129] In complex **49** the R_2 group is located over the region of the diamagnetic field of the aromatic ring, hence, it would be more shielded (upfield shift) than the R_2 in complex **50**. The converse is true for R_1 which would be shifted upfield in **50** but not in **49**.

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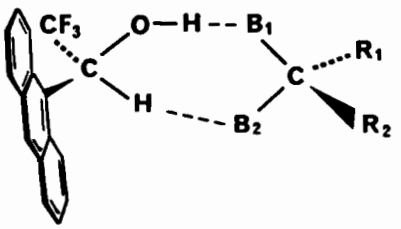
125. W.H. Pirkle and S.D. Beare, *J. Am. Chem. Soc.* **90**, 6250 (1968).

126. W.H. Pirkle and S.D. Beare, *J. Am. Chem. Soc.* **91**, 5150 (1969).

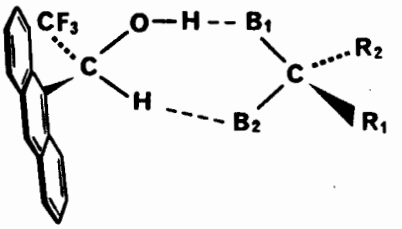
127. W.H. Pirkle and P.L. Rinaldi, *J. Org. Chem.* **42**, 3217 (1977).

128. Replacement of the phenyl group with 9-anthryl provides greater shielding ability leading to a larger degree of nonequivalence.

129. A. Allerhand and P.V.R. Schleyer, *J. Am. Chem Soc.* **85**, 1715 (1963).

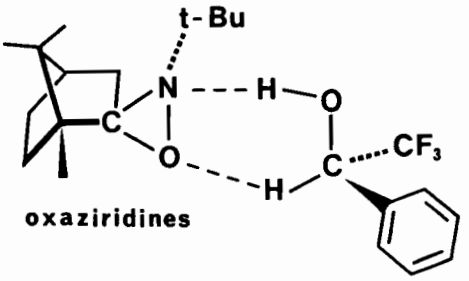


(49)



(50)

For example, in the case of the chiral oxaziridine **51**, the presence of (R)-(-)-2,2,2-trifluorophenylethanol (**48a**) causes opposite senses of nonequivalence for the N-*tert*-butyl (downfield) and the C-10 methyl (upfield) resonances as compared to its enantiomer. These affects are consistent with the above model and are attributed to the formation of a chelate complex between the two compounds[130] (Fig. 10).



(51) [(R)-48a]

Fig 10: Chelate complex of oxaziridine 51 and solvating agent (R)-48a.

130. W.H. Pirkle and P.L. Rinaldi, *J. Org. Chem.* 43, 4475 (1978).

Application of the same model to an ester analogue of natural product **38** would predict an upfield shift of the C-2 proton of the (S) enantiomer, but not of the (R), upon chelation with the (S) enantiomer of the solvating agent (Fig. 11).

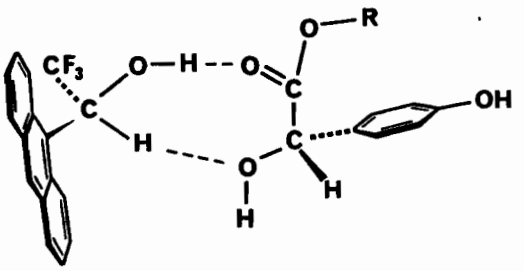


Fig. 11: Chelate complex of (S) p-hydroxymandelate ester and (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol.

Ideally, NMR experiments of this nature should be conducted in nonpolar solvents such as CCl_4 or C_6D_6. Any dipole-dipole interaction, other than those between the compound of interest and the shift reagent, would decrease the effectiveness of the latter. However, given the initial polarity of metabolite **38**, it was expected that neither of these two solvents would be suitable and $CDCl_3$[131] was used instead.

In order to obtain a chloroform-soluble analogue with the same stereochemistry, esterification of compound **38** was carried out.[132] The methyl ester **52**, along with the minor side-product **53**, was first synthesized in refluxing methanol with a catalytic amount of *p*-toluenesulfonic

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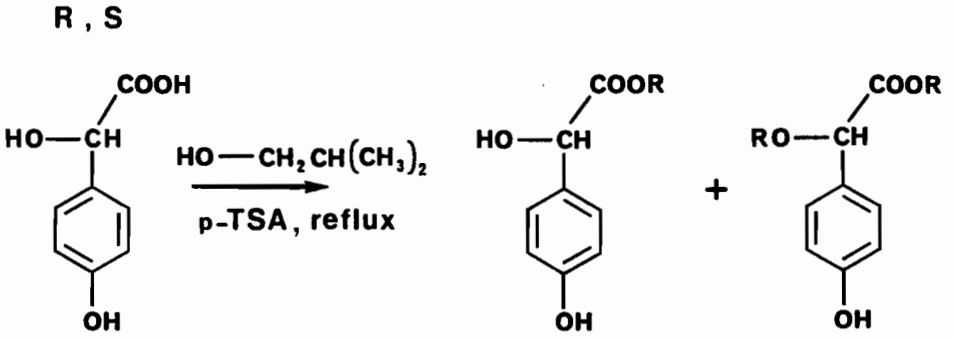
131. $CDCl_3$ is not an ideal solvent for these types of experiments, since it can itself hydrogen bond to the shift reagent. However, the moderate amounts of nonequivalence observed were sufficient for the purpose of this work.

132. A commercial sample was used (see experimental section).

acid (Fig. 12). Since **52** turned out to be practically insoluble in $CDCl_3$, the isobutyl ester (**54**) was prepared using the same procedure (Fig. 12). This reaction was much slower and produced an equivalent amount of the corresponding side product (**55**).



(52) R= CH_3 **(53)** R= CH_3

(54) R= $CH_2CH(CH_3)_2$ **(55)** R= $CH_2CH(CH_3)_2$

Fig. 12: Esterification of p-hydroxymandelic acid (38).

The ability of solvating agent (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol (**48b**) to induce a useful degree of nonequivalence between the two enantiomers of ester **54** was confirmed through the ^1H NMR (Fig.13a-c) of a racemic mixture[132] which showed two equally intense signals for the C-2 proton ($\Delta\delta$ =0.03 ppm), one for each enantiomer (Fig. 13a).[133] According to the model, the upfield signal corresponds to the (S) enantiomer and the downfield corresponds to the (R). Unexpectedly, nonequivalence was also observed with the -$COOCH_2$- protons but assignments could not be made with respect to the two enantiomers. It was speculated that deshielding of the -CH_2- protons of the (S) enantiomer could occur if these protons were placed close to the paramagnetic field of the 9-anthryl ring and almost on the same plane. Molecular modeling calculations were carried out in order to gain relevant information regarding the conformation of each molecule in the chelate complex.

133. Coupling between the C-2 H and the C-2 OH was not always observed due to the hygroscopic nature of the compound and the presence of a phenol.

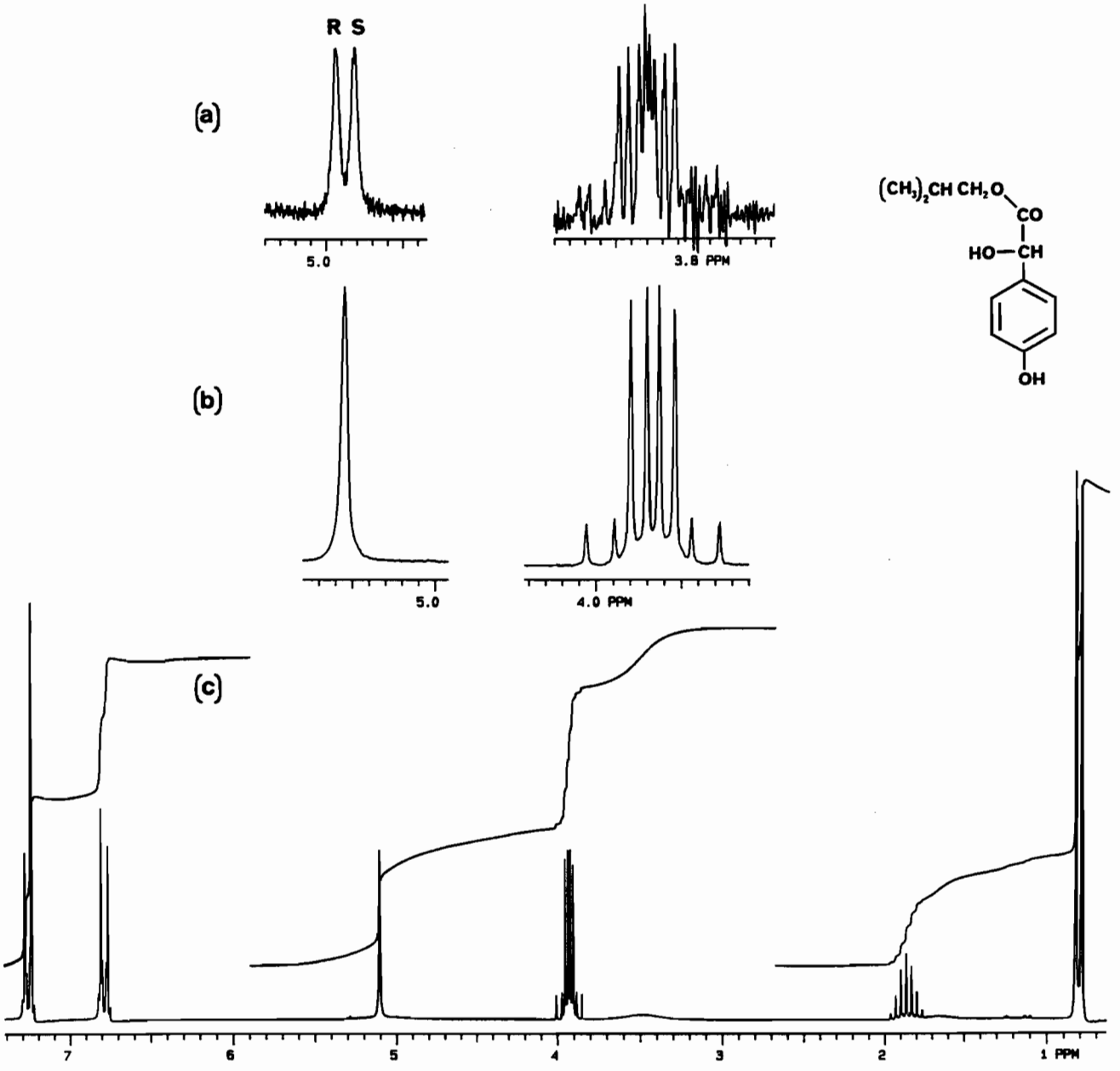


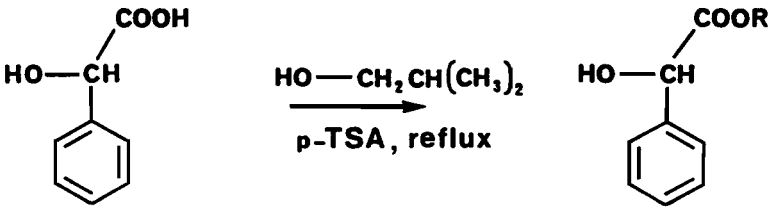
Fig. 13: ^{1}H NMR (200 MHz, $CDCl_3$) of racemic compound 54.

Expanded spectrum of C-2 H and -$COOCH_2$- region: a) 54 plus (S)-48b, b) pure 54.

c) Complete spectrum of pure 54.

Before proceeding, however, it was felt necessary to verify the applicability of Pirkle's model to compounds which are structurally related to metabolite **38** and which have known absolute stereochemistry. Hence, the closely related natural product mandelic acid (**56**) was chosen as a model compound. It was postulated that any observed ^1H NMR differences between optically pure (R) and (S) mandelic acid, in the presence of the solvating agent **48b**, should be analogous to those expected for compound **38**.

Commercially available samples of optically pure (R) and (S) mandelic acid [**56a** (R), **56b** (S))] were converted to their respective isobutyl esters (**57a, 57b**). The reaction was carried out in refluxing isobutyl alcohol with a catalytic amount of *p*-toluenesulfonic acid. The (R) and (S) products (**57a, 57b**) were formed cleanly and quantitatively (Fig. 14).



56a (R), 56b (S) **57a (R), 57b (S)**

Fig. 14: Esterification of (R) and (S) mandelic acid.

The ^1H NMR of the pure esters **57** (**a** or **b**) in $CDCl_3$ (Fig. 15b) showed a doublet of doublets for the two methyls at 0.80 ppm, a multiplet at 1.86 ppm for the C-8 H, a doublet at 3.53 ppm for the C-2 OH, a multiplet at 3.93 ppm for the C-2 H and a multiplet between 7.32 and 7.44 ppm for the aromatic protons. Figure 15a is an expansion of the spectral region which was predicted to change in the presence of the solvating agent.

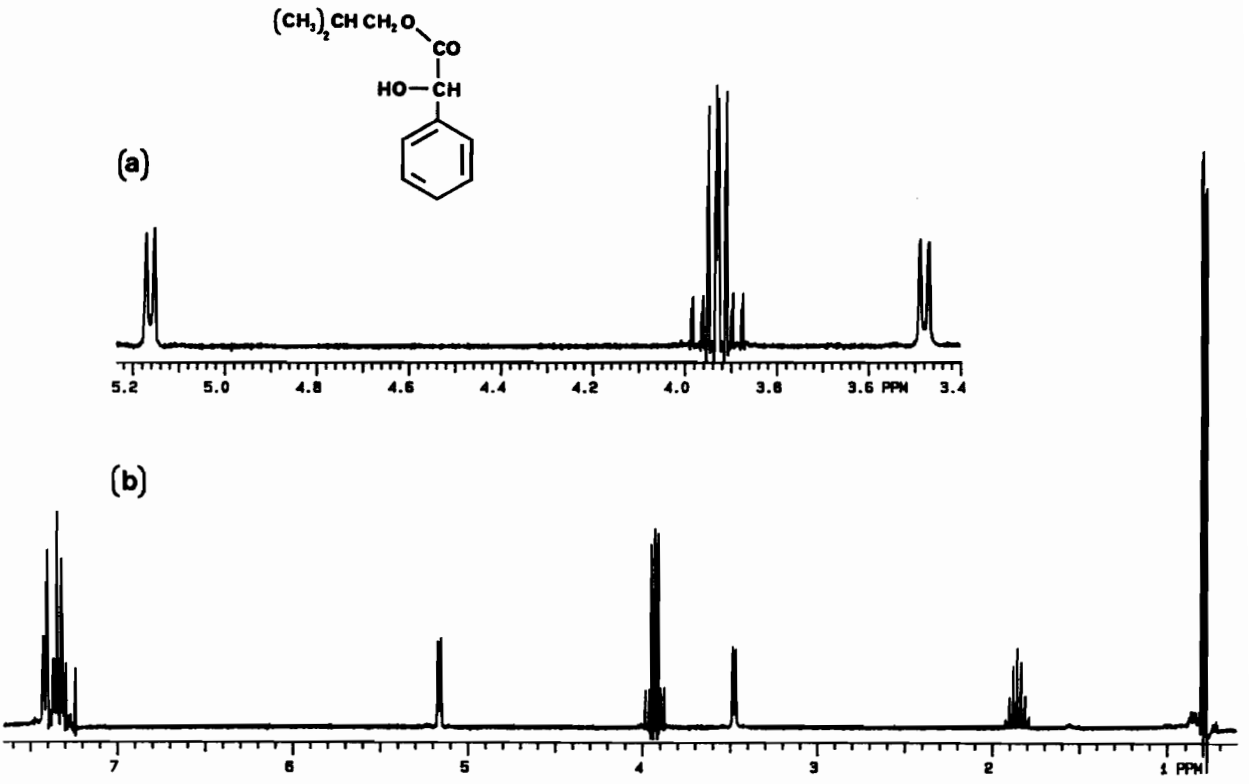


Fig. 15:^{1}H NMR (200 MHz, $CDCl_3$) of compound 57
a) Expansion of relevant region, b) Complete spectra.

To a known enantiomeric mixture of compound **57** (R/S=3.7, 100mg sample), (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol (**48b**) was added in portions of 0.2eq (20 mg). Fast proton exchange between the shift reagent and compound **57** was suggested by the disappearance of coupling between C-2 H and C-2 OH at low concentrations of shift reagent (Fig. 16, b *vs* c). At ratios of 1:1, two C-2 H singlet peaks were observable with the lower intensity signal upfield, corresponding to the (S) enantiomer as expected (Fig. 16a). Similarly, the C-7 protons of the (S) enantiomer appeared downfield from those of the (R) (Fig. 16a).

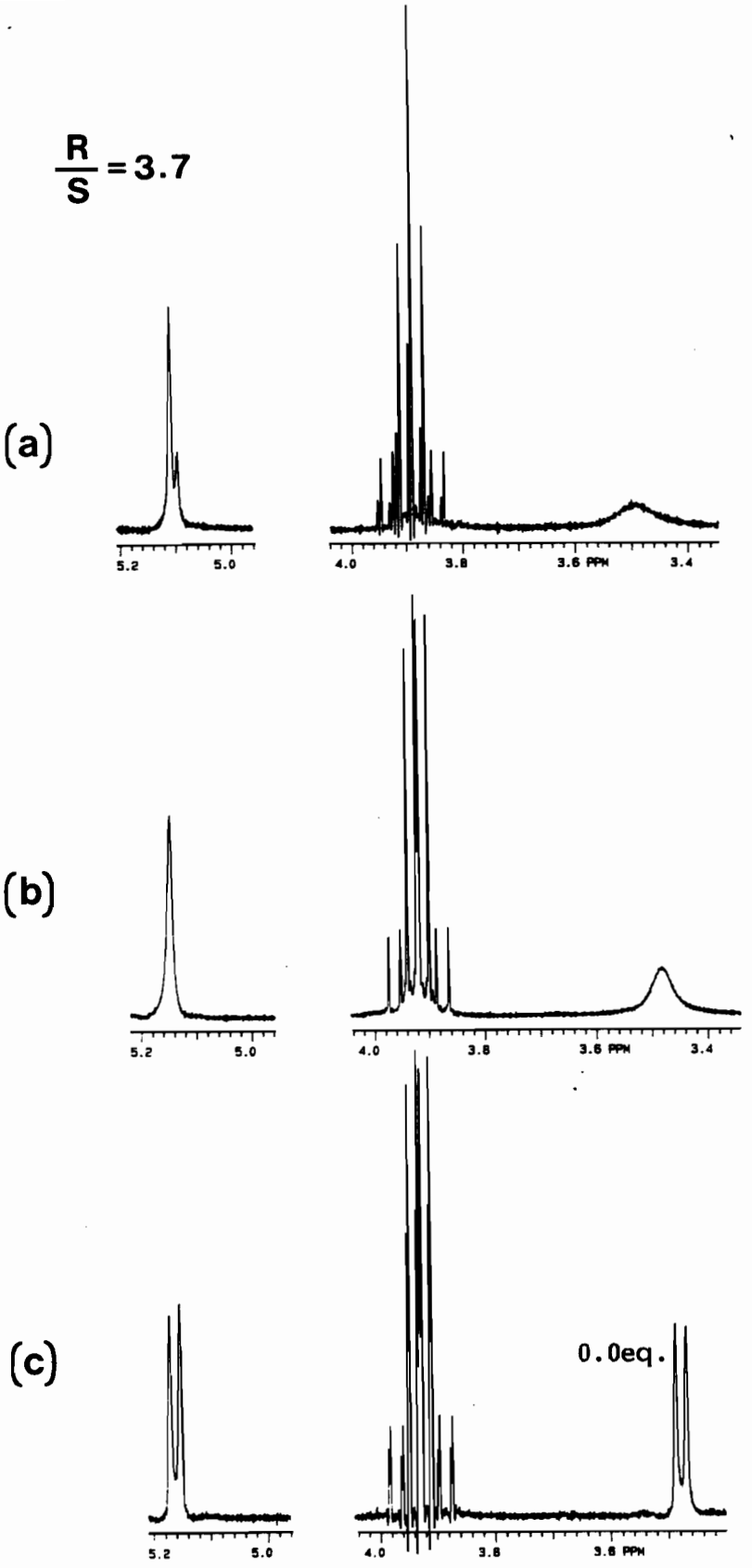


Fig. 16: ^{1}H NMR (300 MHz, $CDCl_3$) of compound 57 with (S)-48b; a) ~1 eq. of (S)-48b, b) ~0.2 eq. of (S)-48b, c) pure compound 57.

Although nonequivalence between the two enantiomers was observed at equimolar ratio, optimum results were achieved at excess concentrations of solvating agent (1.5-2eq). Under these conditions, coupling between the C-2 H and the C-2 OH was once again evident (Fig. 17), indicating maximum chelation through hydrogen bonding. These results are consistent with those observed by Pirkle, who noted that maximum nonequivalence was obtained at a 2-3 fold excess of solvating agent to the compound of interest.[134] Although the induced shift difference was only 0.014 ppm the integration ratio of the two non overlapping peaks of each doublet (peaks **R** and **S** in Fig. 17) was very close to the actual ratio of 3.7:1.

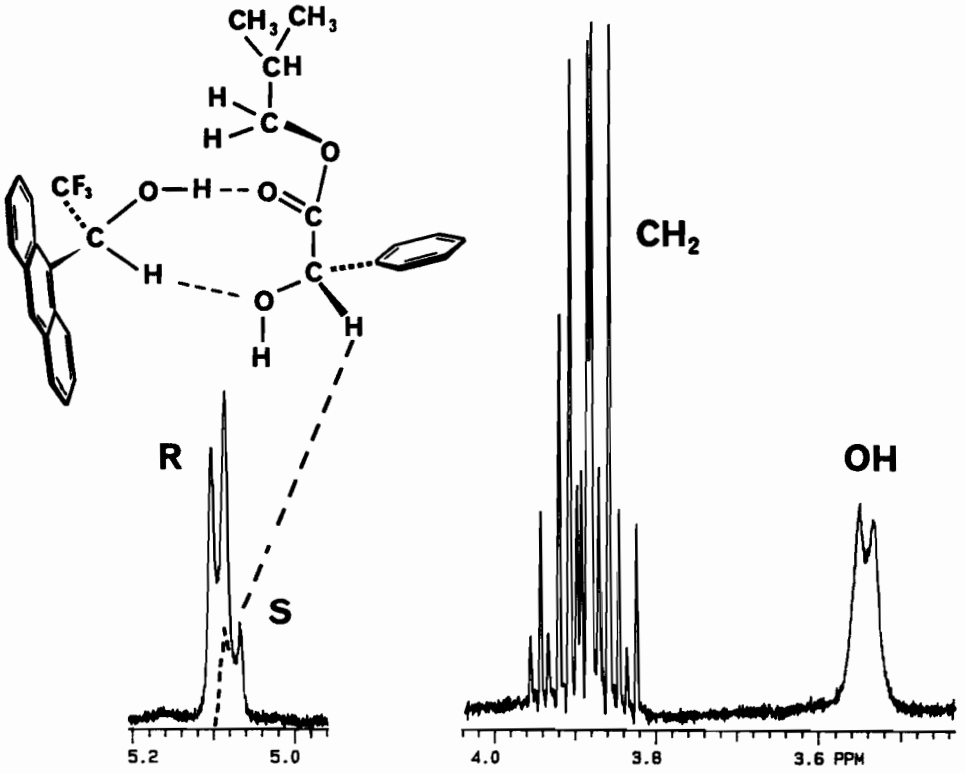
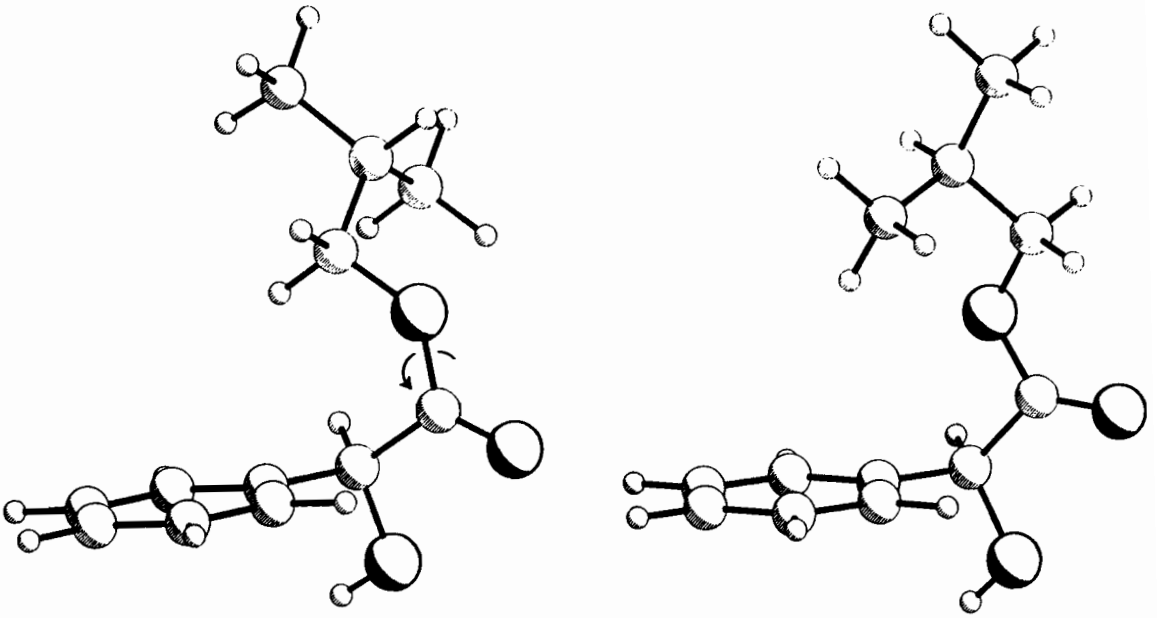


Fig. 17: ^{1}H NMR (300 MHz ,$CDCl_3$) of the chelate-complex formed between compounds 57 and (S)-48b.

134. W.H. Pirkle, S.D. Beare and R.L. Muntz, *Tetrahedron Lett.*, 2295 (1974).

Minimization of the conformational energies of compound **57**, using PCMODEL *via* MMX molecular mechanics,[135] indicated that the cis ester (**57-II**, C-7 cis to the carbonyl) was ~8 Kcal lower in energy that the trans (**57-I**). The solvating agent (S)-**48b** was first minimized separately [(S)-**48b-III**] and then two molecules were minimized as a hydrogen-bonded complex [**57**--(S)-**48b-IV**]. Although "hydrogen bonding" between the α-hydroxyl of **57** and the carbinyl hydrogen of the solvating agent was not recognized by the program, the two molecules were still placed close enough to give a distance of 3.97 Å between the C-7 and the aromatic protons. Theoretical calculations[136] predicted that at such a distance, a downfield shift of about 0.2 ppm should be observed for the C-7 protons of the (S) enantiomer, which was consistent with the shift observed in Figure 17.



17.08 Kcal

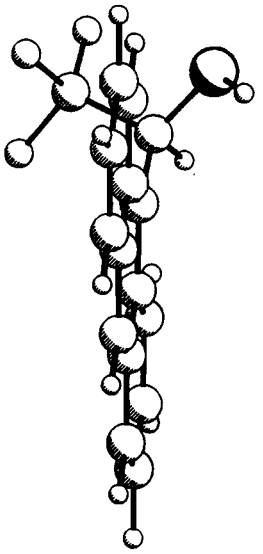
9.1 Kcal

(57-I)

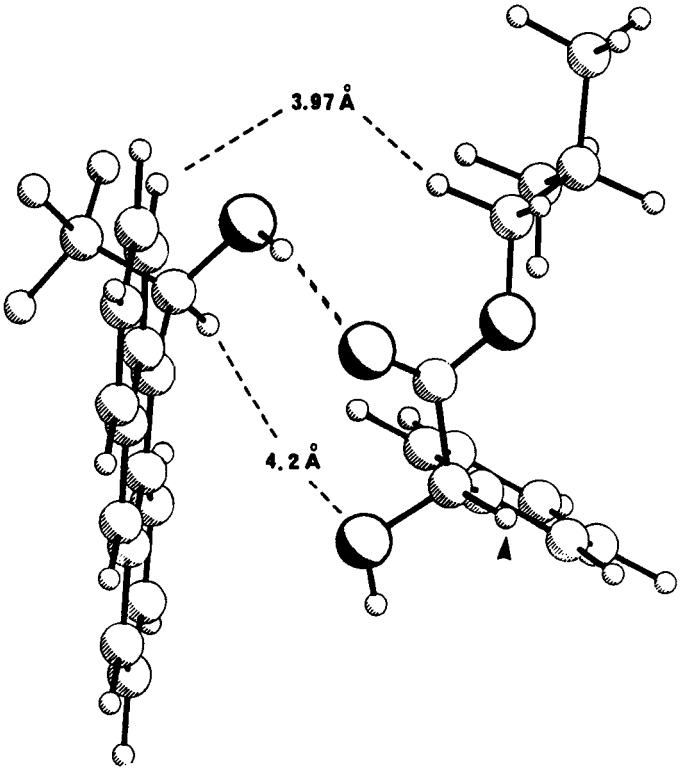
(57-II)

135. Dr. K. Steliou (Univ. of Montreal) and Warren Chew are gratefully acknowledged for their advice on the use of PCMODEL. This program was obtained from Serena Software, P.O. Box 3076, Bloomington, Indiana, USA 47402-3076.

136. C.E. Johnson, Jr. and F.A. Bovey, *J. Chem. Phys.* **29**, 1012 (1958).



[(S)-48b-III]



[57--(S)-48b-IV]

Since the experimental data for mandelic acid was in good agreement with the predictions made using Pirkle's model it was decided to apply the same theory for the determination of the absolute configuration of metabolite **38**. The amount of available natural product, however, was extremely small (5-10mg) and in an effort to esterify 6 mg of it, only product **55** was obtained (100% yield). The formation of compound **55** involves a carbonium ion intermediate at C-2 which would cause total racemization. It is believed that this unsucessful and disasterous result was caused by the presence of excess amounts of *p*-toluenesulfonic acid; determination of what would constitute an effective "catalytic amount" of the acid for 6 mg of the substrate was difficult. The synthesis of optically pure *p*-hydroxymandelic acid was then attempted with the intention to: a) determine the absolute stereochemistry of the natural product and b) obtain sufficient amounts of each enantiomer in order to carry out a variety of biological tests.

2. 4. 2 Synthesis of (R)- and (S)-p-hydroxymandelic acid

The simplest way to obtain *p*-hydroxymandelic acid (**38**) in high enantiomeric purity would have been *via* a stereospecific reduction of the easily obtainable α-keto acid or its ester derivative. The sodium salt of the keto acid **37** is commercially available and its protonated form can be easily obtained through chromatography on an H^+ ion-exchange column.

2. 4. 2a Enzymatic reduction of α-keto acids to α-hydroxy acids

In the last few years, highly stereospecific reduction reactions have been successfully carried out using enzymes as catalysts. These types of enzymes, collectively referred to as dehydrogenases, catalyze the reversible conversion of carbonyl compounds to alcohols of known stereochemistry. L-Lactate dehydrogenase is one such enzyme which in the presence of the coenzyme NADH catalyzes the reduction of pyruvate to L-lactate *in vivo*, with absolute enantiospecificity for the (S)-enantiomer[137] (Fig. 18). An extensive survey by Kim and Whitesides[138] has revealed, however, that

137. J.J. Holbrook, A. Liljas, S.J. Steindal, M.G. Rossmann, In *The Enzymes*, 3rd ed., Ed. P.D. Boyer, Academic, New York (1975), and references therein.

138. M.-J. Kim and G.M. Whitesides, *J. Am. Chem. Soc.* **110**, 2959 (1988).

although this enzyme possesses a broad substrate specificity and high enantioselectivity for the production of the (S)-enantiomer, it could not catalyze the reduction of benzoylformic acid to mandelic acid (R = C_6H_5).

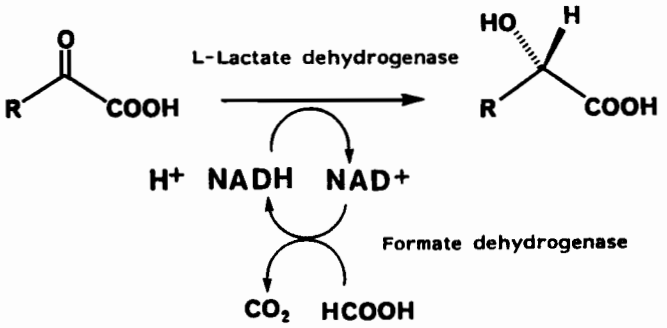


Fig. 18: L-Lactate dehydrogenase-catalyzed synthesis of (S)-2-hydroxy acids.

Another dehydrogenase, which is commercially available and known to have broad specificity, is the enzyme alcohol dehydrogenase.[139] This enzyme, usually extracted from yeast or horse liver, reduces a wide range of aliphatic and aromatic carbonyl compounds (e.g. benzaldehyde) using NADH and Zn^{2+} ions. Attempts to reduce either the sodium salt of *p*-hydroxybenzoylformic acid or its methyl ester using alcohol dehydrogenase were unsuccessful, hence, the enzymatic approach was dismissed and a chemical, stereospecific reduction of the keto acid to the alcohol was pursued. However, in a very recent publication[140] yeast lipase was shown to catalyze the stereospecific hydrolysis of racemic methyl or butyl O-acetylmandelate esters to their respective (R)-α-hydroxy esters. Although the reaction times reported were very long (7-23 days) this approach may have been useful to our project.

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139. a) E. Horjales and C.-I. Brandén, *J. Biol. Chem.* **260**, 15445 (1985).
b) J.B. Jones and J.F. Beck, in *Applications of Biochemical Systems in Organic Chemistry*, Eds. J.B. Jones, C.J. Sih and D. Perlman, John Wiley and Sons, New York, part 1, 107 (1976).

140. H.S. Bevinakatti, A.A. Banerji and R.V. Newadkar, *J. Org. Chem.* **54**, 2453 (1989).

2. 4. 2b Chemical stereospecific reduction of isobutyl keto ester (58) to the (R) or (S) α-hydroxy acid (54)

A wide variety of reagents have been developed for the stereospecific reduction of prochiral carbonyl compounds.[141] The majority among them utilize metal hydride reagents bearing chiral natural products or natural product derivatives.[142] The limitation with most of these reagents is that, although they can be used for the formation of a chiral compound in high enantiomeric excess, the same reagent can not also be used to obtain the other enantiomer. Reagents which have been specifically used for the reduction of α-keto esters to chiral alcohols include rhodium(I) complexes with chiral phosphine ligands *via* hydrosilation,[143] model compounds of the dehydrogenase coenzyme NAD(P)-H[144] and chiral borohydrite agents.[145] In addition, since chiral α-hydroxy alcohols are important synthons for a large number of natural products other synthetic methods are also available.[146]

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141. a) J.D. Morrison and H.S. Mosher, in *Asymmetric Organic Reactions*, Prentice-Hall, Englewood-Cliffs, N.J., 160 (1971).
b) D. Valentine Jr. and J.W. Scott, *Synthesis*, 329 (1978).
c) J.W. ApSimon and R.P. Seguin *Tetrahedron* **35**, 2797 (1979).

142. a) S.R. Landor, B.J. Miller and A.R. Tatchell, *J. Chem. Soc. C*, 1822 (1966); 197 (1967).
b) S. Yamaguchi and H.S. Mosher, *J. Org. Chem.* **38**, 1870 (1973).
c) J.P. Vigneron and I. Jacquet, *Tetrahedron* **32**, 939 (1976).
d) S. Terashima, N. Tanno and K. Koga, *Chem. Lett.*, 981 (1980).
e) A.I. Meyer and P.M. Kendall, *Tetrahedron Lett.*, 1337 (1974).
f) S. Yamaguchi, F. Yasuhara and K. Kabuto, *J. Org. Chem.* **42**, 1578 (1977).
g) E.D. Lund and P.E. Shaw, *Ibid* **42**, 2073 (1977).
h) T.H. Johnson and G. Rangarajan, *Ibid* **44**, 3966 (1979).
i) T. Mukaiyama, M. Asami, J. Hanna and S. Kobayashi, *Chem. Lett.*, 783 (1977).
j) M. Asami and T. Mukaiyama, *Heterocycles* **12**, 499 (1979).
k) N. Cohen, R.J. Lopresti, C. Neukom and G. Saucy, *J. Org. Chem.* **45**, 582 (1980).
l) H. Suda, S. Kanoh, N. Umeda, T. Nakajo and M. Motoi, *Tetrahedron Lett.* **24**, 1513 (1983).
m) T. Sato, Y. Gotoh, Y. Wakabayashi and T. Fujisawa, *Ibid* **24**, 4123 (1983).

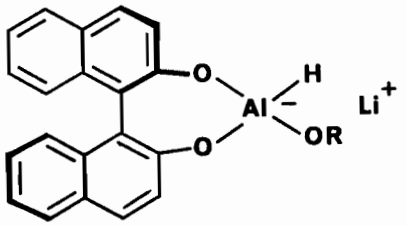
143. a) I. Ojima and T. Kogure, *Tetrahedron Lett.*, 1889 (1974).
b) I. Ojima, T. Kogure and M. Kumagai, *J. Org. Chem.* **42**, 1671 (1977).

144. A. Ohno, M. Ikeguchi, T. Kimura and S. Oka, *J. Am. Chem. Soc.* **101**, 7036 (1979) and references therein.

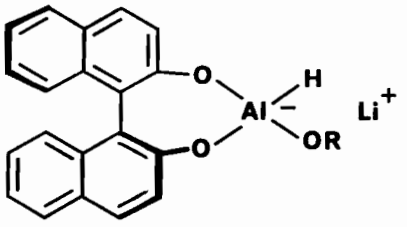
145. a) H.C. Brown, W.S. Park and B.T. Cho, *J. Org. Chem.* **51**, 1934 (1986).
b) H.C. Brown, B.T. Cho and W.S. Park, *J. Org. Chem.* **51**, 3396 (1986).
c) H.C. Brown and G.G. Pai, *J. Org. Chem.* **50**, 1384 (1985) and references therein.

146. a) D.A. Evans, M.M. Morrissey and R.L. Dorow, *J. Am. Chem. Soc.* **107**, 4346 (1985).

Recently Noyori[147] and coworkers designed a new enantioselective reducing agent by attaching optically active binaphthol to lithium aluminum hydride. The methodology developed by these researchers permits the conversion of a prochiral carbonyl compound to either its (R) or (S) enantiomer depending on the chirality of the binaphthol auxiliary attached to the aluminum. The high degree of stereoselectivity observed is attributed not only to the presence of chiral binaphthol but to the minimization of reactive species to only one; i.e. only one hydride equivalent per mole of reagent. The preparation of the (R)- or (S)-2,2'-dihydroxy-1,1'-binaphthyl lithium aluminum hydride reagent (**BINAL-H, 59, a** and **b**) involves the titration of lithium aluminum hydride with one equivalent of alcohol (methanol or ethanol) followed by one equivalent of optically pure binaphthol.



(59a)



(59b)

Noyori proposed[147] that the reduction mechanism proceeds *via* a six-membered ring transition state in the chair conformation. This permits the prediction of the product's chirality by taking into consideration the steric and electronic effects which would give the most favorable energy for that particular transition state (Fig. 19). For example, reduction of the aromatic ketones **60 (a-c)**, with the **(S)-BINAL-H (59b)** would proceed *via* transition state **61** to give the (S) enantiomer of alcohol **62** (Fig. 19). In the transition state the aromatic substituent is placed at the equatorial

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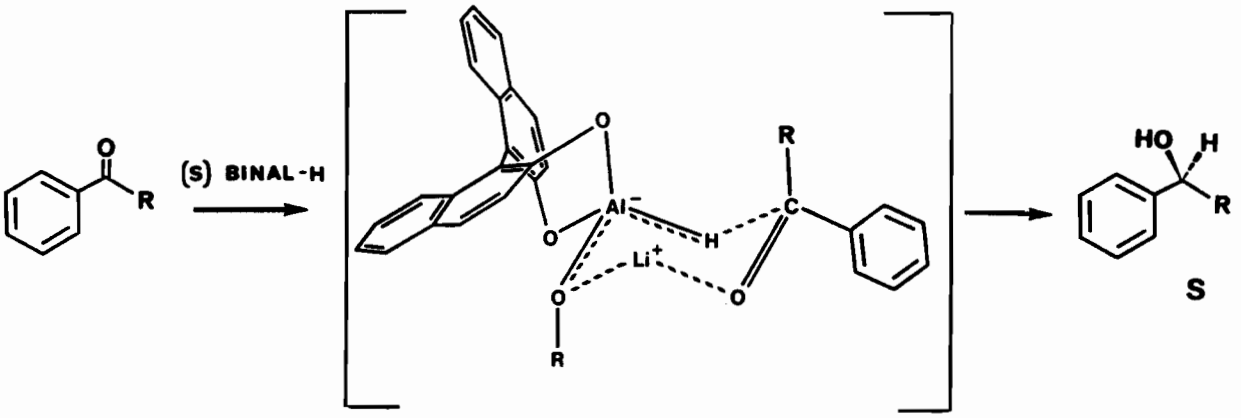
b) W.H. Pearson and M.-C. Cheng, *J. Org. Chem.* **51**, 3746 (1986).
c) J.K. Whitesell, R.M. Lawrence and H.-H. Chen, *J. Org. Chem.* **51**, 4779 (1986), and references therein.

147. a) R. Noyori, Y. Tanimoto and M. Nishizawa, *J. Am. Chem. Soc.* **106**, 6709 (1984).
b) R. Noyori, I. Tomino, M. Yamada and M. Nishizawa, *J. Am. Chem. Soc.* 106, 6717 (1984).

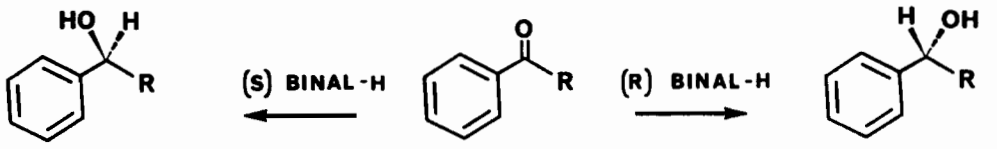
position in order to avoid electronic repulsion between the aromatic ring and the lone pair of electrons of the binapthol oxygen. However, the 1,3-diaxial steric repulsion becomes increasingly significant as the bulkiness of the R substituent increases, which leads to both low reactivity and low enantioselectivity (Tab. 4).



(60) (61) (62)

Fig. 19: (S)-BINAL-H reduction of acetophenone 60.

Table 4: BINAL-H stereospecific reduction of aromatic ketons.



[62 (S)] (60) [62 (R)]

R=	% ee	Reaction conditions:
$-CH_2CH_2CH_3$	~100	-100°C for 3h and -78°C for 16h.
$-CH(CH_3)_2$	71	-100°C for 3h and -78°C for 16h.
$-C(CH_3)_3$	44	start at -100°C, gradually warm-up to 23°C over a period of 10h.

The BINAL-H reagent has been shown to reduce a large number of aromatic, acetylenic and olefinic ketones with excellent stereoselectivity. In addition, potentially labile substrates, containing a lactone and/or ester[148] as part of their structure, have been reduced sucessfully using this reagent (Fig. 20).

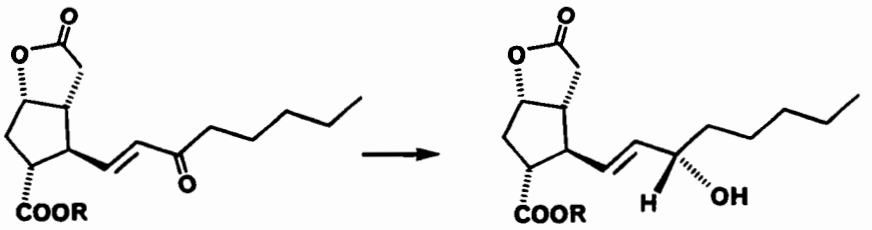


Fig. 20: BINAL-H asymmetric reduction of the ω-enone PG side chain to the 15 (S) allylic alcohol.

148. E.J. Corey, N.M. Weishenker, T.K. Schaaf and W. Huber, *J. Am. Chem. Soc.* 91, 5675 (1969).

Quantitative yields of the isobutyl *p*-hydroxybenzoylformate ester (**58**) were obtained from the free acid **37** when refluxed, overnight, in isobutyl alcohol with catalytic amounts of *p*-toluenesulfonic acid. The esterification reaction was also attempted using the commercialy available sodium salt of **37** as the starting material. In such cases the yields of the desired product were very low due to a competing decarboxylation reaction leading to the formation of *p*-hydroxybenzaldehyde (**63**).

The (S)-BINAL-H reagent[149] was then prepared following the Noyori procedure,[147] and it was used in 3 fold excess to reduce the isobutyl *p*-hydroxybenzoylformate ester (**58**) to the (R) enantiomer of alcohol **54** at -100°C and -78°C. In both cases, after reaction periods of 3.5 and 2 hours respectively, an insignificant amount of product was detected and the unreacted starting material was recovered. Noyori reported that the 3 fold excess of **BINAL-H** reagent is critical for the completion of these types of reactions in reasonable rates. Hence, taking into consideration that the phenolic proton will immediatly be extracted by one equivalent of hydride,[150] a four-fold equivalence of reducing agent to compound **58** was used subsequently. When the reaction was carried out with 4 equivalence of (S)-BINAL-H reagent at -78°C for 2h, the (R) enantiomer of compound **54** (**54a**) was formed at approximately 35% ee. In addition, the primary alcohol **64** was formed as a major side product (Tab. 5); the isolated yields after column chromatography were 7% of **54**, 29% of **64** and ~50% starting material.

The chirality of product **54a**, obtained from the above reaction, was investigated through its ^{1}H NMR in the presence of the chiral alcohol (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol (**48b**). The C-2 H and C-7 CH_2 proton shifts for pure compound **54**, its racemic mixture in the presence of the solvating agent **48b** and the product obtained from the **BINAL-H** reduction in the presence of **48b** were compaired. The (R) enantiomer of alcohol **54** was evidently formed in approximately 35% enantiomeric excess. Although a low degree of stereospecificity was obtained, the overall results were consistent with the proposed mechanism. It was speculated that a decrease of the %ee value could be caused either by the bulkiness of the isobutyl ester group, which would decrease the stability of the expected transition state **61**, or racemization of the chiral center C-2 in the presence of base. It

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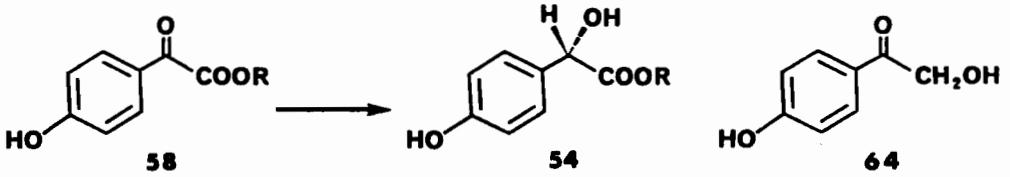
149. The optically pure binaphthol used was a generous gift of Dr. R.J. Kazlauskas; R.J. Kazlauskas, *J. Am. Chem. Soc.* **111**, 4953 (1989).

150. The ionization of the phenol to the phenoxide ion was evident immediatly upon addition of the substrate to the reagent by the change in colour of the reaction mixture from white to intense yellow.

should be noted that strongly basic conditions result upon quenching of the excess hydride at the end of the reaction. This could cause not only racemization but low chemical yields due to possible hydrolysis of the ester. This possibility was investigated by synthesizing the (S) enantiomer (**54b**), using the (R)-BINAL-H reagent, and quenching the reaction mixture with approximately 3 equivalents of glacial acetic acid. The pH of the reaction mixture was adjusted to about 6.5 before the temperature was allowed to rise above 0°C. After column chromatography the (S) enantiomer of compound **54** was isolated in 15% chemical yield with a 60% ee, as estimated once again by ^{1}H NMR in the presence of chiral solvating agent (S)-**48b** (Fig. 21a). Although it is likely that the reaction conditions could still be improved so as to allow complete conversion of starting material to products and better stereocontrol, the information thus far obtained was sufficient for the purpose of this project. The determination of the absolute configuration of natural product **38** could now be assigned once both esters **54a** and **54b** were hydrolyzed and their optical rotation measured.

Table 5: BINAL-H reduction of compound 58



TEMP	RXN TIME	BINAL-H	YIELDS	
-100°C	3.5 h[a]	3 eq. (S)	---	---
-78°C	2.0 h[a]	3 eq. (S)	<5%	---
-78°C	2.0 h[a]	4 eq. (S)	7% (R/S=2)	29%
-78°C	3.5 h[b]	4 eq. (R)	15% (S/R=4)	10%

[a]Reaction quenched with CH_3OH/H_2O, [b]Reaction quenched with ~3eq. of CH_3COOH

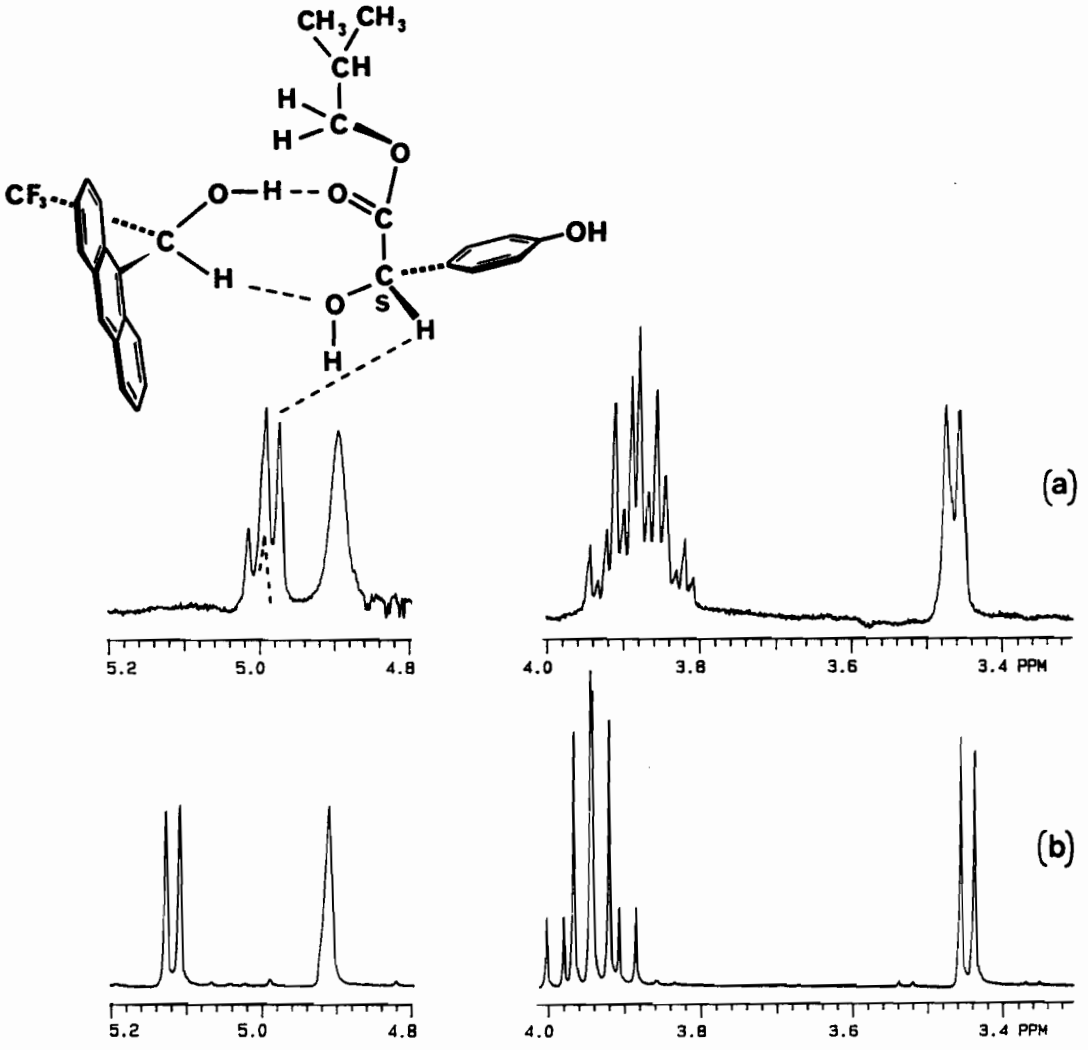


Fig. 21: ^{1}H NMR (300MHz, $CDCl_3$) of compound 54b; a) with solvating agent (S)-48b, b) pure.

Ester hydrolysis was carried out with 2 equivalents of hydroxide base in dioxane. The reaction was almost complete after a period of 12 hours at room temperature without racemization. This was indicated by the fact that deuterium exchange was not observed in the ^1H NMR of the carboxylic acid obtained when the reaction was carried out in a solution of 50% dioxane-50% D_2O . Measurements of the optical rotation of natural product **38** along with both enantiomers of *p*-hydroxylmandelic acid (Tab. 6) strongly suggested that metabolite **38** has the (R) absolute stereochemistry. However, the [α] values obtained were not consistent. This was most likely caused by the autocatalysed racemization of the compound upon standing in solution at room temperature (Tab. 6).

Table 6: Optical rotation properties of p-hydroxymandelic acid

Compound	[α]
natural **38**	-2.7°
synthetic **38a***	-10.2° (decreased to -6.1° after 24h in aqueous solution at 23°C)
synthetic **38b***	+26.3°

*Reaction products from table 5.

2. 5 The antifungal antibiotic properties of metabolites 37 and 38, and some of their structural analogues.

Ectomycorrhizal Basidiomycete[151] fungi are known to provide protection to forest trees against disease-causing microorganisms. Mycelial ensheathed roots of host plants are characteristic of their symbiotic association and are believed to act as a physical barrier against infectious pathogens. However, the marked resistance of trees to aggressive pathogens, often noted, has been shown to involve the production of extracellular metabolites having antibiotic activity. In 1969 Marx[110a] demonstrated the *in vitro* presence of a chemical antibiotic barrier formed by the ectomycorrhizal fungus *Leucopaxillus cerealis*. He demonstrated antibiotic activity in root tissues quite distant from sites of ectomycorrhizal colonization (ensheathed roots). In a review article he then later reported[152] the production of antibiotics, *in vitro*, by Basidiomycetes in over 100 mycorrhizal fungi.

Although, the use of ectomycorrhizal fungi in the protection of roots has since been extensively investigated, few metabolites with antibiotic activity have been isolated and characterized. The compounds chloromycorrhizin A (**65**) and mycorrhizin A (**66**) are two such compounds. These metabolites were isolated from the fungus *Monotropa hypopitys*[153] and shown to have strong activity against the root rotting fungus *Fomes annosus*; a microorganism responsible for large economic losses in Swedish forestry.[153]

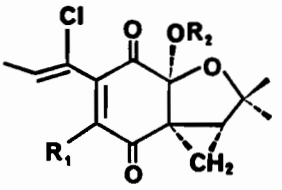
151. Type of fungi which possesses hyphae having cross walls. Reproduce sexually by making spores known as **Basidiospores**, borne externally on a club-shaped or cylindrical cell. In some members the **basidia** are grouped together in a highly-organized fruit-body, e.g. mushroom or puff-ball.

152. D.H. Marx, *Ectomycol.*, 351 (1973).

153. J. Trofast and B. Wickberg, *Tetrahedron* 33, 875 (1977).

(65) R_1= Cl, R_2= H

(66) R_1= R_2= H



The beneficial effects of *Pisolithus tinctorius* to forest trees has been attributed mostly to its production of phytohormones and its ability to supply nutrients to its host *via* its large network of mycelia. Its antifungal properties were discovered only recently by Kope and Fortin[98] who showed that sterile, cell-free extracts of this fungus can cause bursting of the hyphal tips of several phytopathogenic and dermatopathogenic fungi. In a collaborative effort, the two antifungal antibiotics *p*-hydroxybenzoylformic acid (37) and (R)-(-)-*p*-hydroxymandelic acid (38) were isolated from the liquid culture of *P. tinctirius* and their biological activity was investigated.

Through the course of isolation and structure determination of the active compounds, hyphal lysis and inhibition of spore germination was followed using the phytopathogenic fungus *Truncatella hartigii* as the test organism. This microorganism was chosen for its relatively high sensitivity to the antibiotics and its ease of handling.

Plugs (5 mm^2) of actively growing colonies of *Truncatella hartigii*, cultured on Potato Dextrose Agar (PDA), were exsized and placed into wells of a MULTIWELL tissue culture plate to which 300 μL of Potato Dextrose Broth (PDB) at 50% dilution was added. The cultures were incubated at 25°C and after 24 hours were inspected for good growth. The PDB was then removed and a sterile water solution (300 μL) of the test sample was added. Overall growth, based on the final mycelial dry mass and hyphal morphology, was assessed after an incubation period of 7 days. Crude extracts which caused mycelial growth to be significantly reduced or completely arrested, in addition to lyses of the newly formed hyphi, were further purified. All tests were done in multiple runs of 10 for concentrations ranging from 250 μg/mL to 8 μg/mL (6 dilutions). Bioassays on spore-germination inhibition were carried out in runs of 10 samples with 10 conidia each (100 conidia total) and the average number of germinated conidia was compared to that of the control. The controls were also run in 10 conidia per 10 samples; since the biology of germination is not absolute, variations

in the control were taken into consideration. In a typical control the average number of germinated conidia was 9.3-9.5 (i.e. 93%-95%). Growth Inhibition Concentration 50 (GIC 50) was defined to be the minimum concentration at which the average number of germinated conidia in a test solution was **less than half** of the average of the control (i.e. less than ~4.5-4.7) after an incubation period of 5-7 days.

Once the active metabolites had been completely purified and characterized, they were tested against a large number of plant and animal pathogens for their ability to inhibit growth. Some representative examples are given in table 7 along with the concentrations at which less than 50% growth (as compare to the control) and changed hyphal morphology was observed. Especially noteworthy are the plant pathogens *Rhizoctonia solani* and members of the species *Pythium*, *Phytophthora, and Fusarium*. These fungi are responsible for large economic losses to nurseries as they cause dampingoff diseases (rotting of seedlings) which represents the most important diseases of tree seedlings. In addition the fungi of the species *Microsporum* and *Trichophyton* are known for their ability to infect animals and sometimes humans, causing dermatitis.

Table 7. Growth Inhibition Concentration (GIC 50) of metabolite 37 for mycelial growth.

Phytopathogenic fungi	**GIC 50* (μg/mL)**
Phytophora sp.	50
Rhizoctonia solani	50
Fusarium solani	250
Pythium debaryanum	50
Pythium ultimum	50
Verticillium dahliae	50
Pyrenochaeta terrestris	50
Cochliobolus sativus	500
Septoria musiva	250
Brunchorstia pinea	125
Dermatopathogenic fungi	
Microsporum gypseum	50
Trichophyton equinum	50

* Changed hyphal morphology was also observed at these concentrations.

Biological testing of both metabolites **37** and **38** against *Truncatella hartigii* showed hyphal lysis at concentrations of 125 μg/mL within 4 hours, however, even at 62 μg/mL some lysis was observed. In spore germination bioassays the GIC 50 value was set at 62 μg/mL, even though at 31 μg/mL the extent of spore germination was 50-60% of the control. Interestingly, the synthetic (S) enantiomer of metabolite **38** turned out to have stronger antifungal activity than the natural product itself. The biological activity of several other structural analogues was also investigated.

The commercially available compounds, sodium *p*-hydroxybenzoylformate, racemic *p*-hydroxymandelic acid, benzoylformic acid, and both the (R) and (S) enantiomers of mandelic acid were subjected to the same biological testing as the natural products **37** and **38**. The GIC 50 concentrations for spore germination, using *T. hartigii* as the test organism, are given in Table 8.

Table 8. Inhibition of Spore Germination Bioassay.

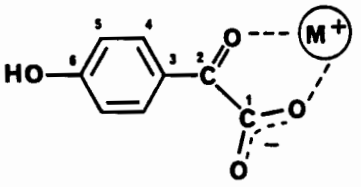
Sample	GIC 50 (μg/mL)	% S (μg/mL)
p-hydroxybenzoylformic acid	120	--
benzoylformic acid	140	--
(R/S)-*p*-hydroxymandelic acid	56	28
(R)-*p*-hydroxymandelic acid, 33% ee	70	23
(S)-*p*-hydroxymandelic acid, 60% ee	30	24
(R) mandelic acid, >95% ee	125	
(S) mandelic acid, >95% ee	30	

The results obtained from all α-hydroxy acid samples consistantly indicate that the (S) enantiomer is more active than the (R). Surprisingly, the sodium salt of compound **37** was found to be biologically inactive even at high concentration (500 μg/mL). However, when the commercial compound was converted to its free acid *via* an ion exchange column the same biological results as with the natural product were obtained. It was then speculated that "the mode of action" of these compounds may be similar to that of ionophore-type antibiotics involving the chelation of metal ions essential to the normal growth of the susceptible microorganisms. In such an event, the carboxylate ion would play a key role in the metal binding process. The presence of sufficient metal ions (eg.

sodium) could "associate" all of the carboxylate ions leading to a loss of antibiotic effect. Ionophore-type activity would not be observed when the carboxylate ion is unavailable for binding (complex 67) with the metal(s) involved in the microbial growth. Lack of biological activity was also observed with the sodium salts of metabolite 38 and mandelic acid.

A thorough investigation of the mode of action of these compounds was beyond the scope of this thesis. However, some NMR data was obtained which, along with literature information, supports the notion that they may chelate metal ions. NMR spectroscopy has been used in the past for the investigation of metal-ligand bonding formation.[154] The ^{13}C NMR chemical shifts of metabolite 37 in the free acid form and the lithium, sodium and potassium salts were compared (Tab. 9a).

Table 9a. ^{13}C NMR Shifts[155] (300 MHz, DMSO) of metabolite 37



(67)

C-1	C-1	C-2	C-3	C-4	C-5	C-6
-COOH	167	187	123	132	116	164
$-COO^-Li^+$	170	195	126	132	115	162
$-COO^-Na^+$	171	195	126	132	115	162
$-COO^-K^+$	171	195	124	132	116	166
$-COO^-Ag^+$	171	194	125	132	115	163

154. Sir G. Wilkinson, FRS, in *Comprehensive Coordination Chemistry*, Eds. R.D. Gillard and J.A. McCleverty, Pergamon Press, vol. 2 "*Ligands*" (1987), and references therein.

155. All chemical shifts are in values of ppm, using DMSO as the reference, and they are rounded to the nearest three significant figure number.

A down field shift of ~4 ppm was observed for the formation of the carboxylate ion as expected. However, a significant down field shift (~8 ppm) of the α-carbon (C-2) was also observed, possibly due to the formation of the metal-ligand complex (**67**) which would decrease the electron density of the C-2 carbon. In addition, it was noted that among all the salt compounds, there was very little difference in the chemical shifts except for the aromatic carbons C-3 and C-6 of the potassium salt which showed a smaller and larger change in shift (respectively) from the corresponding carbons of the other salts. These results suggested the formation of a stronger metal-ligand chelate between carboxylate **37** and the potassium ion. The absence of any significant difference between all ortho (C-4) carbons indicated that the phenol does not contribute to the electon density of the C-2 carbon *via* resonance. However, the existence of a dipole inductive effect through the ring would be most pronounced on carbons C-3 (increased electron density) and C-6 (decreased electron density). Molecular modeling calculations indicated that the α-ketone and the carboxylate ion are co-planar and at a 53° angle from the plane of the aromatic ring (Fig. 22), which confirmed a significant reduction of resonance.

The silver salt of metabolite **37** was also prepared in order to investigate the ionic radii-chelate complex strength formation. However, the results obtained were unreliable due to the occurrence of a redox reaction between the metal and the ligand during the course of the NMR experiment, leading to deposition of elemental silver.

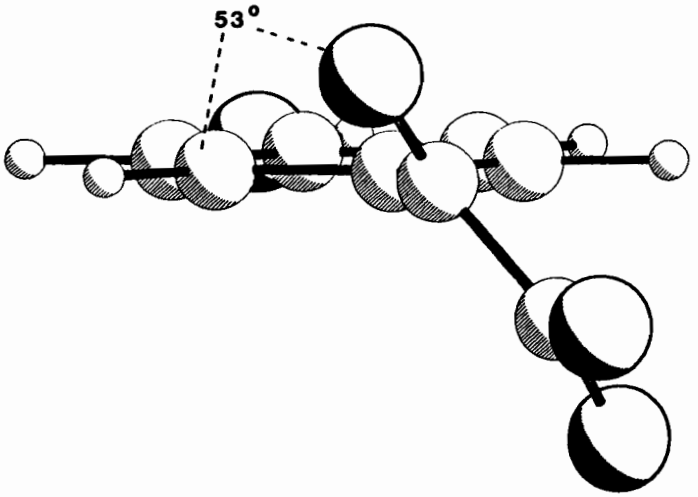


Fig. 22: Conformational analysis of the p-hydroxybenzoylformate ion.

The strong electron-withdrawing effect of the metal ions on the C-2 carbonyl was evident even when the ^{13}C NMR was observed in fairly dilute D_2O solutions (Tab. 9b). Similar results were observed when the ^{13}C NMR of metabolite **38** in the free acid form was compaired to that of its metal salts.

Table 9b. ^{13}C NMR Shifts of p-Hydroxybenzoylformate salts in D_2O (300 MHz).

C-1	C-1	C-2	C-3	C-4	C-5	C-6
-COOH	167	187	123	132	116	164
$-COO^-Na^+$	174	196	125	133	117	163
$-COO^-K^+$	175	196	124	134	118	166

Although references to α-keto acid-metal chelates are scarce, there is an abundance of literature regarding the formation of metal complexes with α-hydroxy acids.[156] Mandelic acid itself has been shown to form chelates with a large number of metals, including nickel(II),[157] zirconium(IV) and hafnium(IV),[158] several lanthanide ions[159] and transition metals.[154,160] A number

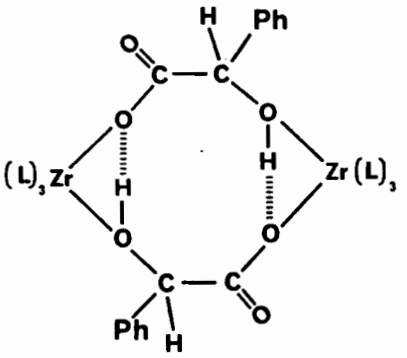
156. J.D. Pedrosa de Jesus, Hydroxy Acids in *Comprehensive Coordination Chemistry*, Pergamon Press, vol. 2 "Ligands", 461 (1987).

157. P.V. Khadikar and R.L. Ameria, *J. Indian Chem. Soc.* **49**, 717 (1972).

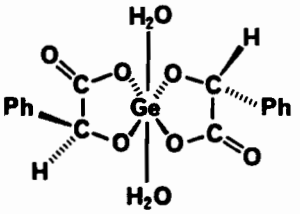
158. E.M. Larsen and E. H. Homeier, *Inorg. Chem.* **11**, 2687 (1972).

159. A. Dadgar and G.R. Choppin, *J. Inorg. Nucl. Chem.* **34**, 1297 (1972).

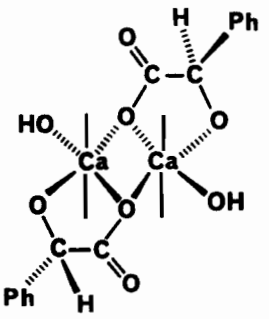
of complexes involving polymeric bridged dimers (complex **68**) or bidentate ligands coordinating *via* the deprotonated form of the α-hydroxyl and the carboxylate ion have been proposed (complexes **69**, **70**).



(68)



(69)



(70)

It is also noteworthy that a number of α-hydroxy acid biomolecules, such as malic and citric acid, are well known for their ability to chelate metals. Both malic and citric acid are commonly used in the food industry as preservatives. Their metal-complexing ability prevents fats and oils from becoming rancid due to air oxidation which is catalysed by trace metals. The coordination chemistry

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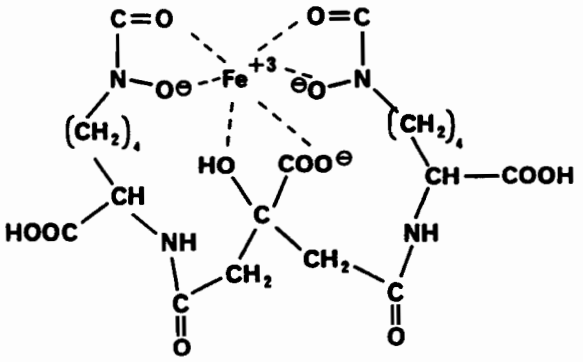
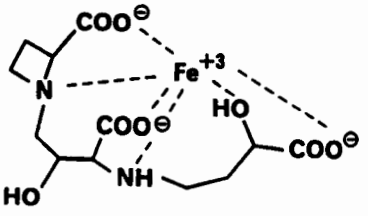
160. a) R.E. Tapscott, in *Transition Metal Chemistry*, Eds. G.A. Melson and B.N. Figgs, Dekker, New York, vol. 8, 253 (1982).
b) C. Sterling, *J. Inorg. Nucl. Chem.* **29**, 211 (1967).

of citric acid[161] in particular has attracted a great deal of attention due to its involvement in the "Krebs cycle". This rather simple compound was first isolated from lemon juice in 1784 by Scheele and since then it has been found in most plant and animal tissues, playing an important role in the transport of inorganic ions in biofluids.

Another class of biomolecules containing as part of their structure an α-hydroxy acid are the iron-chelating compound, siderophores.[162] Iron is an essential element for almost all biosystems, however, the solubility of ferric ions at physiological pH is about 10^{-18} M which is many orders of magnitude lower than what is required by either microbial or plant cells. Hence, through evolution this problem has been circumvented by the production of the chelating agents siderophores. The chemical structure of these compounds is very complex, but the parts of the ligand which are known to bind to the iron are almost always α-amino acids, α-hydroxy acids or hydroxamates. The structural formulas of the microbial siderophore, aerobactin[163] (71) and the plant siderophore mugineic acid A[164] (72) are representative of these types of compounds.



(71)



(72)

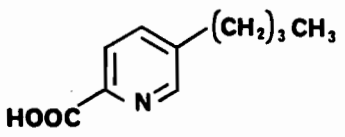
161. J.P. Glusker, *Acc. Chem. Res.* 13, 345 (1980) and references therein.

162. F. Oida, N. Ota, Y. Mino, K. Nomoto and Y. Sugiura, *J. Am. Chem. Soc.* 111, 3436 (1989) and references therein.

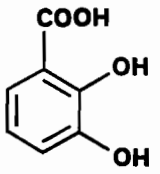
163. K.N. Raymond and C.J. Carrano, *Acc. Chem. Res.* 12, 183 (1979).

164. K. Nomoto, H. Yoshioka, M. Arima, S. Fushiya, S. Takagi and T. Takemoto, *Chimia* 35, 249 (1981).

The involvement of iron in the biological activity of fungal metabolites is not unprecedented in the literature. For instance, Oku and Nakanishi[44] have shown that both the phytotoxic and the antibiotic effects of the β-keto-/ β-hydroxy acid ascohitine (13) are antagonized by the presence of an equal molar concentration of ferric ions. The phytotoxicity of fusaric acid (73), a strong wilting agent from the *Fusarium oxysporum* fungus, can be eliminated[165] by the addition of Fe^{+3}, Cu^{+2} or Mn^{+2} ions. Similarly, Ayer[166] has proposed that the blue complex formed when iron is chelated by the fungal metabolite 2,3-dihydroxybenzoic acid (74) is involved in the staining of the sapwood of lodgepole pines infected with blue stain fungi. However, in the case of the metabolites from *P. tinctorius*, the addition of Fe^{+3} ions to the test solutions of the antifungal activity bioassays had no apparent effect.



Fusaric acid (73)



(74)

In conclusion, total lack of biological activity for both the sodium and potassium salts of metabolites 37 and 38 along with the ^{13}C NMR data strongly suggest, but far from prove, that the mechanism of action of these two antibiotics may involve the chelation of metal ions.

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165. K. Tamari and J. Kaji, *J. Agr. Chem. Soc. Japan* **27**, 249 (1953) and references therein.

166. W.A. Ayer, L.M. Browne, M.C. Feng, H. Orszanka and H. Saeedi-Ghomi, *Can. J. Chem.* **64**, 904 (1986).

CHAPTER 3

THE PRODUCTION OF ETHYLENE FROM THE ECTOMYCORRHIZAL FUNGUS SUILLUS CAVIPES

3. 1. Introduction

A unique characteristic in ectomycorrhizal root systems of pine trees (genus: *Pinus*) is the development of dichotomously branched roots,[167] a morphological effect which refers to symmetrical fork-branching of root tips caused by the division of localized active plant-cells (apical meristems) into two equal parts.[168] Although this phenomenon does occur spontaneously to a limited extent, it increases markedly in response to mycorrhizal infections.[169] In the past, cell free filtrates of liquid media, in which ectomycorrhizal fungi had been grown, were shown to enhance dichotomous branching of excised pine roots.[170] It is believed that the plant hormone ethylene could induce such a phenomenon. For instance, studies on the root development of Mugo pines have shown that the presence of either auxins or exogenous ethylene in the culture medium produces the same morphological changes.[171,172] The addition of auxins to the growth culture of plants is known to induce the endogenous (in tissue) production of ethylene. Hence, the direct or indirect supply of ethylene to a host plant by a symbiotic microorganism, would lead to morphological changes of its root system. The microbial production of ethylene[23,24,26,27] and its role in plant biochemistry[25,173] is

167. M. Faye, M. Racillac and A. David, *Sol. New Phytol.* **87**, 557 (1980).

168. A.B. Hatch and K.D. Doak, *J. Arnold Arbor.* **14**, 85 (1933).

169. Y. Piche, J.A. Fortin, R.L. Peterson and U. Posluszny, *Can. J. Bot.* **60**, 1523 (1982).

170. V. Slankis, *Physiol. Plant.* **1**, 390 (1948).

171. L.A. Rupp and K.W. Mudge, *Physiol. Plant.* **64**, 316 (1985).

172. L.A. Rupp, K.W. Mudge and F.B. Negm, *Can. J. Bot.* **67**, 477 (1989).

173. J. Weyers, *New Scientist* **17**, 9 (1984).

now well documented and it was briefly described in the introduction of this thesis. In higher plants, the biosynthetic precursors and the reactions involved in the production of ethylene are well understood, but in fungi and other microorganisms the exact pathways are yet unknown.

The ectomycorrhizal fungus *Scillus cavipes* was found to strongly promote the formation of roots and root primordia[174] on hypocotyl cuttings of the coniferous tree species[175] *Larix laricina*. Increased root development and dichotomous branching was also observed upon prolonged periods of hypocotyl incubation in a medium containing cell free extracts of *S. cavipes* (Fig. 23). The economic value of these types of trees is quiet considerable, and presently, their vegetative propagation by means of cuttings is very difficult at best.[176] Therefore, *S. cavipes* presented an interesting and potentially beneficial micro-organism; thus, a part of this thesis was devoted towards the investigation of its biologically active metabolites.

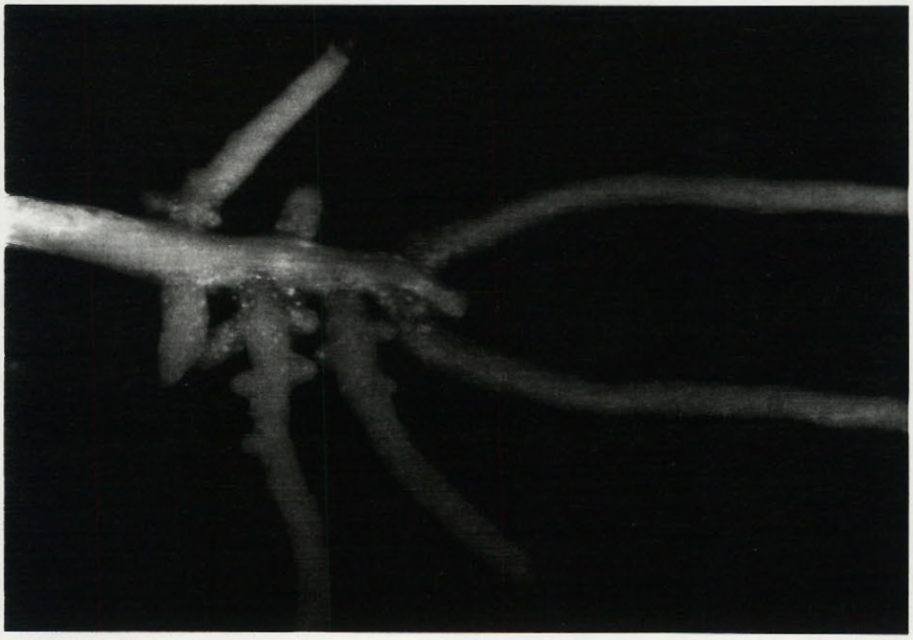

Fig. 23: Root development of hypocotyl cutting exposed to metabolite mixtures of S. cavipes.

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174. Primordial consist of actively dividing cells which have not yet begun to show morphological differentiation.

175. J.A. Fortin and A. Stein, unpublished results, private communication.

176. R.L. Mott, in *Cloning Agricultural Plants via in vitro Techniques*, Ed. B.V. Conger, CRC Press, Boca Raton FLA., 217 (1981).

3. 2. Discussion

3. 2. 1. Attempts towards the isolation of ethylene or indolyl-3-acetic acid from S. cavipes.

Soil microorganisms which produce ethylene gas have been known for a number of years.[177] Among them are a large number of fungal species including ectomycorrhizal fungi which release ethylene in their growth culture.[26,27,178] Several other research groups have reported enhancement of lateral root formation and dichotomous branching in coniferous trees caused by ethylene.[171,179] These literature results were very similar to those observed with the hypocotyl cuttings of *L. laricina* pine in the presence of extracts from the growth media of *Suillus cavipes*. Hence, the possibility that exogenous ethylene was responsible for the morphological effects induced by this fungus was considered. However, biological testing of freeze-dried cultures and cultures evaporated to dryness at 40°C under high vaccum (for prolonged periods of time) indicated that there was no loss of biological activity, confirming that a non gaseous metabolite had to be involved.

Alternatively, ethylene could still be involved indirectly, through the release of auxins by the fungus. The endogenous production of ethylene in plants is controlled by auxins, as mentioned previously. A large number of ectomycchorizal fungi have been shown to produce these hormones[180] and their exogenous application is known to enhance root development and ethylene biosynthesis in tree seedlings.[171,181] Hence, efforts were redirected towards the possible isolation of such a compound from the cultures of *S. cavipes*.

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177. J.M. Lynch, *Nature* **240**, 45 (1972).

178. L.A. Rupp, H.E. DeVries II and K.W. Mudge, *Can. J. Bot.* **67**, 483 (1989).

179. a) J.H. Graham and R.G. Lindermann, *Can. J. Bot.* **59**, 149 (1981).
b) E.R.L. Wilson and R.J. Field, *New Phytol.* **98**, 465 (1984).

180. a) Iwan Ho, *Can. J. For. Res.* **17**, 31 (1987).
b) J.M.Jr. Frankenberger and M. Poth, *Appl. Environ. Microbiol.* **53**, 2908 (1987).
c) J.M. Ulrich, *Physiol. Plant.* **13**, 429 (1960).
d) E. Strzelczyk, J.M. Sitek and S. Kowalski, *Acta Microbiol. Pol.* **26**, 255 (1977).

181. C.M. Baser, H.E. Garrett, R.J. Mitchels, G.S. Cox and C.J. Starbuck, *Can. J. For. Res.* **17**, 36 (1987).

Liquid cultures of *S. cavipes* grown at room temperature for periods of 6-7 weeks were analyzed for the presence of auxin-like metabolites. Following literature procedures for the general purification of indolic compounds, the freeze-dried culture media was extracted with aqueous methanol[182] or acidified to pH 3 and extracted with large volumes of ethyl acetate.[183] The isolated crudes did not exhibit any biological activity, indicating that auxin-type compounds (e.g. indolyl-3-acetic acid) were likely not involved. In addition, the affects of indolyl-3-acetic acid (3) on the *L. laricina* pine hypocotyl cuttings were examined and found to be different from those obtained with the liquid cultures of *S. cavipes*.

At this point, the morphological changes observed on the seedling cuttings exposed to the crude metabolite mixture of *S. cavipes* were re-evaluated, and once again the involvement of ethylene was implicated. Interestingly, very similar results were obtained when the *L. laricina* cuttings were incubated in sterile media containing ethephon,[184] but not when incubated with indolyl-3-acetic acid. Hence, the search for a biosynthetic precursor of ethylene was initiated.

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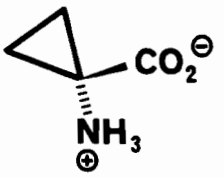
182. S. Horemans, H.A. Van Onckelen, P. Rudelsheim and J.A. De Greef, *J. Exper. Bot.* 35, 1832 (1984).

183. R. Rouillon, G. Gay, J. Bernillon, J. Favre-Bonvin and G. Bruchet, *Bot.* 85, 153 (1983).

184. a) Ethephon is an ethylene producing compound, which is briefly discussed in the introduction of this thesis.
b) A. Stein, M. Sc. Thesis, Laval University, Quebec, Que. (1988).

3. 2. 2 Attempts towards the isolation of a biosynthetic precursor of ethylene from Suillus cavipes

The possibility that *S. cavipes* was producing a biosynthetic precursor of ethylene was conceivable, since several such compounds are known in the literature. The best known example, is the amino acid 1-aminocyclopropane-1-carboxylic acid (ACC, **75**). It was first isolated by Burroughs[185] in 1957 during his investigation of the amino acid content of cider apples and pear juice. Although its metabolic role was not recognized at the time, he observed that this new amino acid appeared only during the last three to four weeks of the fruits ripening period. The isolation of ACC from cowberry berries was again reported a few months later by Finnish researchers,[186] who observed that the amino acid was not at all present in the unripe berries.



ACC (75)

In was not until 1979 when Adams and Yang[187] showed ACC to be the precursor of ethylene in plant tissues. At that time it was already established that methionine was the starting material of the ethylene biosynthetic scheme[188,189] and it was shown that in apple tissue the synthesis of ethylene represented the major metabolic use of methionine.[190] In 1966 Lieberman[191] reported that the *in*

185. L.F. Burroughs, *Nature* **179**, 360 (1957).

186. M.-L. Vahatalo and A.I. Virtanen, *Acta Chem. Scand.* **11**, 741 (1957).

187. D.O. Adams and S.F. Yang, *Proc. Natl. Acad. Sci. USA* **76**, 170 (1979).

188. A.D. Hanson and H. Kender, *Plant Physiol.* **57**, 528 (1976).

189. L.D. Owens, M. Lieberman and A. Kunishi, *Plant Physiol.* **48**, 1 (1971).

190. S.P. Burg and C.O. Clagett, *Biochem. Biophys. Res. Commun.* **27**, 125 (1967).

vivo production of ethylene was derived from the C-3 and C-4 of methionine (corresponding to C-2 and C-3 of ACC). These results were confirmed by Burg and Clagett[190] using the same tissue, and radioactively labeled methionine; DL-[$^{14}C_1$]-methionine, DL-[$^{14}C_2$]-methionine, L-[$^{14}C_{1-4}$]-methionine. In addition, it was reported[190] that the C-1 of methionine (corresponding to the carboxyl of ACC) was metabolized to carbon dioxide, where the sulfur of L-[^{35}S]-methionine and the methyl of L-[$^{14}CH_3$]-methionine were mostly converted to S-adenosylmethionine.[192] S-Adenosylmethionine was then shown to be utilized by the ACC-synthase enzyme as a substrate for the production of ACC and 5'-methylthioadenosine, the latter of which was rapidly hydrolysed to 5-methylthioribose. Finally, Peiser[193] showed that the C-1 of ACC is converted to cyanide and the carboxyl group to carbon dioxide probably through a cyanoformic acid intermediate. The overall biosynthetic scheme for the production of ethylene, in plants, is now well established and it is believed to follow the steps outlined in Figure 24.

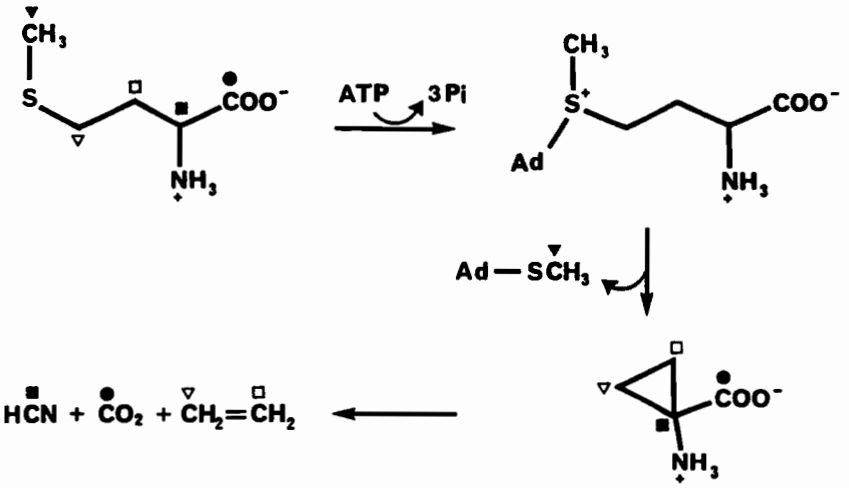


Fig. 24: The biosynthesis of ethylene from methionine

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191. M. Lieberman, A. Kunishi, L.W. Mapson and D.A. Wardale, *Plant Physiol.* **41**, 376 (1966).

192. Y.-B. Yu, D.O. Adams and S.F. Yang, *Arch. Biochem. Bioph.* **198**, 280 (1979).

193. G.D. Peiser, T-T Wang, N.E. Hoffman, S.F. Yang, H.-W. Liu and C.T. Walsh, *Proc. Natl. Acad. Sci. USA* **81**, 3059 (1984).

An ethylene-forming enzyme has not yet been isolated and characterized *in vitro*, due to the fact that it is labile and membrane bound,[194] however, there is little doubt that it exists. Studies on the stereoselectivity of the enzyme using alkyl-ACC and deuterium-labeled ACC analogues were initiated by Baldwin[195] and co-workers and have revealed several aspects of the ethylene-biosynthesis reactions which could not be rationalized by a non-enzymatic process. Observations on the enzymatic conversion of ACC to ethylene have also been reported by Pirrung.[196]

Although much remains to be learned about the ACC and ethylene-forming enzyme, the involvement of a coenzyme, pyridoxal phosphate (vitamin B_6), which is required for the production of ACC is well established. Yu and coworkers[197] have examined the role of pyridoxal phosphate in this process using a crude enzyme preparation from tomatoes. They observed a maximum of a 10-fold increase in the production of ethylene when pyridoxal was present at concentrations of 0.1μM (or higher) as compared to the control (0.0μM). Radioactively labeled S-adenosyl-L-[$^{14}C_{3,4}$]methionine was utilized to verify that the enzymatic conversion involved the production of labeled ACC, which was isolated and identified. In summary, the overall involvement of vitamin B_6 in the biosynthesis of ethylene *via* the formation of ACC is believed to follow the pathway outlined in Figure 25.

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194. D.O. Adams and S.F. Yang, *Trends Biochem. Sci.* **6**, 161 (1981).

195. a) R.M. Adlington, R.T. Aplin, J.E. Baldwin, B.J. Rawlings and D. Osborne, *J. Chem. Soc. Chem. Commun.*, 1086 (1982).
b) R.M. Adlington, J.E. Baldwin and B.J. Rawlings, *J. Chem. Soc. Chem. Commun.*, 290 (1983).
c) J.E. Baldwin, D.A. Jackson and in part R. M. Adlington and B.J. Rawlings, *J. Chem. Soc. Chem. Commun.*, 206 (1985).

196. a) M.C. Pirrung, *J. Am. Chem. Soc.* **105**, 7207 (1983).
b) M. Pirrung and G.M. McGeehan, *Angew. Chem. Int. Ed. Engl.* **24**, 1044 (1985).

197. Y.-B. Yu, D.O. Adams and S.F. Yang, *Arch. Biochem. Biophys.* **198**, 280 (1979).

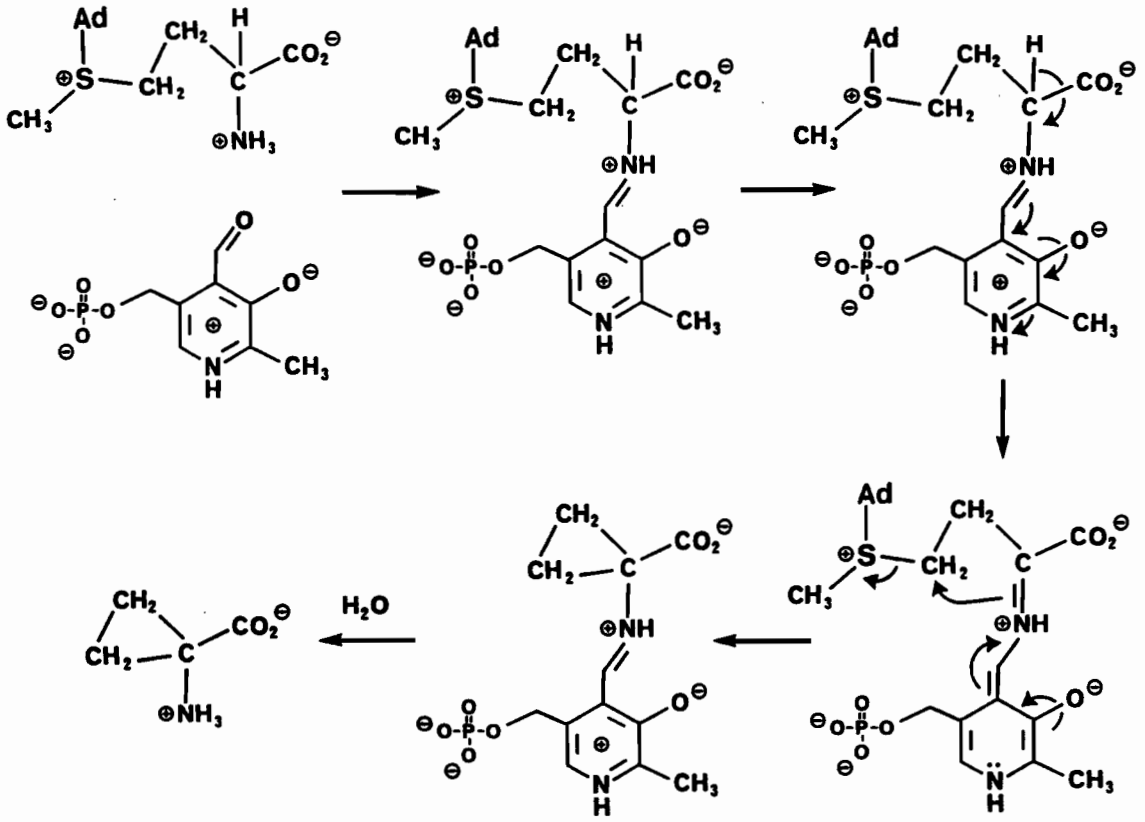
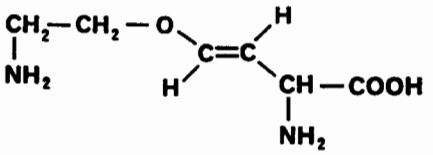


Fig. 25: Biosynthesis of ACC from S-adenosylmethionine.

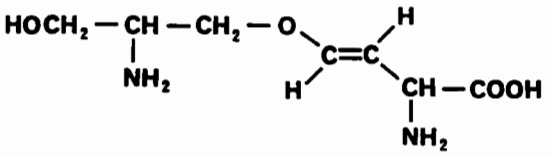
To further test the notion that the formation of ACC is catalyzed by a pyridoxal-linked enzyme, a number of known pyridoxal inhibitors have been tested. Vinylglycine and methoxyvinylglycine were found to be ineffective inhibitors of the enzyme where 2-amino-4-(2'-aminoethoxy)-trans-3-butenoic acid (**76**), also called aminoethoxyvinylglycine (AVG), effectively blocked production of ACC and ethylene.[197,198]

198. a) Y.-B. Yu and S.F. Yang, *Plant Physiol.* **64**, 1074 (1979).
b) T. Boller, R.C. Herner and H. Kende, *Planta* **145**, 293 (1979).

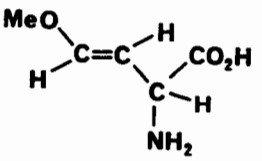


AVG (76)

When a physiological phenomenon is under investigation which is believed to depend on ethylene biosynthesis, such as dichotomous root branching, pyridoxal phosphate inhibitors are often used as probes. Elimination of the physiological effect observed in the control by the addition of a pyridoxal phosphate inhibitor is usually a good indication that the biological activity is ethylene dependent. The mechanism of inhibition is believed to involve the formation of a Schiff base intermediate with the aldehyde form of pyridoxal phosphate.[199] The phytotoxin rhizobitonin (77), a natural product isolated from *Rrizobium japonicum*,[200] and its analogue L-2-amino-4-methoxy-trans-3-butenoic acid (78) were used to describe the inhibition mechanism involved (Fig. 26).



(77)



(78)

199. R.R. Rando, *Science* **185**, 32 (1974).

200. J.P. Scannell, D.L. Preuss, T.L. Demny, L.H. Sello, T. Willims and A. Stempel, *J. Antibiot. Tokyo Ser. A* **25**, 122 (1972).

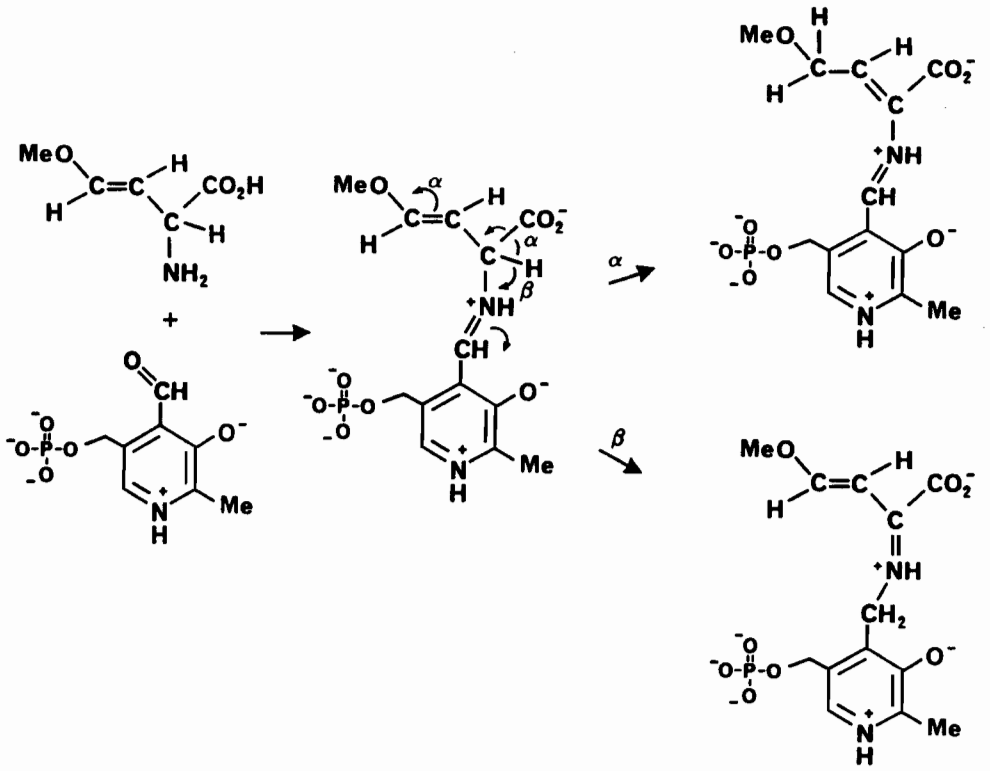


Fig. 26: Inhibition of pyridoxal phosphate with L-2-amino-4-methoxy-trans-3-butenoic acid (78).

In microbial systems there appear to be several alternate routes for ethylene biosynthesis, none of which are yet totally understood. Chou and Yang[201] have reported that α-ketoglutarate and glutarate are the precursors of ethylene in *Penicillium digitatum*. However, Chalutz and Lieberman[202] showed that these two compounds served as ethylene precursors only in static cultures, where cultures incubated on a rotary shaker used methionine as the precursor. In addition, they observed a partial inhibition of ethylene synthesis in the presence of AVG and suggested that the ethylene-forming system in *P. digitatum* may in fact be quite similar to that of higher plants. The same pyridoxal inhibitor (AVG) was shown to have no effect on the production of ethylene in studies conducted with other fungi.[178,203]

Other putative intermediates in the biosynthesis of ethylene from microorganisms are the

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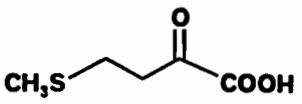
201. T.W. Chou and S.F. Yang, *Arch. Biochem. Biophys.* **157**, 73 (1973).

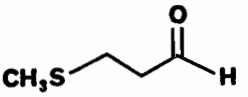
202. F. Chalutz and M. Lieberman, *Plant Physiol.* **60**, 402 (1978).

203. P.E. Axelrood-McCarthy and R.G. Linderman, *Phytopathology* **71**, 825 (1981).

compounds 4-methylthio-2-oxobutanoic acid (MTBA, 79) and 3-(methylthio)propanal (methional, 80). Both compounds have been shown to produce ethylene in model systems involving either peroxidase[204] or flavin and light.[205] Although, their biosynthetic role in the conversion of methionine to ethylene has been disputed by Bauer and Yang,[206] Baker[207] showed that in mature green and pink tomatoes the synthesis of ethylene was stimulated by the addition of MTBA, where the production of [$^{14}C_{1,2}$]-ethylene from L-[$^{14}C_{3,4}$]-methionine was strongly inhibited by unlabelled MTBA. This latter observation suggested that unlabelled MTBA diluted the radioactive MTBA which was synthesized from [^{14}C]-methionine, clearly indicating the role of this compound in the conversion of methionine to ethylene.



MTBA (79)



Methional (80)

Support for the role of MTBA and methional was also provided by the work of Lynch[208] and Primrose.[209] MTBA was isolated and fully characterized as its dinitrophenylhydrazone derivative from the culture fluids of a range of bacteria, the yeast *Saccharomyces cerevisiae* and the fungus

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204. a) L.W. Mapson and D.A. Wardale, *Biochem. J.* **102**, 574 (1967).
b) M.S. Ku, S.F. Yang and H.K. Pratt, *Arch. Biochem. Biophys.* **118**, 756 (1967).

205. a) S.F. Yang, *Arch. Biochem. Biophys.* **122**, 481 (1967).
b) S.F. Yang, H.S. Ku and H.K. Pratt, *J. Biol. Chem.* **242**, 5274 (1967).

206. A.H. Baur and S.F. Yang, *Plant Physiol.* **44**, 1347 (1969).

207. J.E. Baker, M. Lieberman and J.D. Anderson, *Plant Physiol.* **59**, *Suppl.* 45 (1977).

208. J.M. Lynch, *J.Gen. Microd.* **83**, 407 (1974).

209. a) D.C. Billington, B.T. Golding and S.B. Primrose, *Biochem. J.* **182**, 827 (1979).
b) S.B. Primrose, *J. Gen. Microbiol.* **98**, 519 (1977).

Penicillium digitatum. Deuterium label incorporation from [Me-^{2}H]methionine to 4-[Me-^{2}H]methylthio-2-oxobutanoic acid in these cultures was also demonstrated. The possibility that the deuterium labeled MTBA was not the product of an enzymatic process, but an artifact induced chemically by low-molecular weight coenzymes, was rejected since it was shown that chloramphenicol[210] inhibited the conversion of methionine to MTBA, indicating a requirement for protein synthesis. In addition, the use of boiled cells and cell-free culture filtrates failed to convert methionine to MTBA or ethylene.

The effects of pyridoxal phosphate inhibitors on the bio-conversion of MTBA to ethylene were studied by Primrose using L-canaline [L-α-amino-γ-(aminooxy)-*n*-butyric acid]. The addition of L-canaline to the growth culture of *E. coli* at 70μM concentration was found to result in 94% inhibition of the conversion of methionine to ethylene, but only 20% inhibition of MTBA conversion to ethylene. These results suggested two different biosynthetic routes leading to ethylene production, as was also suggested previously in the case of *P. digutatum*.[202]

The possibility that MTBA and/or methional could be metabolic products of *S. cavipes* was investigated. However, treatment of the culture medium with 2,4-dinitrophenylhydrazine (according to literature procedures), followed by purification of the 2,4-dinitrophenylhydrazone derivatives formed, failed to reveal the presence of these two compounds.

The possible role of ACC in the biosynthesis of ethylene from ectomycorrhizal fungi was recently investigated by Rupp and coworkers.[178] They showed that ACC was not a precursor of ethylene in growing cultures of either *Laccaria laccata* or *Hebeloma crustiliniforme*, since its addition to the culture media failed to stimulate ethylene production the way it normally does in the tissues of higher plants.[211] Their results were further supported by the observation that AVG, the biosynthetic inhibitor of ACC, did not effect the conversion of methionine to ethylene in cultures of *L. laccata*.

The role of ACC was also investigated in other microorganisms. For example, Walsh[212] has shown that bacterial cultures of *Pseudomonas* do not metabolize ACC to ethylene but to α-ketobutyrate and ammonia through a deamination reaction (Fig. 27). He showed that this conversion is very efficient, and the micro-organisms can grow on ACC as their sole source of nitrogen. The enzyme responsible for the ACC deamination process in bacteria has been purified to

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210. Chloramphenicol is an antibacterial antibiotic which inhibits protein synthesis.

211. Higher plant tissues produce ethylene at rates of 10-1000 times faster than the controls when treated with ACC.

212. H.-W. Liu, R. Auchus and C.T. Walsh, *J. Am. Chem. Soc.* 106, 5335 (1984).

homogeneity[213] and used to study several other biochemical processes.[214] This enzyme contains tightly bound pyridoxal-5'-phosphate, and the reaction it catalyses seems to be analogous to the reverse process of the S-adenosylmethionine to ACC conversion.

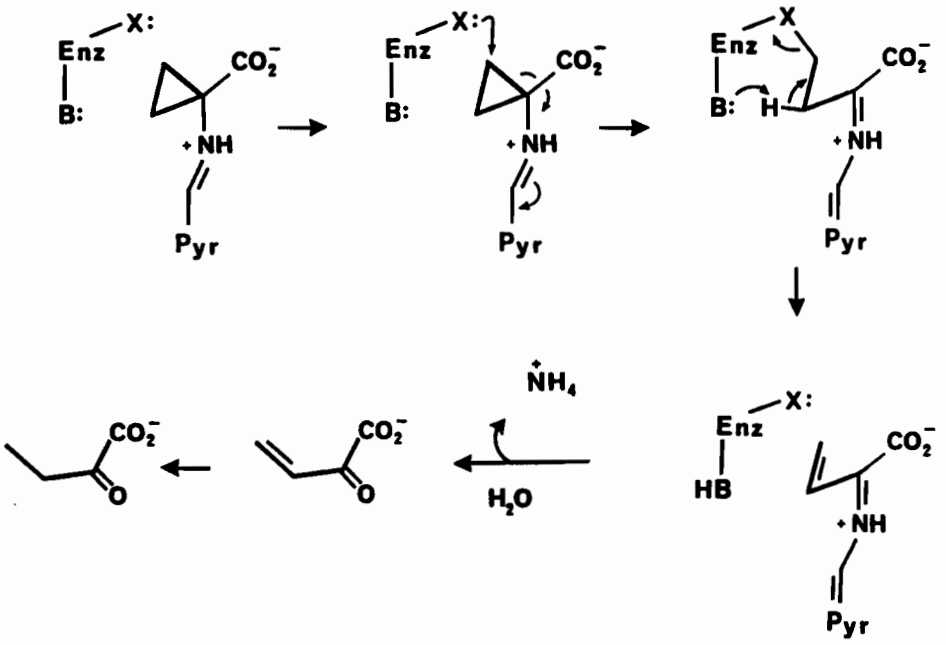

Fig. 27: The bioconversion of ACC to α-ketobutyrate.

213. M. Honma and T. Shimomura, *Agric. Biol. Chem.* 42, 1825 (1978).

214. M. Honma, T. Shinomura, K. Shiraishi, A. Ichihara and S. Sakamura, *Agric. Biol. Chem.* 43, 1677 (1979).

In further investigations of root development of *L. laricina*, the addition of AVG (76) to the growth medium of the hypocotyl cuttings was found to significantly inhibit the morphological changes induced by ethephon and extracts of *S. cavipes*. These results were analogous to those reported with the *P. digitatum* fungi[202] and confirmed the involvement of ethylene one more time.

Several other attempts to isolate a biologically active sample from the culture medium of *S. cavipes* were made, including various types of chromatography, soxhlet extractions of the freeze-dried liguid culture and liquid-liquid extractions. The biological activity of the original culture was often lost at the early stages of most isolation schemes indicating the possible instability of the metabolite of interest. However, the results thus far obtained clearly indicate that the effects of *S. cavipes* on the hypocotyl cutting of *L. laricina* and other conifers, are caused by a metabolite which acts as a precursor in ethylene biosynthesis.

CHAPTER 4

STUDIES ON THE PHYTOTOXICITY OF PHOMOPSIS CONVOLVULUS

4. 1 Introduction

4. 1. 1 The genus Phomopsis

The genus *Phomopsis* includes several pathogenic fungi which cause a variety of disease symptoms to plants, animals and occasionally humans. The majority of these microorganisms have not yet been thoroughly investigated and only a few are known to produce biologically active metabolites. Among these metabolites are the cytochalasins, which are mammalian toxins exhibiting a number of unusual effects on animal cells There are more than twenty cytochalasins[215] which have been isolated thus far from such species as *Aspergillus, Chalara, Helminthosporium, Phoma and Phomopsis*. All of these compounds are characterized by a highly substituted hydrogenated isoindole moiety fused to a macrocyclic ring system. The cytochalasin H toxin (**81**) was first isolated from *Phomopsis paspalli*[216] and shown to inhibit floral development and plant growth in tobacco, wheat and beans. In addition, it has been found to be toxic to animals at very low concentrations.[217]

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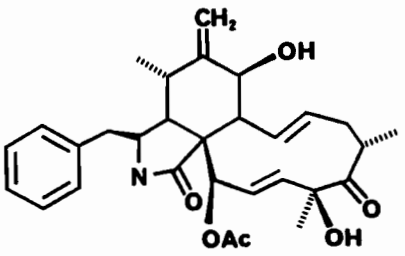
215. a) REVIEW: M. Binder and C. Tamm, *Angew Chem. Int. Ed.* **12**, 370 (1973), and references therein.
b) D.C. Aldridge, J.J. Armstrong, R.N. Speaker and W.B. Turner, *J. Chem. Soc. C*, 1667 (1967), and references therein.
c) G. Buchi, Y. Kitaura, S.-S. Yuan, H.E. Wright, J. Clardy, A.L. Demain, T. Glinsukon, N. Hunt and G.N. Wogan, *J. Am. Chem. Soc.* **95**, 5423 (1973), and references therein.
d) T. Fex, *Tetrahedron Lett.* **22**, 2706 (1981).
e) P.S. Steyn *J. Chem. Soc. Perkins Trans. I*, 541 (1982).

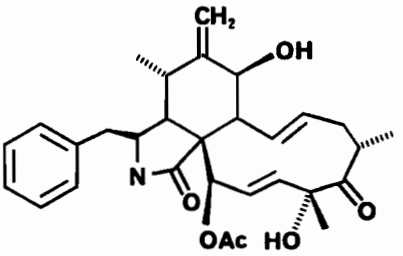
216. a) G.S. Pendse, *Experientia* **30**, 107 (1974)
b) S.A. Patwardhan, R.C. Pandey, S. Dev and G.S. Pendse, *Phytochemistry* **13**, 1985 (1974).
c) M.A. Beno, R.H. Cox, J.M. Wells, R.J. Cole, J.W. Kirksey, and G.G. Christoph, *J. Am. Chem. Soc.* **99**, 4122 (1977).

217. J.M. Wells, H.G. Cutler and R.J. Cole, *J. Can. Microbiol.* **22**, 1137 (1976)

Several other members of this family are known to interfere with cell division,[218] cardiac and smooth muscle contraction, platelet aggregation and the secretion of hormones.[219] Antibiotic activity[220] has also been observed with these compounds and one of them, cytochalasin D (**82**), has been reported to be a selective antitumor agent.[221]



Cytochalasin H (81)



Cytochalasin D (82)

The ionophoric metabolite phomopsin A (**83**) is another potent mycotoxin[222] isolated from a *Phomopsis* fungus (*Phomopsis leptostromi*). *P. leptostromi* is a pathogen of lupin plants[223] and the causative agent of Lupinosis,[224] a severe hepatotoxic disease which afflicts primarily sheep, cattle, pigs and horses grazing on infected lupin lands. The structure of its active metabolite was first

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218. N.K. Wessels, B.S. Spooner, J.F. Ash, M.O. Bradley, M.A. Luduena, E.L. Taylor, J.T. Wrenn and K.M. Yamada, *Science* **171**, 135 (1971).

219. J.G. Schofeil, *Nature, (London), New Biol.* **234**, 215 (1971).

220. V. Betna, D. Micekova and P. Nemec, *J. Gen. Microbiol.* **71**, 343 (1972).

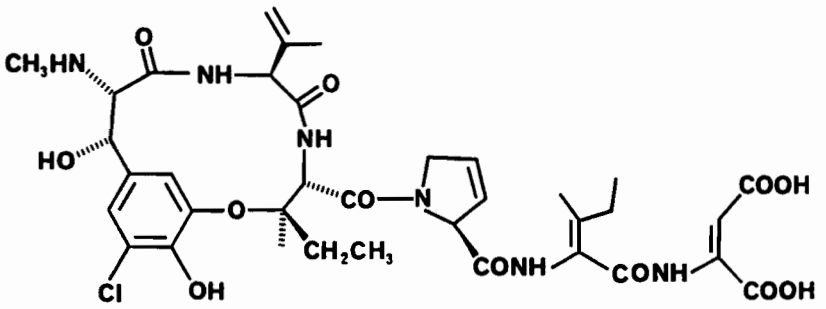
221. K. Katagiri and S. Matsuura, *J. Antibiot.* **24**, 722 (1971).

222. J.L. Frahn, *Toxicon (Suppl. 3)*, 149 (1983).

223. S.A. Ortazewski and H.D. Well, *Plant Dis. Report.* **44**, 66 (1960).

224. P.McR. Wood and D.S. Petterson, *Aust. J. Exp. Agric.* **25**, 164 (1985).

investigated in 1983 and confirmed by X-ray crystallography three years later to be the peptide **83**.[225]



Phomopsin A (83)

The fungus *Phomopsis oblonga* is another interesting member of this species. It is frequently found in the outer bark of healthy elm trees but is known to invade the phloem of trees when they become infected by *Ceratocysis ulmi*, the causative agent of Dutch elm disease.[226] It has been shown that the beetle insects, which are responsible for transmitting the disease, find the *P. oblonga*-infected phloem unsuitable for feeding,[227] hence, the metabolites of *P. oblonga* have been investigated as potential insect deterrents. Several simple aromatic compounds, phthalides and α-pyrones have been isolated from this fungus[228] and shown to possess antibiotic, phytotoxic and insect deterrent properties. Orsellinic acid (**84**), *p*-hydroxyphenylethanol (**85**), 5-methylmellein (**86**), Phomopsolide A and B (**87**, I and II, also known as tiglic esters), and nectriapyrone (**88**) are some characteristic active metabolites of *P. oblonga*.

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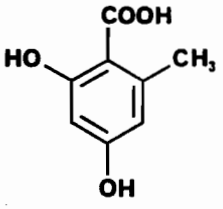
225. a) C.C.J. Calvenor, *J. Chem. Soc. Chem. Commun.*, 1259 (1983).
b) M.F. Mackay, *J. Chem. Soc. Chem. Commun.*, 1219 (1986).

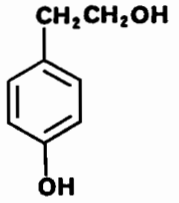
226. a) J.N. Gidds and M.E. Smith, *Ann. Appl. Biol.* **89**, 125 (1978).
b) J.F. Webber and J.N. Gidds, *Trans. Br. Mycol, Soc.* **82**, 348 (1984)

227. J. Webber, *Nature (London)* **292**, 448 (1981).

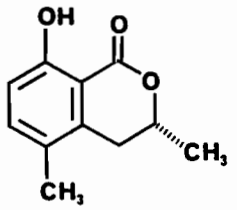
228. N. Claydon, J.F. Grove and M. Pople, *Phytochemistry* **24**, 937 (1985), and references therein.



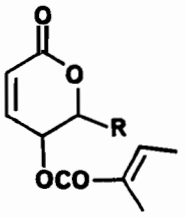
Orsellinic acid (84)



p-Hydroxyphenylethanol (85)



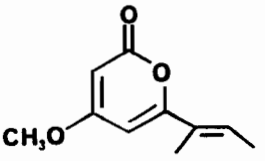
5-Methylmellein (86)



(I) R= $CH_3CH(OH)COCH\overset{Z}{=}CH-$

(II) R= $CH_3CH(OH)CH(OH)CH\overset{E}{=}CH-$

Phomopsolide (87)



Nectriapyrone (88)

4. 1. 2 The plant "field bindweed" and its Phomopsis pathogen

The perennial plant *Convolvulus arvensis*, better known as field bindweed or wild morning glory, is a serious agricultural problem around the world with the exception of the tropics. Field bindweed infestations are encountered along roadsides, in city green spaces and cultivated lands throughout most of Canada[229] and it has been classified as one of the most important weeds worldwide.[230]

Phenoxy herbicides such as (2,4-dichlorophenoxy)acetic acid (2,4-D^{Tm}), (4-chloro-2-methylphenoxy)acetic acid (MCPA^{Tm}) and 3,6-dichloro-2-methoxybenzoic acid (DICAMBA^{Tm}) are presently used for the control of this pest. Although, these simple aromatic compounds are among the most effective and commonly used herbicides[231] they are generally costly and ineffective in suppressing the spreading of bindweed. For example, in California, field bindweed is more prevalent now than 20 years ago in spite of the intensive control programs used.[232]

In the early 1970's, a number of independent studies on the biological control of field bindweed were initiated. Among them, insects[233] and gall mites were considered but were found to be inadequately host-specific causing damage to crop plants in addition to the weeds.

Fungi which are pathogenic to field bindweed present an alternate possibility for its biological control. *Phomopsis convolvulus*, an organism which was isolated and classified recently,[234]

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229. J.F. Alex, in *Biology and Ecology of Weeds*, Eds. Holzner and M. Numata, W. Junk Publishers, The Hague, 309 (1982).

230. L.G. Holm, D.L. Plunknett, J.V. Pancho and J.P. Herberger, in *The World's Worst Weeds*, University Press of Hawaii, Honolulu, Hawaii, Chapter 12 (1977).

231. a) W.P. Anderson, in *Weed Science: Principles*. 2nd ed., West Publishing Co., St. Paul (1983).
b) *Herbicide Handbook*, Weed Science Society of America, 5th ed., Weed Science Society of America, Champaign (1983).

232. S.S. Rosenthal, *Calif. Agric.* **37**, 16 (1983).

233. a) S.S. Rosenthal and J. Carter, *Envir. Entomol.* **6**, 155 (1977).
b) S.S. Rosenthal, *Entomophaga* 23, 367 (1978).

234. a) J. Ormeno-Nunez, R.D. Reeleder and A.K. Watson, *Plant Dis.* **72**, 338 (1988).
b) J. Ormeno-Nunez, R.D. Reeleder and A.K. Watson, *Can. J. Bot.* **66**, 2228 (1988).

is one such pathogen. It has been shown to infect field bindweed causing brown lesions which some times are surrounded by yellow hallows characteristic of phytotoxin production.

A preliminary investigation into crude metabolite-mixtures of *P. convolvulus* further supported the existence of phytotoxic compounds. Leaf-puncture bioassays[235] using these crudes were found to cause the formation of brown spots in the tissue of young bindweed leaf cuttings.

The production and yields of secondary metabolites, such as phytotoxins, almost always depends on the growth conditions of the microorganism. Knowledge of the identity of an active metabolite in a culture medium is necessary in order to optimize the conditions for its production. The onset of this project, however, coincided with the initial stages of characterization and the development of fermentation conditions for *Phomopsis convolvulus*. Consequently, in a collaborating effort with the research group of Dr. A.K. Watson, Department of Plant Science, McGill University, a number of different media were investigated. In the absence of knowledge of the phytotoxic metabolites involved, it was decided to investigate the conditions necessary for optimum production of infectious conidia of this fungus.[236] A variety of solid and liquid media were used and conidia viability, morphology and yields were observed. The crude mixture of metabolites from each of these media were analyzed by thin layer chromatography and their phytotoxicity to field bindweed leaf-cuttings was compared. The overall results of these tests indicated that pearl barley and modified Richard's (V-8) media[236] were the optimum solid and liquid media, respectively, for the culturing of *P. convolvulus*. However, the production of conidia may not be proportional to yields of phytotoxic metabolites, hence, these two aspects of microbial growth need to be examined further and independently of each other.

The purpose of one part of this thesis was to initiate a thorough investigation into the active metabolites of *Phomopsis convolvulus*. Cultures grown on moist pearl barley grains were chosen for this purpose. The steroids ergosterol (**89**) and ergosterol peroxide (**90**), the phthalides, 4-carboxy-3-hydroxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (**91**), 4-(hydroxymethyl)-7-methoxy-6-methyl-1(3H)-isobenzofuranone (**92**), 4-carboxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (**93**), and the α-pyrone, 3-[4-methoxy-3-methyl-α-pyron-6-yl]-2-methyl-2-butenoic acid (**94**) were identified to be metabolites of *Phomopsis convolvulus*. The compounds **90**, **91** and **92** were shown to possess phytotoxic activity against field bindweed and *Lemna* plants.

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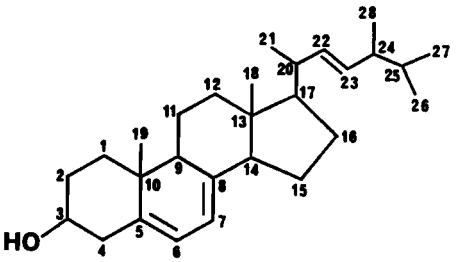
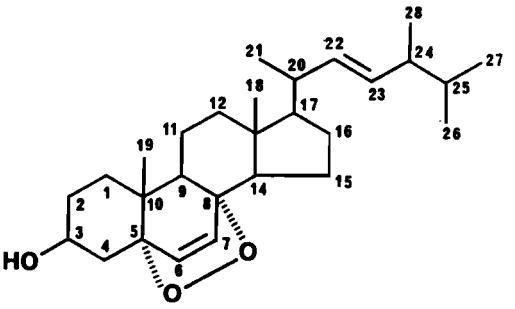
235. F. Sugawara, G. Strobel, R.N. Strange, J.N. Siedow, G.D. Van Duyne, and J. Clardy, *Proc. Natl. Acad. Sci. USA* **84**, 3081 (1987).

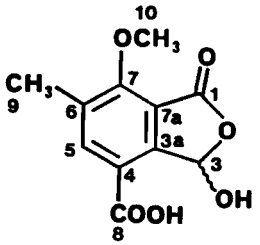
236. L. Morin, M.Sc. thesis, McGill University, Macdonald College, Ste-Anne-de-Bellevue, Quebec, Canada.



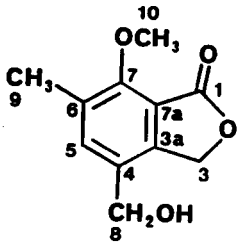
Ergosterol (89)



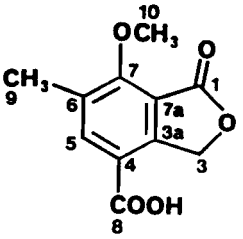
Ergosterol peroxide (90)



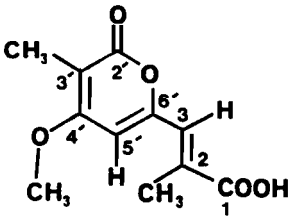
(91)



(92)



(93)



(94)

4. 2 General isolation scheme for the metabolites of P. convolvulus

Moist barley grains were inoculated with a conidia suspension of *Phomopsis convolvulus* and incubated at room temperature for 28-30 days. At the end of the growth period the infected grains were washed for several hours with excess water in order to extract the extracellular metabolites of this fungus. The overall general scheme outlined in Figure 28 was subsequently followed in order to eliminate biologically inactive components and partition the remaining crude into a non-polar fraction, containing a number of steroid compounds, and a polar fraction, containing most of the phytotoxic metabolites.

The aqueous crude was filtered through a few layers of cheese cloth and centrifuged to remove the grains, mycelia and newly formed conidia. The liquid culture was then reduced to about 10% of its original volume and freeze-dried to give a very fine, light-grey, powder. Upon extraction with methanol this powder was partitioned into a biologically phytotoxic methanolic extract and a white solid void of any observable activity. The methanolic crude was evaporated to a brown gum, which was suspended in a small volume of water, acidified to pH ~3 and extracted several times with ethyl acetate. The ethyl acetate extract was found to exhibit strong phytotoxic activity. Insignificant activity was observed with the aqueous extract.

Several attempts to isolate the active metabolites from the above ethyl acetate crude *via* flash column chromatography using a variety of different solvent systems were unsuccessful, leading to poor separation of the components and loss of biological activity. It was, however, noted that a number of metabolites in this crude had an "acidic" character, giving long strikes of high R_f values, on TLC eluted with ethyl acetate, but Rf values of almost zero when a trace amount of triethylamine was added to the solvent system.

This crude was then suspended in methylene chloride and extracted with a saturated solution of $NaHCO_3$. The bicarbonate layer was re-acidified with dilute HCl solution to pH ~2.5-3, reduced in volume under high vacuum and extracted with ethyl acetate. Leaf-puncture bioassays indicated than most of the biological activity was extracted into the basic aqueous layer and only very weak activity was observed with the methylene chloride extract. This results confirmed the acidic nature of the main phytotoxic metabolites.

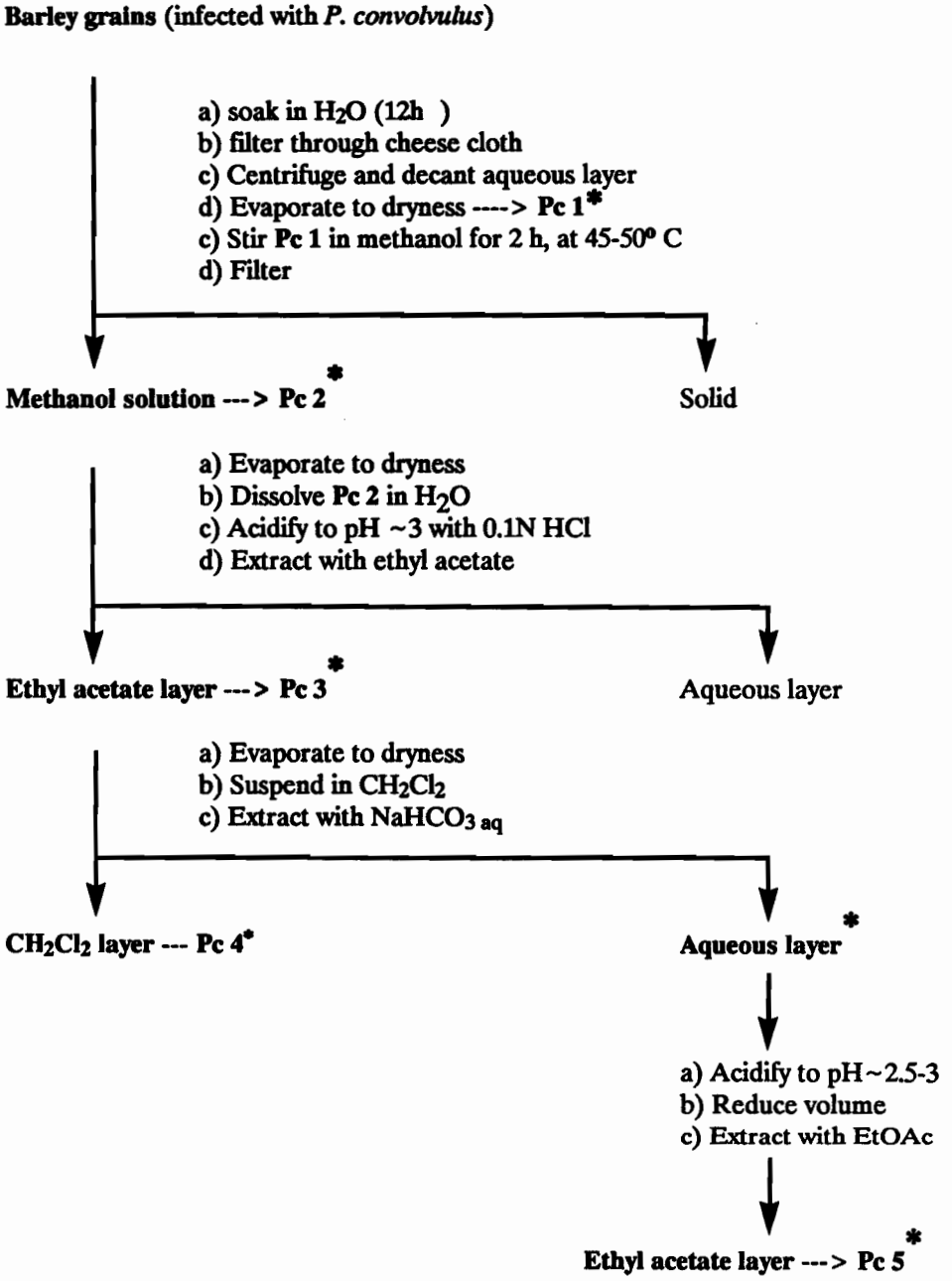


Fig. 28: General isolation scheme of Phomopsis metabolites

4. 3. Isolation and structure determination of steroid metabolites from P. convolvulus

4. 3. 1. Isolation and identification of ergosta-5,7,22-trien-3-ol (ergosterol, 89)

The methylene chloride extract **Pc 4** (Fig. 28) was found to be weakly phytotoxic to field bindweed. Analysis of its metabolite mixture *via* thin layer chromatography indicated the presence of two major compounds among a number of minor spots. Both of these compounds gave a dark red or violet color upon reaction with vanillin/sulfuric acid on TLC, indicating a possible steroid or terpenoid chemical nature. Flash column chromatography, using petroleum ether-ethyl acetate (1:1) as the eluting solvent, led to the separation of the above two compounds from the **Pc 4** metabolite mixture. The remaining recovered material was found to be void of any biological activity, hence, further investigation of its components was not actively pursued.

Pure and crystalline (m.p. 163-164°C) metabolite **89** was isolated after a second flash column chromatography using a petroleum ether-ethyl acetate (5:1) solvent mixture. Ammonium ion chemical ionization mass spectrometry gave a base peak at 379 (due to loss of H_2O from the molecular ion), a molecular ion at 397 and an $M^+ + NH_4^+$ peak at 414. The uv absorption spectra (Fig. 29) showed maxima at 274, 284 and 294nm. Solution IR in dry $CDCl_3$ showed two sharp -OH peaks at 3600 and 3700 cm^{-1}.

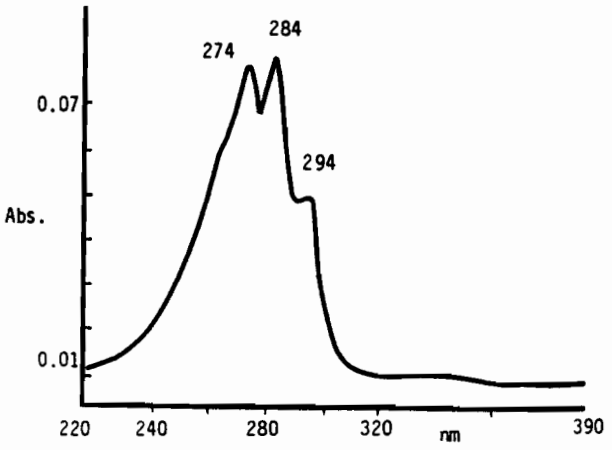


Fig. 29: UV absorption spectra of metabolite 89 in ethanol.

The proton NMR (Fig. 30) revealed the presence of four olefinic protons at 5.20 ppm (m, 2H), 5.38 ppm (m, 1H) and 5.58 ppm (dd, 1H). A broad multiplet at 3.53 ppm and a broad D_2O exchangeable singlet at 1.51 ppm suggested the presence of a CH-OH group. In addition six methyl groups were observed, two quaternary at 0.63 ppm (s, 3H) and 0.94 ppm (s, 3H) and four attached to a methine carbon at 0.81 ppm (d, 3H), 0.83 ppm (d, 3H), 0.91 ppm (d,3H) and 1.05 ppm (d, 3H). Finally, a large number of complex overlapping signals were observed between 1.2 and 2.5 ppm which could not be identified giving a total integration of approximately 20-22 protons. Although the actual structure of this compound could not yet be determined, its ^1H NMR was consistent with that of a steroidal type of natural product.

^{13}C NMR (Fig. 31) of metabolite 89 contained 26 observable carbon signals. Six of these carbons had to be part of a double bond; two di-substituted (141.38 and 139.78 ppm) and four mono-substituted (135.57, 131.97, 119.59 and 116.28 ppm). A signal appearing at 70.47 ppm was assigned to a C-OH carbon. However, the 19 other signals between 10 and 60 ppm could not easily be characterized. Hence, Distortionless Enhancement by Polarization Transfer ^{13}C NMR, which can be set so as to produce separate spectra for methyl, methylene and methine carbons was utilized in order to identify these signals.

The presence of six methyl groups (12.06, 16.29, 17.61, 19.65, 19.96 and 21.11 ppm), which was previously suggested by the ^1H NMR, was confirmed by the DEPT spectra obtained (Fig. 32a). In addition, seven methylene carbons (21.11, 23.00, 28.30, 31.99, 38.37, 39.08 and 40.79 ppm) and six methine (33.09, 40.44, 42.82, 46.25, 54.56 and 55.72 ppm) were clearly identified. Finally, Attached Proton Test (APT) NMR revealed the presence of four quaternary carbons (141.32, 139.73, 42.82 and 37.15) two of which could not be detected under normal ^{13}C NMR conditions due to the overlap of signals (Fig. 32b).

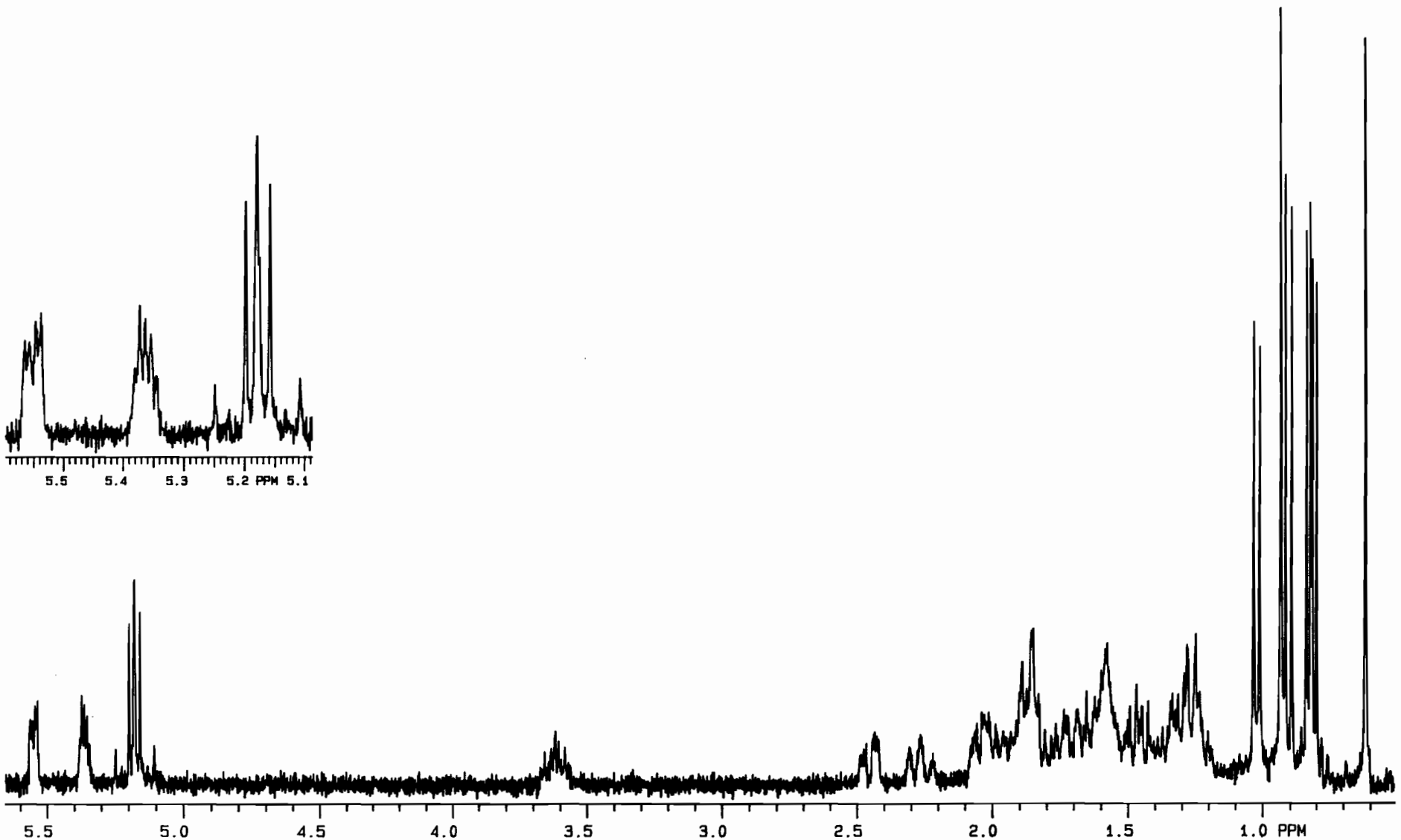


Fig. 30: ^{1}H NMR (200 MHz, $CDCl_3$) of metabolite 89.

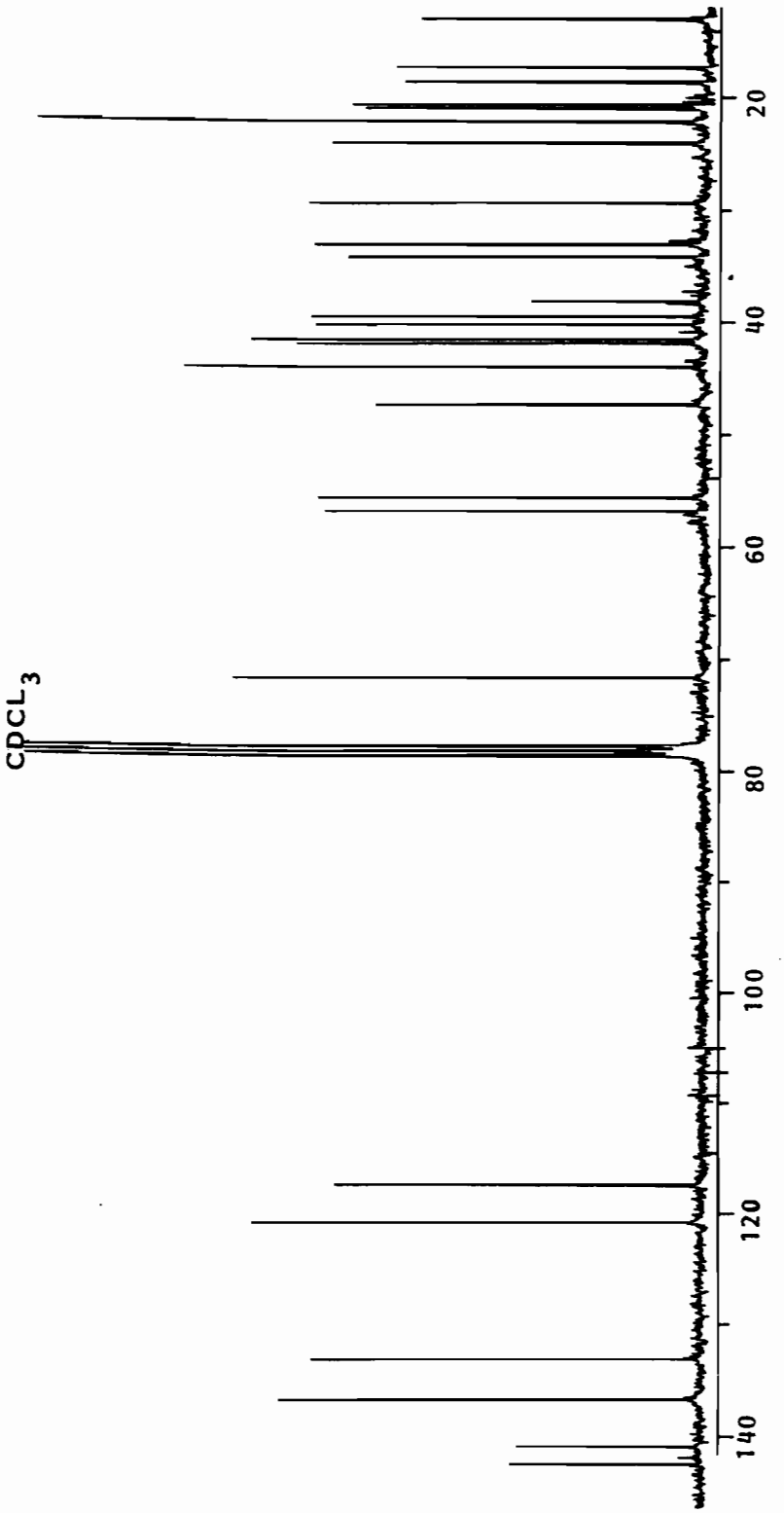


Fig. 31: ^{13}C NMR (300 MHz, $CDCl_3$) of metabolite 89.

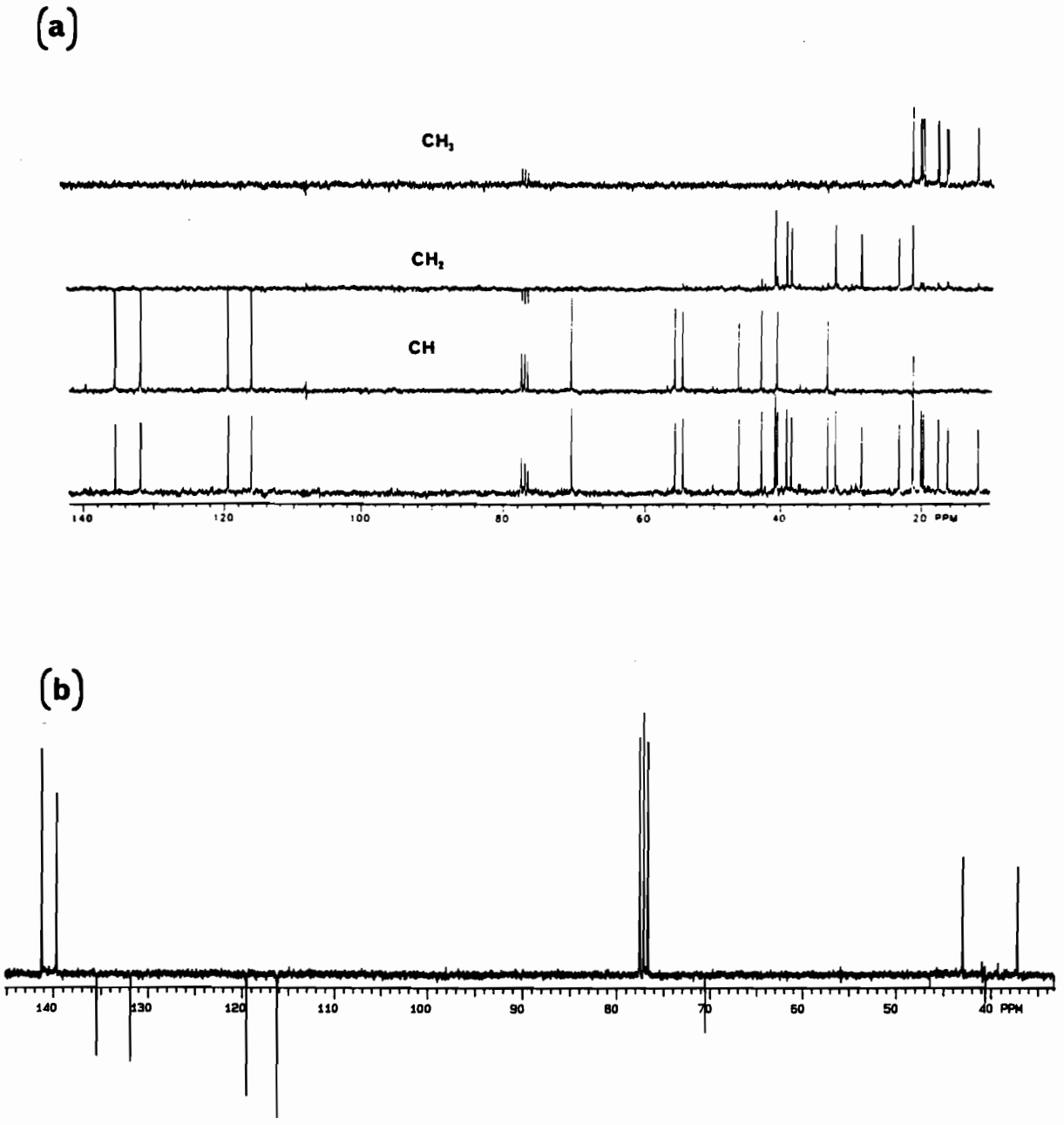
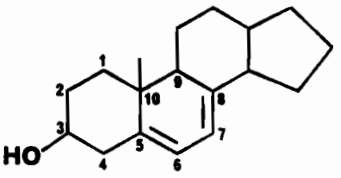


Fig. 32: ^{13}C NMR (300 MHz, $CDCl_3$) of metabolite 89; a) DEPT b) APT.

The coupling between ^{1}H-^{1}H and ^{13}C-^{1}H was also investigated through COSY (Fig. 33) and HETCOR (Fig. 34) NMR experiments. All results obtained suggested a steroid type structure possessing a -CH-OH moiety coupled to both protons (non-equivalent protons) of a -CH_aH_b- at 2.26-2.27 and 2.41-2.49 ppm. One of these protons (2.26-2.27 ppm) was also coupled to the olefinic proton at 5.54-5.56 ppm which in turn was coupled to the olefinic proton at 5.35-5.38 ppm. The other two olefinic protons, at 5.16-5.20 ppm, were coupled to each other and to methine protons remote from those already mentioned. COLOC NMR data indicated the presence of long-range coupling (^{3}J coupling) between the olefinic proton at 5.54-5.56 ppm and the quaternary carbon at 37.10 ppm and the -CH_aH_b methylene carbon mentioned before. These results suggested a cholesterol type nucleus (**95**) where there is an -OH group at C-3, the -CH_aH_b moiety would be C-4 and the olefinic proton at 5.54-5.56 ppm would be that on C-6 giving a ^{3}J coupling with C-10 in a COLOC experiment.

(**95**)



At this point, literature search of common steroidal fungal metabolites lead to the well-known natural product ergosterol (**89**). This natural sterol was first isolated in 1889 from the ergot fungus by Charles Tanrer, a Parisian apothecary who named the compound, ergosterol.[237]

The ^{1}H NMR spectral data of the metabolite isolated from *P. convolvulus* was compared with that of an authentic sample of ergosterol and found to be identical. Similarly, the ^{13}C NMR literature values for ergosterol[238] where almost identical to those of metabolite **89** (Tab. 10).

Although ergosterol is an important metabolite of *P. convolvulus* and the major component of the weakly phytotoxic crude **Pc 4**, it did not show activity in leaf-cutting bioassay with field bindweed.

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237. a) C. Tanret, *C.R. Acad. Sci. (Paris)* **108**, 98 (1889).
b) L.F. Fieser and M. Fieser, in *Steroids*, Reinhold Publishing Corporation, New York, Chapman and Hall, LTD, London (1959), and references therein.

238. W.B. Smith, *Org. Mag. Res.* **9**, 644 (1977).

Fig. 33: COSY NMR (300 MHz, $CDCl_3$) of metabolie 89

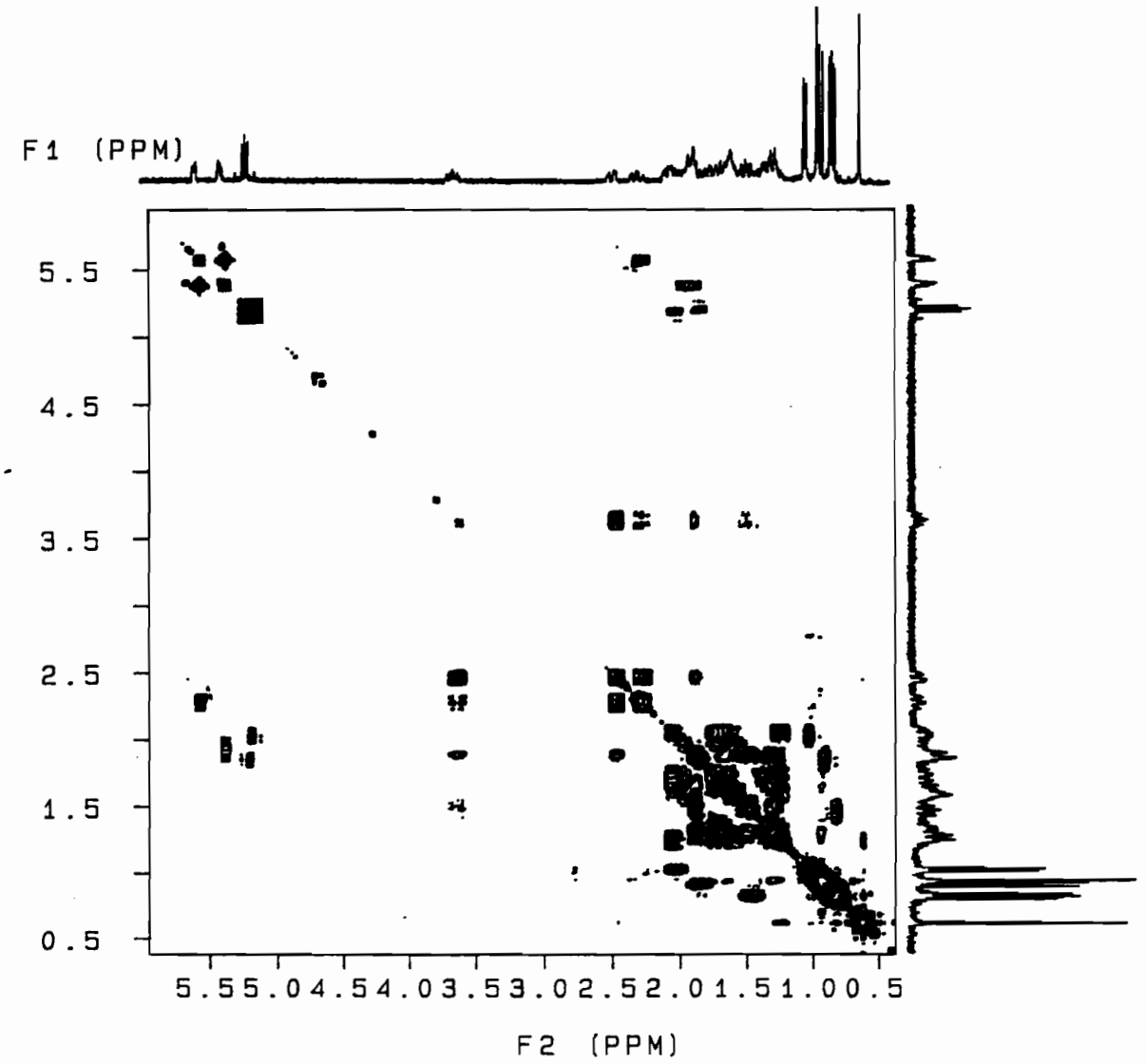


Fig. 34: HETCOR NMR (300 MHz, $CDCl_3$) of metabolite 89

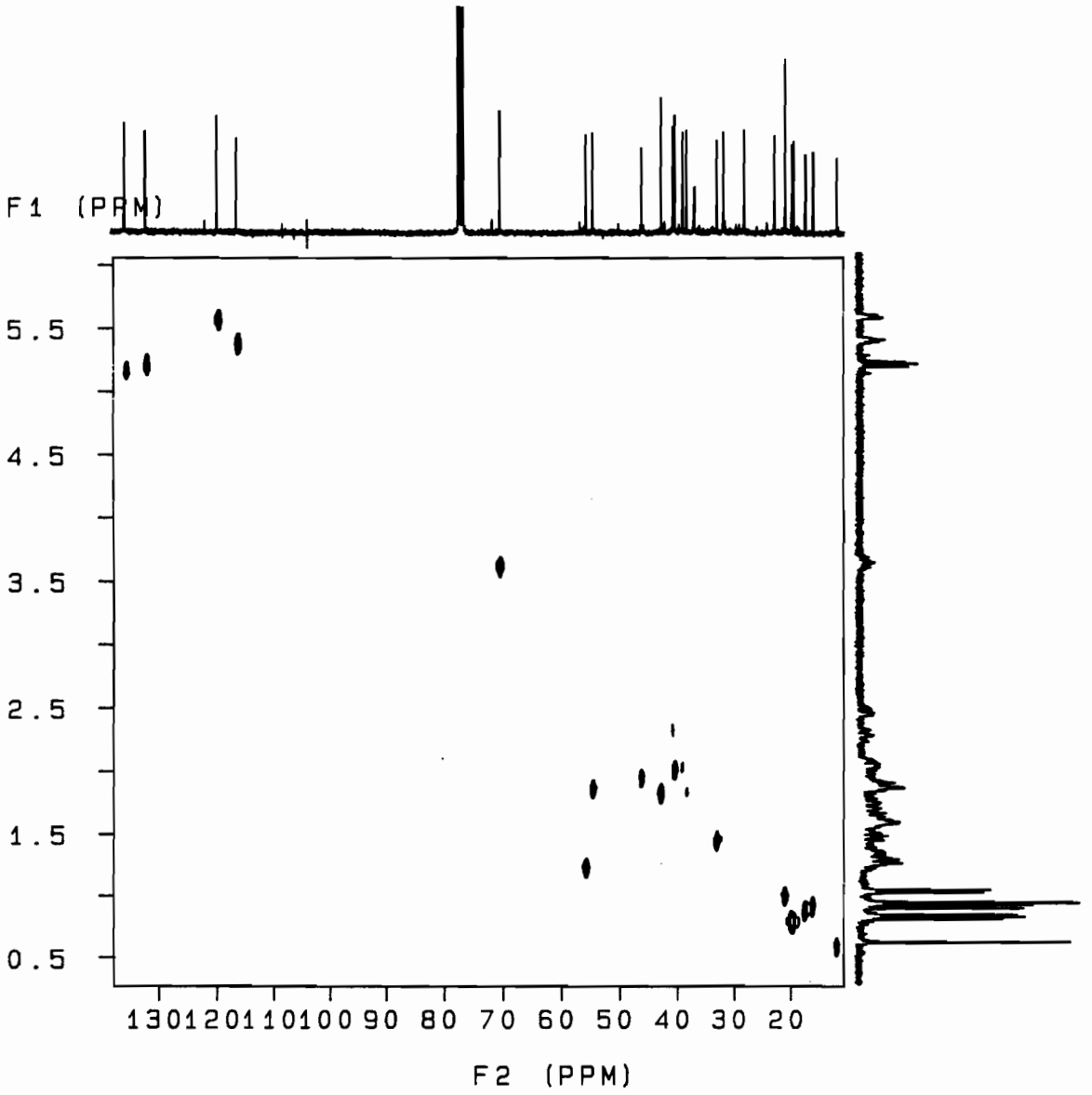
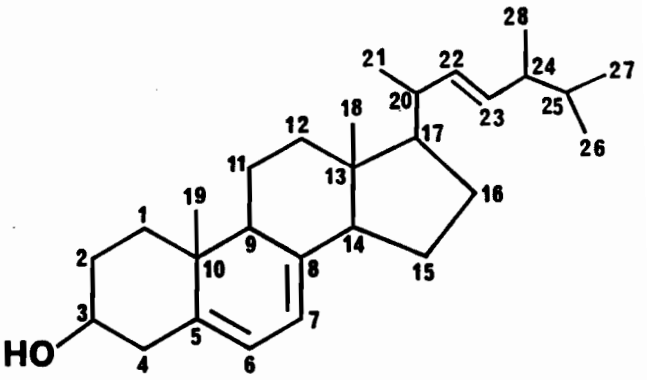


Table 10: ^{13}C NMR chemical shifts of ergosterol in $CDCI_3$.

Carbon	Literature[238]	Experimental
1	38.43	38.37
2	32.06	31.99
3	70.46	70.47
4	40.87	40.79
5	139.77	139.78
6	119.65	119.59
7	116.36	116.28
8	141.37	141.38
9	46.33	46.25
10	37.10	37.03
11	21.15	21.11
12	39.15	39.08
13	42.88	42.82
14	54.61	54.56
15	23.03	23.01
16	28.26	28.30
17	55.84	55.72
18	12.09	12.06
19	16.31	16.29
20	40.41	40.44
21	21.15	21.11
22	135.60	135.57
23	132.06	131.97
24	42.88	42.83
25	33.14	33.10
26	19.65	19.65
27	19.98	19.96
28	17.64	17.62



4. 3. 2 Isolation and identification of ergosta-6,22-dien-5α, 8α-epidioxy-3-ol (ergosterol peroxide, 90)

The second metabolite which was isolated from the flash column chromatography of the Pc 4 crude, was further purified on preparative thin layer silica gel plates, developed twice with a solvent mixture of petroleum ether - ethyl acetate (1:1). The crystalline compound obtained was fairly pure and it exhibited ^{1}H NMR signals (Fig. 35) very similar to those of ergosterol. The most characteristic difference was the downfield shift of the olefinic protons H6, H7 and the simultaneous loss of long-range coupling between H6 and H4, and between H7 and H14. Comparison of normal ^{13}C, DEPT and APT (Fig. 36) NMRs further supported the change of the two disubstituted olefinic carbons, C-5 and C-8, to quaternary carbons attached to oxygen (chemical shifts 79.5 and 82.2 ppm). In addition, high resolution mass spectrometry gave an exact mass of 429.33687 corresponding to a molecular ion of $C_{28}H_{44}O_3$ (calculated mass 429.33685). Hence, the structure of metabolite 90 was assigned to be that of ergosta-6,22-dien-5a, 8a-epidioxy-3-ol, better known as ergosterol peroxide.

Ergosterol peroxide was first isolated in 1947 from the mycelium of *Aspergillus fumigatus*[239] and since then it has been found in several other fungi,[240] lichens[241] and marine organisms.[242] This biologically active compound has also been isolated from the Chinese mushroom *Polyporus umbellatus* (Cho-Rei) and shown to cause aggregation of rabbit platelets.[243] Consequently, it was suggested that it may be responsible for the therapeutic effects of "Cho-Rei" which is used in traditional Chinese medicine for the treatment of hematuria.

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

240. a) G. Bauslaugh, G. Just and F. Blank, *Nature* 202, 1218 (1964).
b) J. Arditti, R. Ernst, M.H. Fisch and B.H. Flick, *J. Chem. Soc. Chem Comm.*, 1217 (1972).
c) J.D. Weete, *Phytochemistry* 12, 1843 (1973).
d) G. Kusano, H. Ogawa, A. Takahashi, S. Nozoe and K. Yokoyama, *Chem. Pharm. Bull.* 35, 3482 (1987).

241. T. Hirayama, F. Fujikawa, I. Yosioka, I. Kitagawa, *Chem. Pharm. Bull.* 23, 693 (1975).

242. a) A.A.L. Gunatilata, Y. Gopichand, F.J. Schmitz and C. Djerassi, *J. Org. Chem.* 46, 3860 (1981), and references therein.
b) M. Guyot and M. Durgeat, *Tetrahedron Lett.* 22, 1391 (1981).

243. W. Lu, I. Adachi, K. Kano, A. Yasuta, K. Toriizuka, M. Ueno and I. Horikoshi, *Chem. Pharm. Bull.* 33, 5083 (1985).

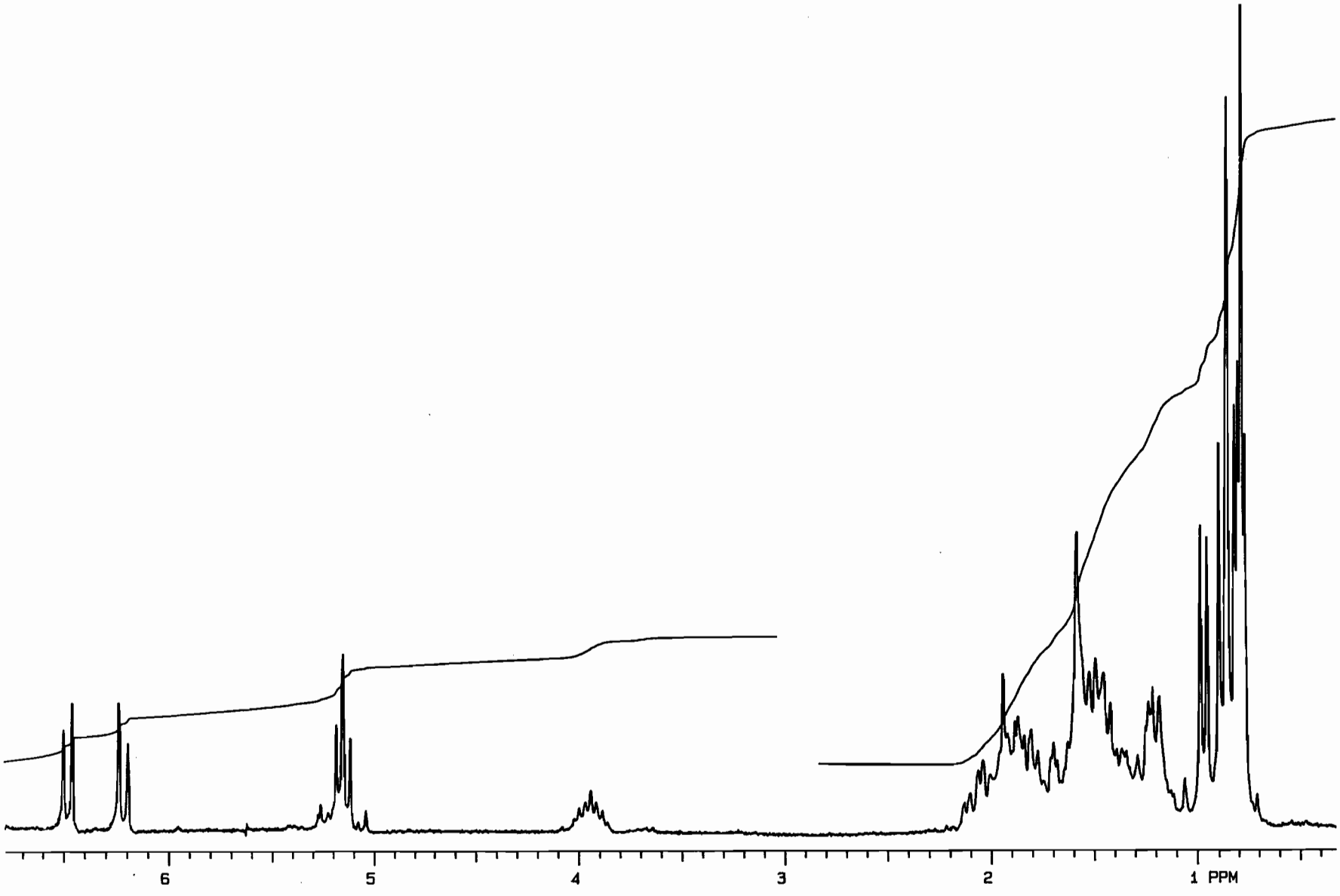

Fig. 35: ^{1}H NMR (200 MHz, $CDCl_3$) of ergosterol peroxide (90).

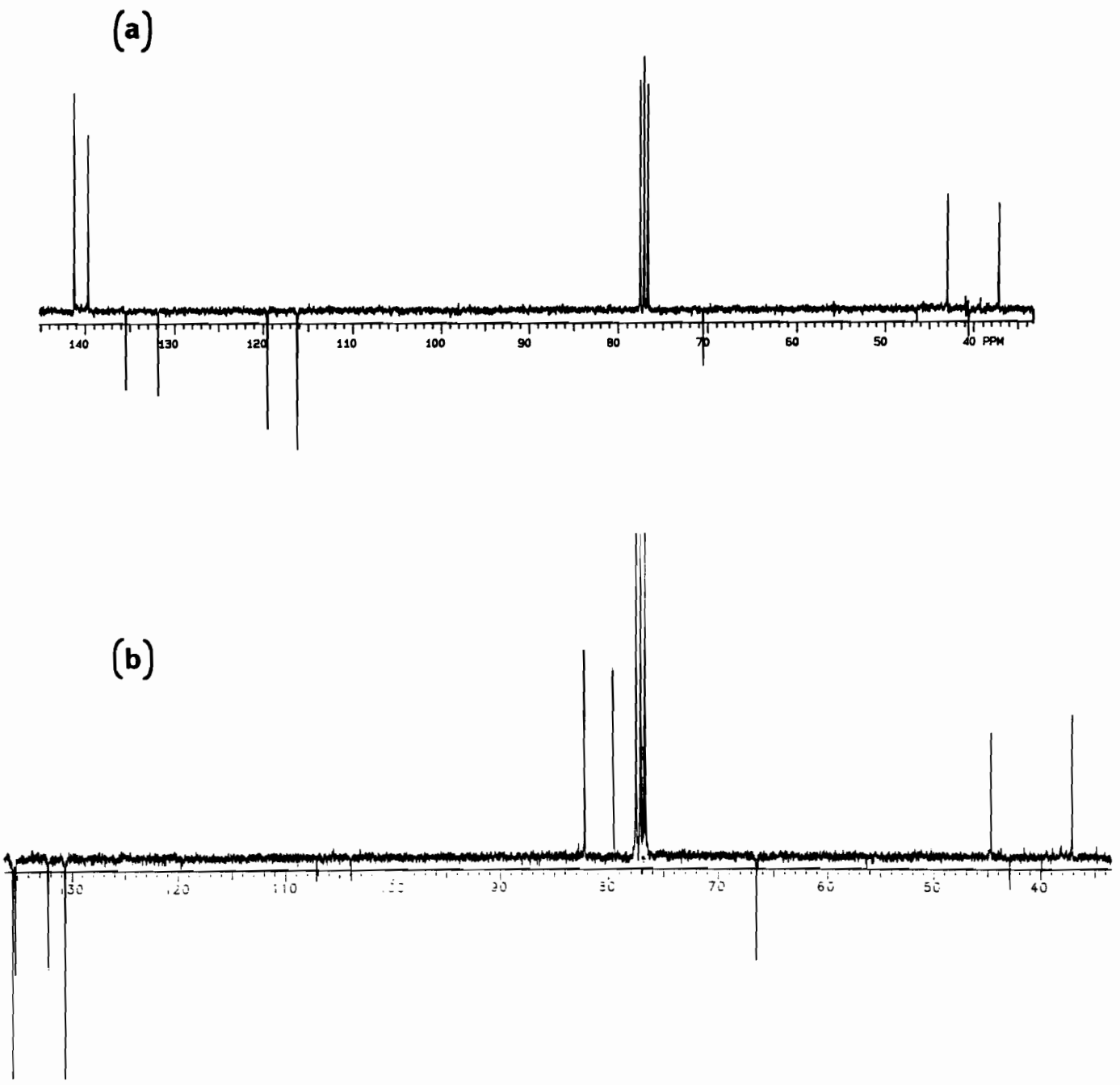

Fig. 36: APT ^{13}C NMR (300 MHz, $CDCl_3$) of a) ergosterol (89) and b) its peroxide (90).

All of the spectral data and physical properties of metabolite **90** were in agreement with literature information on ergosterol peroxide. Biological testing of this compound showed weak toxicity to both *Lemna* plants and field bindweed leaf-cuttings, indicating that it may be involved in the phytotoxic effects of *P. convolvulus*.

4. 5. Isolation of metabolites having a phthalide or α-pyrone structure from P. Convolvulus

4. 5. 1. Isolation and HPLC analysis

Purification of the phytotoxic crude **Pc 5** (Fig. 28) proved to be exceedingly difficult and totally unsuccessful using either silica or cellulose chromatography. Flash column chromatography, however, using reverse phase silica gel led to a moderate separation of the components. The reverse phase silica was prepared using the methodology developed by Evans and co-workers.[244]

Normal phase silica gel was reacted with *n*-octadecyltrichlorosilane in a suspension of dry CCl_4 over a period of two hours. The bonded silica gel was then washed free of unreacted silane and any residual chlorines (of the attached silane) were subsequently converted to methoxy groups with dry methanol. Removal of any unreacted silanol groups (capping of the silica) was achieved through a second reaction of the bonded silica with trimethylchlorosilane. The fully deactivated silica gel was then used for flash column chromatography of the *P. convolvulus* crude extracts.

The effectiveness of reverse phase flash column chromatography in the separation of complex natural-product mixtures has been demonstrated before.[245] The crude sample is usually first dissolved in an organic-aqueous solvent mixture and then adsorbed onto the silica by evaporation of all the organic solvents to obtain an aqueous slurry. The column, packed in methanol and equilibrated to water, is eluted with a gradient of solvent mixtures ranging in polarity from pure water to pure methylene chloride.

The metabolite mixture **Pc 5**, from *P. convolvulus*, was thus partitioned *via* reverse phase flash chromatography using the solvent gradient outlined in Table 11. All fractions (of ~20 mL volume) were assayed for biological activity using leaf-puncture bioassays and *Lemna* plants. The results obtained are also noted in Table 11.

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244. M.B. Evans, A.D. Dale and C.J. Little, *Chromatographia* **13**, 5 (1980).

245. J.W. Blunt, V.L. Calder, G.D. Fenwick, R.J. Lake, J.D. McCombs, M.H.G. Munro and N.B. Perry, *J. Nat. Prod.* **50**, 290 (1987).

Table 11: Reverse phase flash chromatography of crude Pc 5

Solvent (v/v in mL)	Activity	Solvent (v/v in mL)	Activity
H_2O (25)	-	CH_3OH (25)	-
H_2O (25)	-	CH_3OH/CH_2Cl_2 (23/2)	-
H_2O/CH_3OH (23/2)	-	CH_3OH/CH_2Cl_2 (20/5)	-
H_2O/CH_3OH (20/5)	-	CH_3OH/CH_2Cl_2 (15/10)	-
H_2O/CH_3OH (15/10)	++	CH_3OH/CH_2Cl_2 (10/15)	-
H_2O/CH_3OH (10/15)	+++	CH_3OH/CH_2Cl_2 (5/20)	-
H_2O/CH_3OH (5/20)	+	CH_3OH/CH_2Cl_2 (2/23)	-
H_2O/CH_3OH (2/23)	-	CH_2Cl_2 (25)	-

Biological activity observed: - none, + weak, ++ moderate, +++ strong

In hope of achieving better separation of the phytotoxic metabolites, the above procedure was repeated collecting much smaller volume fractions (~8 mL) and changing the polarity of the solvent system even more gradually. Due to the small amounts of available sample, the new fractions were not assayed for biological activity. Instead, their chemical composition was compared to the phytotoxic fractions previously isolated (Tab. 11) by thin layer chromatography. Fractions 18 to 21 appeared to have the same composition as the crudes found to be active, hence, they were further analyzed by HPLC chromatography.

Metabolites **91** and **92** were isolated from the combined fractions 18-19 after HPLC chromatography on a C18 reverse phase column eluted with 59.8% H_2O, 40% CH_3OH, 0.2% CH_3COOH. Their respective retention times (**91, 92**) were 22-23 minutes and 16-17 minutes after the void volume, at a flow rate of 2 mL/min. Metabolite **92** was re-chromatographed using the same column and flow rate but a solvent mixture of 54.1% H_2O, 45.8% CH_3OH, 0.1% CH_3COOH in order to obtain a high purity sample. Under these conditions metabolite **92** eluted 10-11 minutes after the void volume.

Metabolites **93**, **94** and **96** were isolated from the combined fractions of 20-21 after HPLC chromatography on a C18 reverse phase column. The two compounds were eluted using a solvent

mixture of 54.4% H_2O, 45.4% CH_3OH, 0.2% CH_3COOH at a flow rate of 2 mL/min. The retention times of **94**, and **93** were 20-22, and 27-29 minutes after the void volume respectively. An additional minor compound (**96**) eluted as a shoulder peak from metabolite **93** (at 25-26 min.). This latter metabolite had to be chromatographed twice, under the same conditions, in order to achieve complete separation from metabolite **93**.[246]

4. 5. 2 Identification of the metabolite 4-carboxy-3-hydroxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (91)

The ^1H NMR of metabolite **91** strongly suggested an aromatic or other unsaturated type of molecule. There were only four easily distinguishable substituents, a methyl group at 2.35 ppm, a methoxy group at 4.14 ppm and two single protons at 6.91 and 8.09 ppm. The solubility of this compound in non-protic NMR solvents was very poor, hence, information on the presence of any exchangeable protons could not be easily obtained.[247]

Since coupling was not observed between any of the protons on the molecule, NMR experiments using Nuclear Overhauser Enhancement effects were carried out (Tab. 12) in an attempt to gain information on the relative positions of the four substituents. A positive NOE effect was observed between the methoxy and the methyl groups, and between the methyl and the proton at 8.09 ppm. The proton at 6.91 ppm did not exhibit an NOE effect with any of the other protons, suggesting that its position on the molecule was remote from the other substituents.

---

246. ^1H NMR and CI (NH_3) mass spectroscopy of this minor metabolite suggested a very similar structure to metabolites **91**, **92** and **93**. However, the amount of available material did not permit complete stucture determination. The data on this metabolite is given in the experimental section of the thesis.

247. The amount of available sample from each compound was extremely small and had to be saved for biological testing experiments at the end of this project. Hence, ^1H NMR experiments in DMSO were not carried out as recovery of the pure metabolites without any loss of mass would have been difficult.

Table 12: Nuclear Overhauser Enhancement in the ^1H NMR (300 MHz, acetone-d_6) spectrum of 91.

Saturated Signal (δ)	Observed Enhancement (δ)
2.35 (9-CH_3)	4.14 (10-OCH_3, ~10%), 8.09 (H5, ~4%)
4.14 (10-OCH_3)	2.35 (9-CH_3, ~14%)
8.09 (H5)	2.35 (9-CH_3, ~14%).

The ^{13}C NMR of metabolite **91** (Fig. 37) showed the presence of eleven different types of carbons. The presence of a methyl (15.7 ppm) and a methoxy (62.6 ppm) group, both attached to an sp^2-hybridized carbon, were confirmed. APT NMR showed seven of them to be quaternary carbons at 166.4, 165.7, 160.5, 149.4, 134.3, 121.9 and 118.3. The remaining two signals (139.3 and 97.4 ppm) were shown to be tertiary, through a DEPT experiment, and were assigned to the carbons carrying the single protons at 8.09 and 6.91 ppm respectively.

The chemical shift of the carbon at 97.4, as well as the chemical shift of its proton (6.91 ppm), indicated a -O-CH-O- moiety as a possible part of this molecule. From the quaternary carbons, the two signals at 166.4 and 165.7 were thought to be carbonyl carbons of either an acid or an ester. The downfield signal at 160.5 ppm is characteristic of aromatic carbons attached to a hydroxy or methoxy group, hence, it was tentatively assigned as such and connected to the methoxy substituent. The rest of the carbons were tentatively assigned to four carbons of a benzene ring.

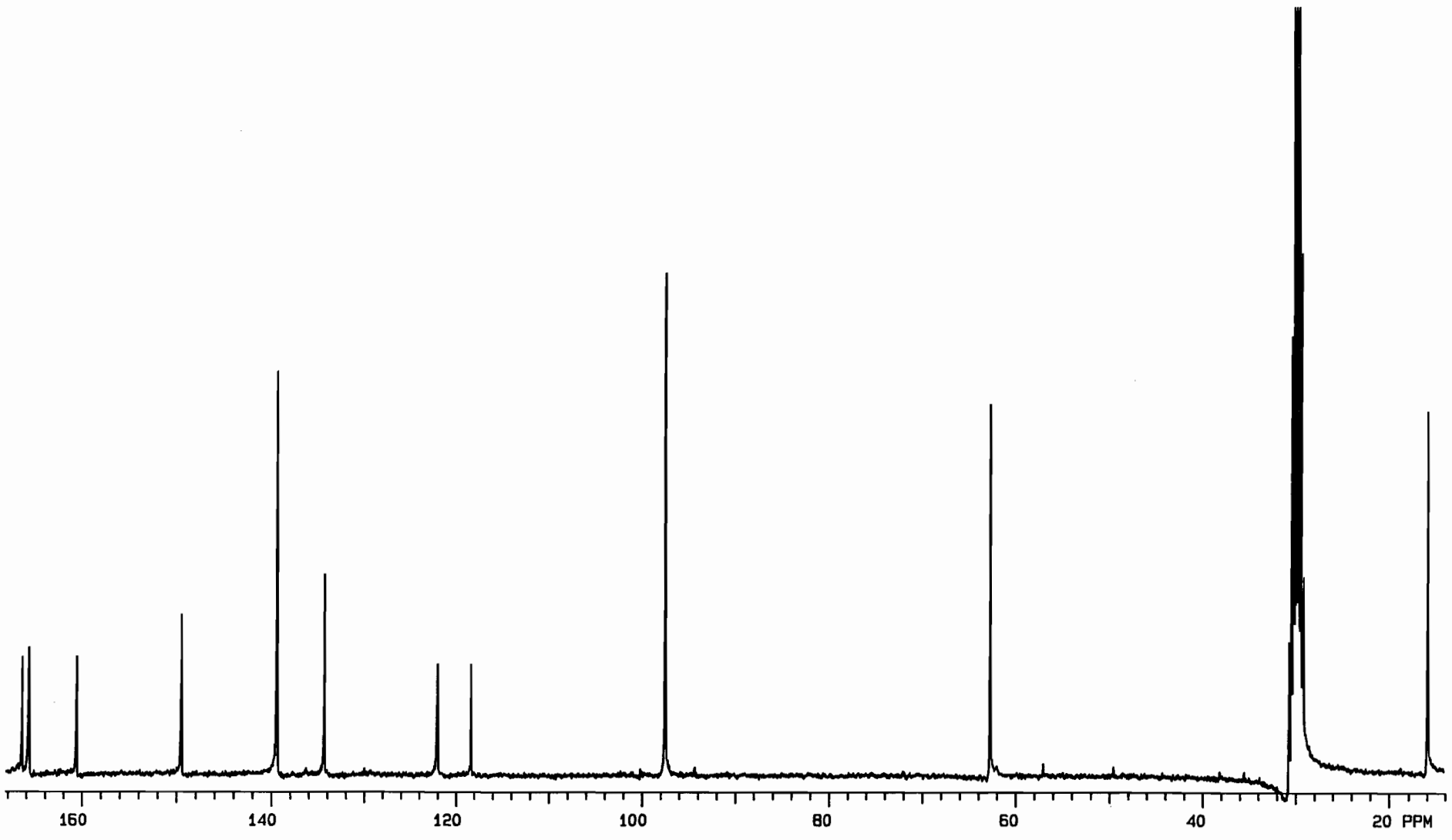
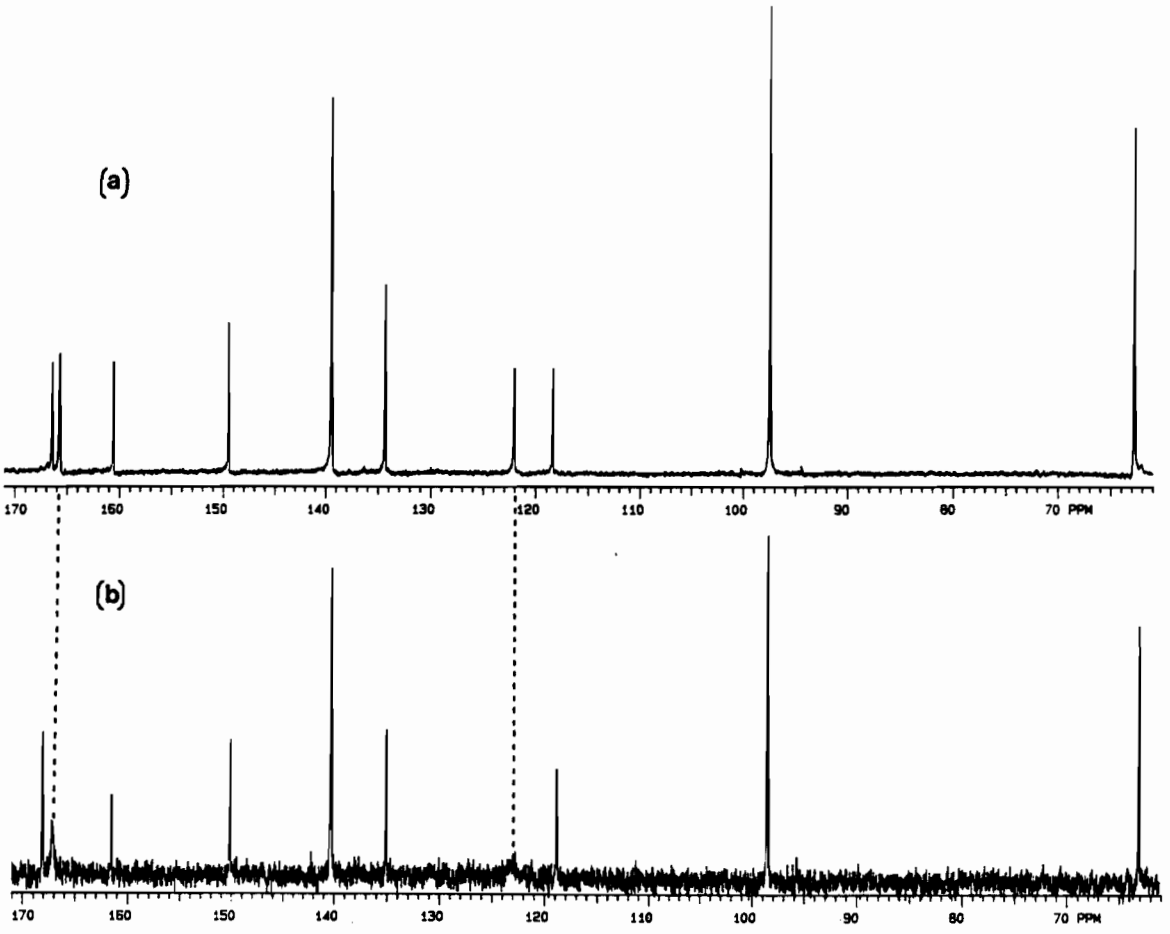


Fig. 37: ^{13}C NMR (300 MHz, acetone-d_6) of metabolite 91 at -52° C

Most of the ^{13}C NMR experiments had to be conducted at very low temperatures (-45° to -55° C) since it was noted that the signals at 165.7 and 121.9 ppm were very broad when the normal ^{13}C NMR spectra (Fig 38b) was recorded at room temperature and they were almost nonobservable in the APT NMR (Fig. 39b). However, in low temperature NMR experiments (-45° to -55° C) these signals were observed without any difficulty (Fig. 38a and Fig. 39a).

Fig. 38: Downfield region of the ^{13}C NMR (300 MHz, acetone-d_6) of metabolite 91
a) at -52° C, b) at room temperature



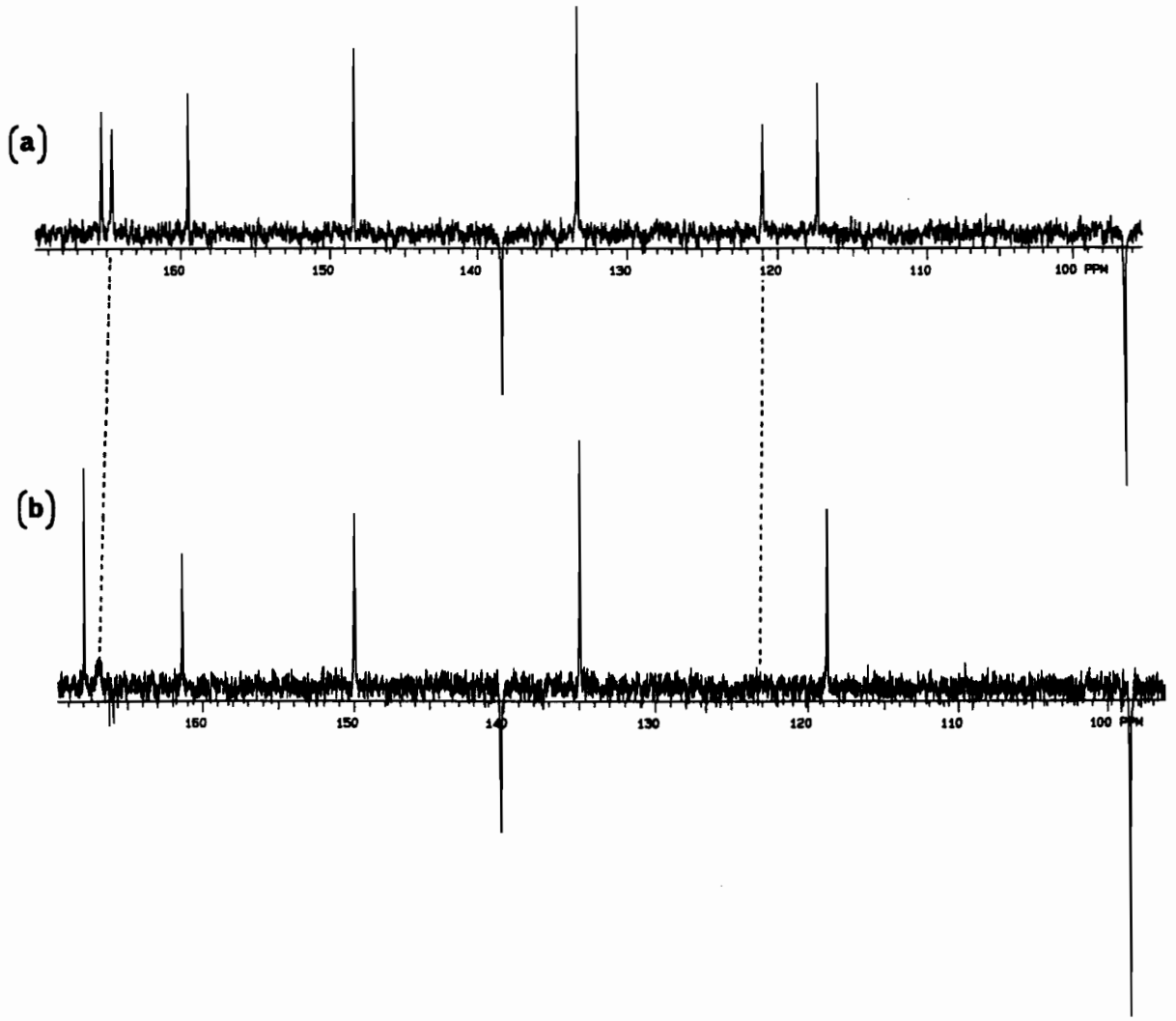
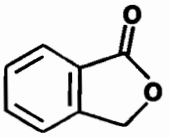


Fig. 39: Downfield region of the APT NMR (300 MHz, acetone-d_6) of metabolite 91
a) at -52° C, b) at room temperature

Such an effect usually indicates the existence of two equilibrium structures with a fast exchange taking place at room temperature In cases where the equilibrium constant permits both structures to exist in significant amounts, two separate sets of signals may be observed by carrying out the NMR experiments at sufficiently low temperature. This was not the case with metabolite **91**. The presence of a carboxylic acid moiety attached to an aromatic carbon was proposed as a possible explanation for the results obtained with this compound. In such molecules a very small degree of dissociation of the acidic proton often causes the broadening of both the carbonyl carbon (165.7 ppm) and the carbon to which it is directly attached (121.9 ppm).

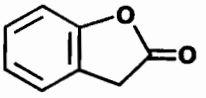
The IR spectra of metabolite **91** showed an OH absorption at 3381 cm^{-1}, -C=C- absorption at 1613 cm^{-1}, and two carbonyl absorptions at 1728 and 1773 cm^{-1}, possibly due to a carboxylic acid and a five-membered ring conjugated lactone. Literature IR values of lactones fused to a benzene ring (e.g. structures **96A-C**) seemed to support a phthalide type structure (**96A**).

IR values of carbonyl:



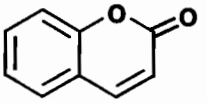
(96 A)

~1750-1780 cm^{-1}



(96 B)

~1760-1820 cm^{-1}

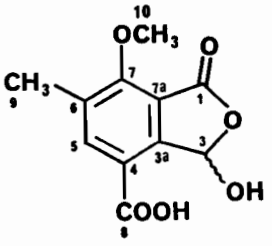


(96 C)

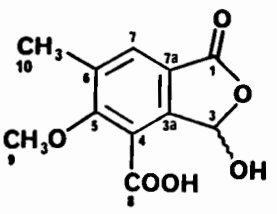
~1700-1720 cm^{-1}

Chemical ionization (NH_3) mass spectrometry gave an ion at 256 ($M^+ + NH_3$) with relative intensity of 100%, a molecular ion of 239 ($M^+ + 1$) with relative intensity of 53%, and two other ions both produced by the loss of -CHO at 227 [$M^+ + NH_3$-(-CHO)] and at 209 [M^+-(-CHO)]. These results supported an elemental composition of $C_{11}H_{10}O_6$ for this compound.

Based on all of the above data, two structures, (a) and (b), were considered possible for metabolite **91**. Final proof, however, in support of structure (a) was obtained through the data of low temperature ^1H-^{13}C coupled NMR experiments and literature ^{13}C NMR values of related compounds.



(a)



(b)

In the fully coupled spectra of metabolite 91 (300 MHz, at -52°C), large coupling constants characteristic of ^1J values were observed for the signals at 139.3, 97.4, 62.6 and 15.7 ppm as expected. The signal at 139.3 ppm appeared as a doublet of quartets due to additional long-lange coupling with the protons of the methyl group (^1J=162 Hz, ^3J=5 Hz). Similarly, the methyl group at 15.7 ppm appeared as a quartet of doublets (^1J=128 Hz, ^3J=5 Hz) which collapsed to a simple quartet upon decoupling of the aromatic proton at 8.09 ppm. The expected results were also obtained with the signal at 97.4 ppm, which was assigned to the C-3 carbon. This carbon (C-3) appeared as a doublet (^1J=179 Hz) in the coupled spectra but changed to a singlet when the proton at 6.91 ppm was decoupled. The quaternary carbons at 121.9 and 118.3 ppm were singlets in all spectra and they were assigned to C-4 and C-7a respectively. Although, the reverse assignment could be argued for these two signals, the broadness of the 121.9 peak in the room temperature NMR spectrum, is believed to be due to the attached ionizable carboxylic acid. The quaternary multiplets at 134.2 and 160.5 ppm were assigned to C-6 and C-7 respectively in structure (a) or C-6 and C-5 in structure (b), since both of them showed long-range coupling with the methyl and methoxy protons.

The splitting pattern of the remaining quaternary carbons were anticipated to show enough differences between structures (a) and (b) so as to provide proof for the identity of metabolite 91. Several small but important differences in the coupling patterns of these two structures were expected. For example, coupling between the aromatic proton (H5 or H7) and the lactone carbonyl was believed unlikely in structure (a) but it was expected in structure (b). ^3J coupling constants between substituents of a benzene ring are usually of the order of 5-7 Hz. The opposite results were anticipated for the coupling of the carboxylic acid carbon and the aromatic proton. ^3J coupling would be expected in structure (a) between H5 and C-8. However, such coupling would be unlikely in structure (b), H7 to C-8, since coupling between protons and carbons separated by more than three bonds is rare and their coupling constants decrease in value with increasing distance (e.g. ^4J=0-1.5 Hz). On the other hand, ^3J coupling between the aromatic proton (H5 or H7) and C-3a, and between H3 and C-1 (the lactone carbonyl) would most likely be observed with either structure. The coupling constant of the latter signal (C-1 signal coupled to H3) is less predictable and, as in all other cases, would depend on the dihedral angle between H3 and the lactone carbonyl.

Both of the carbonyl carbons and the C-3a signal appeared as doublets in the fully coupled spectra (Fig. 40c). Selective decoupling of the aromatic proton (8.09 ppm) led to the collapse of the doublet at 149.4 ppm (C-3a) as expected, and of the carboxylic acid doublet at 165.7 ppm (Fig. 40a) but had no affect on the doublet of the lactone (166.4 ppm, 3J=4 Hz). However, selective decoupling of the proton at 6.91 ppm (H3) affected both carbonyl carbons, changing the lactone into a sharp singlet and the acid into a broad singlet (Fig. 40b). The broadness of the latter signal was due to the

coupling between the acid and the aromatic proton (H5). These results were in strong support of (a) as the correct structure for metabolite 91. A summary of all the data obtained from these experiments is given in Table 14.

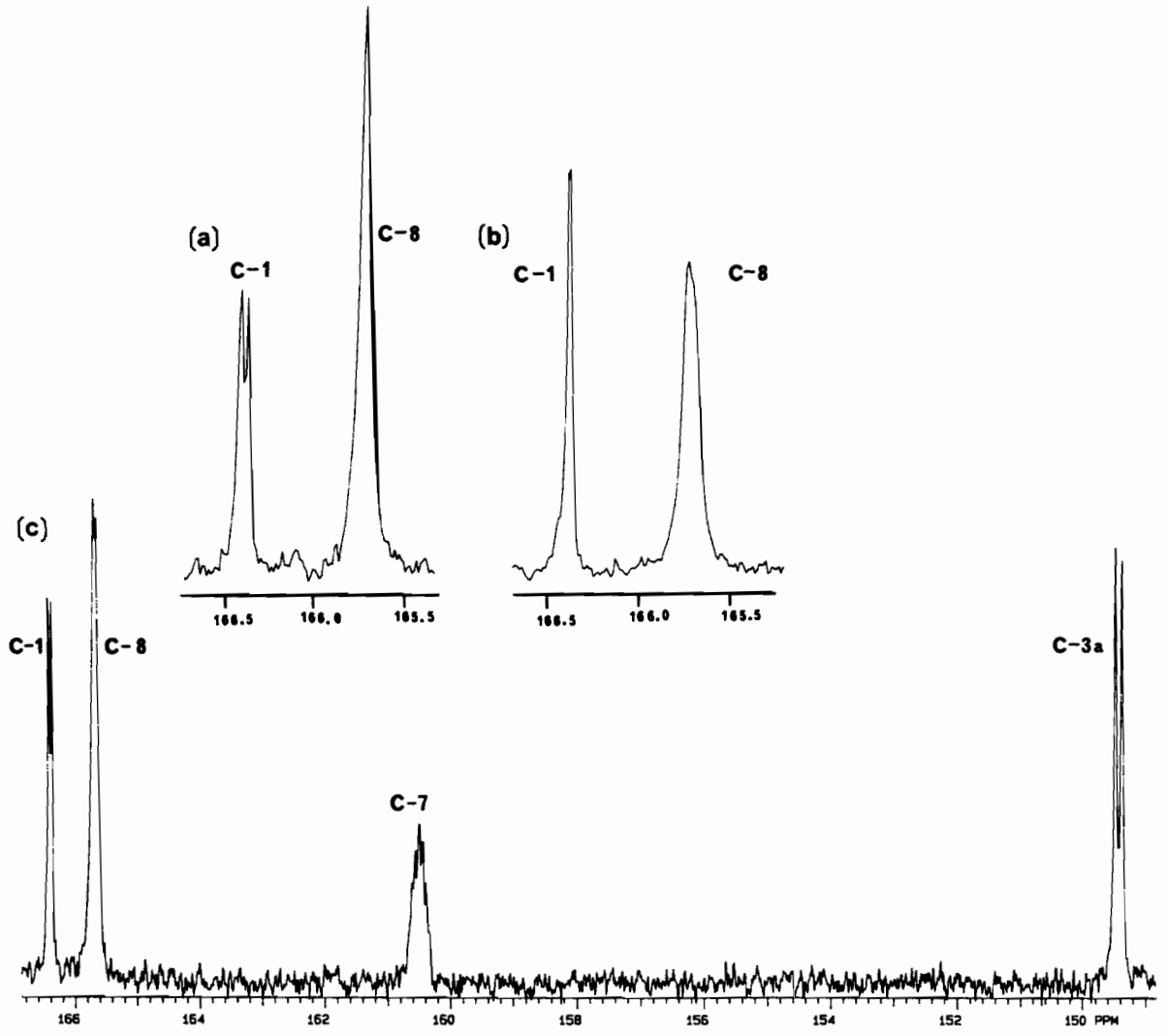


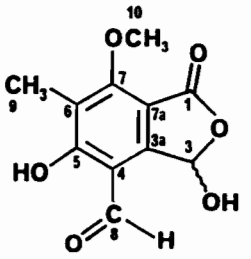
Fig. 40: ^{1}H-^{13}C coupled NMR (300 MHz, acetone-d_6, -52° C) signals of metabolite 91:

a) effects of H5 decoupling on C-1 and C-8 signals,

b) effects of H3 decoupling on C-1 and C-8 signals,

c) fully coupled signals of C-1, C-8, C-7 and C-3a.

A literature search for compounds structuraly related to metabolite **91** revealed, among others, the natural product 4-formyl-3,5-dihydroxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (**97**). This metabolite was isolated by Achenbach[248] and co-workers from the fungus *Aspergillus duricaulis* and shown to possess antibacterial properties.



(97)

Considering the close structural similarities between metabolite **91** and compound **97**, it was assumed that approximate ^{13}C chemical shifts for metabolite **91** could be calculated from those of **97**. The empirical parameters normally used for the calculation of chemical shifts in a substituted benzene ring (Tab. 13) were added to the reported values for compound **97**. For example, the reported chemical shift for C-5 of compound **97** was 166.0 ppm. To this value, the affects of an -OH group directly attached (-26.9 ppm), and of an *ortho* aldehyde (-1.3 ppm) were removed, where the affect of an *ortho* carboxylic acid (+1.5 ppm) was added. The empirical chemical shift obtain from this calculation (139.3 ppm) was identical to that observed for the C-5 carbon of metabolite **91**. All other calculated values were within 0 to 3 ppm from actual shifts and they are shown in Table 14.

It is also interesting to note, that metabolite **91** did not exhibit any optical activity. The spontaneous racemization of 3-hydroxylactones through ring-chain tautomerism[249] is a well known phenomenon which has been reported for many compounds, including metabolite **97**.[248] In an

248. H. Achenbach, A. Muhlenfeld, B. Weber, W. Kohl and G.-U. Brillinger, *Z. Naturforsch* **37B**, 1091 (1982).

249. D.H. Kim and D.N. Harpp, *Chem. and Indust.*, 183 (1965), and references therein.

analogous fashion, metabolite 91 is believed to exist in equilibrium with its aldehyde-carboxylic acid tautomer.

Table 13: Empirical parameters for the calculation of chemical shifts in substituted benzenes[250]

- R	C-1	*ortho*	*meta*	*para*
- H	0	0	0	0
- CH_2OH	+ 12	- 1.0	0	- 1.0
- CH_3	+ 9.3	+ 0./8	0	- 2.9
- COO^-	+ 8.0	+ 1.0	0	+ 3.0
- COOH	+ 2.1	+ 1.5	0	+ 5.1
- CHO	+ 8.6	+ 1.3	+ 0.6	+ 5.5
- OH	+ 26.9	- 12.7	+ 1.4	- 7.3
- OCH_3	+ 31.4	- 14.4	+ 1.0	- 7.7

The ^{13}C NMR data of several naturally occuring phthalides[251] and synthetic compounds[252] was also considered. On the bases of all the evidence, the structure of 91 was assigned to be that of (a) (p. 121).

250 F.W. Wehrli and T. Wirthlin, in *Interpretation of Carbon-13 NMR Spectra*, Heyden, Heyden and Son Ltd. (1978).

251. a) F.C. Fischer and M.J.M. Gijbels, *Planta medica*, 77 (1987).
b) M. Fujita, M. Yamada, S. Nakajima, K.-I. Kawai and M. Nagai, *Chem. Pharm. Bull.* 32, 2622 (1984).

252. E. Solcaniova, P. Hrnciar and T. Liptaj, *Collection Czechosiov. Chem. Commun.* 45, 2772 (1980).

Table 14: ^{13}C NMR (300 MHz, acetone-d_6, - 52°C) of metabolite 91

C	decoupled δ	calculated δ	coupled	H3 coupled	H5 coupled
1	166.4	~ 167	d; $^3J_{C\text{-}H3}$ = 4 Hz	s	d
8*	165.7*	---	d; J = ~ 2.5 Hz	s(br)‡	s(sh)
7	160.5	161.6	m;	m	m
3a	149.4	152.5	d; $^3J_{C\text{-}H5}$ = 7.7 Hz	d	s
5	139.3	139.3	d, q; ^1J = 162 Hz d; $^3J_{C\text{-}H9}$ = 5 Hz	d, q	q
6	134.2	133.6	q; $^2J_{C\text{-}H9}$ = 6 Hz	q	q
4*	122*	119-124	s	s	s
7a	118.3	117.7	s	s	s
3	96.0	96.0	d; ^1J = 179 Hz	s	d
10	62.7	63.1	q; ^1J = 146 Hz	q	q
9	15.7	---	d, q; ^1J = 128 Hz $^3J_{C\text{-}H5}$ = 5 Hz	d, q	q

* broad signals at room temperature

‡ broad signal due to ^4J coupling between C-8 and H3 of ~ 1 - 2 Hz

4. 5. 3 Identification of the metabolite 4-(hydroxymethyl)-7-methoxy-6-methyl-1(3H)-isobenzofuranone (92)

Metabolite **92** was sufficiently soluble in deuterated chloroform to permit a complete proton spectra to be observed (Fig. 41). As in the case of metabolite **91**, a methyl and a methoxy group were present, at 2.26 and 3.90 ppm respectively. An exchangeable, very broad signal (1H) was observed at 2.6-2.7 ppm which was coupled to a doublet (2H, J=6 Hz) at 4.66 ppm. The extreme broadness of this signal (believed to be an -OH) indicated further, long-range coupling to some other proton, although all other signals appeared as singlets. Selective irradiation of a proton at 7.31 ppm led to moderate sharpening of this peak (~2.6 ppm) and change into a triplet (J=~6 Hz). In addition, the irradiation of the doublet (4.66 ppm) led to the collapse of the exchangeable proton into a broad singlet, as it was expected. The ^{1}H NMR of metabolite **92** contained only one other signal at 5.22 ppm, integrating to two protons.

Close similarities between **92** and **91** were also observed in the ^{13}C NMR spectra. A total of eleven carbons were observed (Fig. 42), one of which was a sp^2-hybridized -CH (137.2 ppm), two were secondary carbons (-CH_2, 62.2 and 70.1 ppm), one methoxy (62.5 ppm) and one methyl (15.4 ppm), as it was indicated by its DEPT NMR (Fig. 43). From the remaining six quaternary carbons only one had the appropriate chemical shift for a carboxylic acid or lactone (171.2), four signals had chemical shifts very close to those of C-3a, C-6, C-7 and C-7a of metabolite **91** and one was approximately 10 ppm downfield from the C-4 carbon of metabolite **91**. It was also noted, that the room temperature ^{13}C NMR spectra of **92** did not show any broad signals neither was it any different when recorded at low temperature. This observation, coupled with the fact that a carbonyl carbon was lost and a -CH_2-OH gained in going from the spectra of metabolite **91** to that of **92** suggested the reduction of the carboxylic acid to a primary alcohol. The 10 ppm shift of the C-4 carbon would also be expected if that was the case (Tab. 16).

The IR ($CHCl_3$) data of metabolite **92** was consistent with the proposed loss of the carboxylic acid and showed absorptions for only one carbonyl, that of the lactone at 1762 cm^{-1}, of an alcohol at 3630 and 3540 cm^{-1} and of double bonds at 1600 cm^{-1}.

High resolution, chemical ionization (NH_3), mass spectrometry gave a molecular ion of 209.0814 ($M^+ + 1$) suggesting an elemental complosition of $C_{11}H_{12}O_4$ for metabolite **92** (calculated mass: 209.0814).

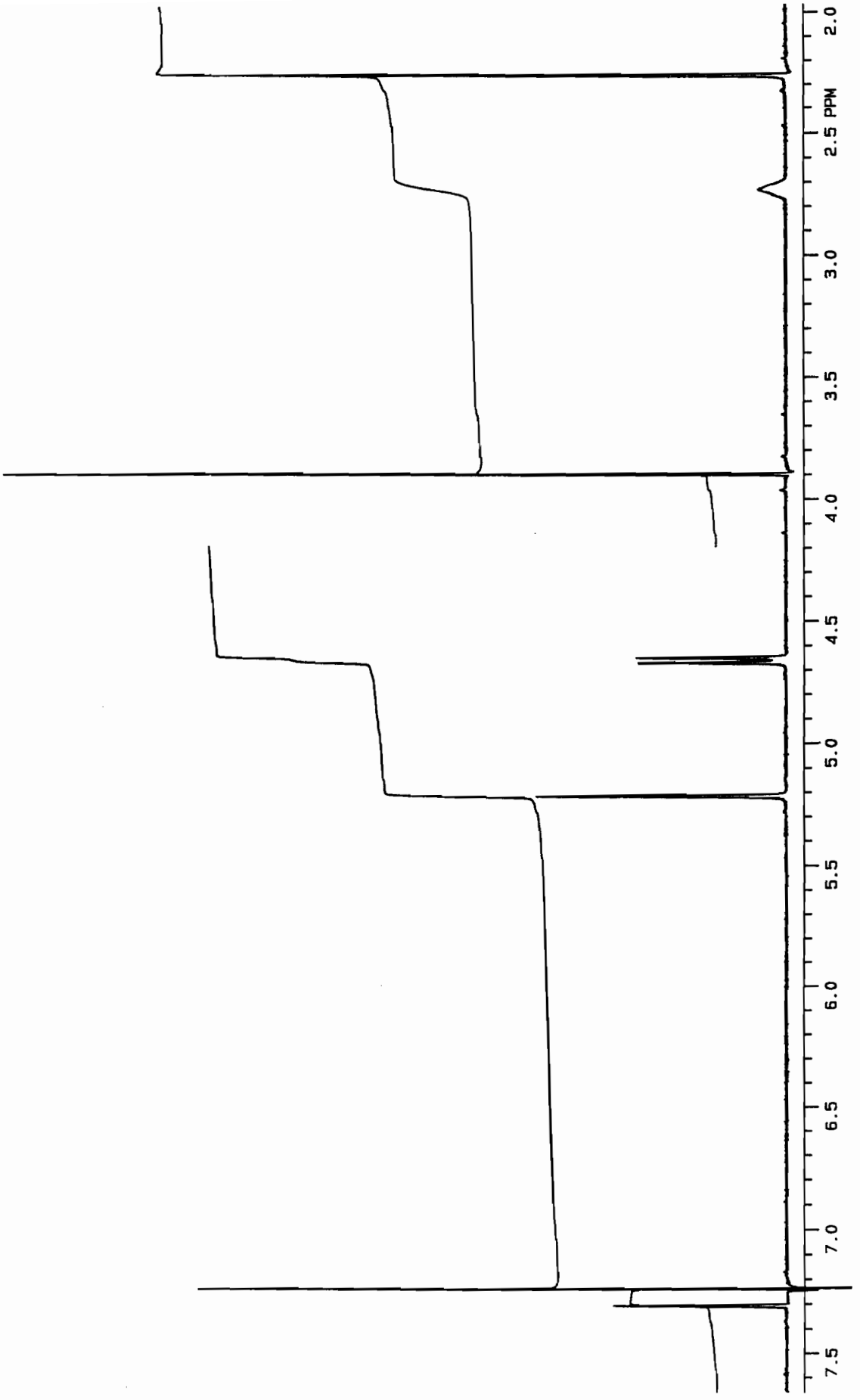


Fig 41: ^{1}H NMR (300 MHz, $CDCl_3$) of metabolite 92

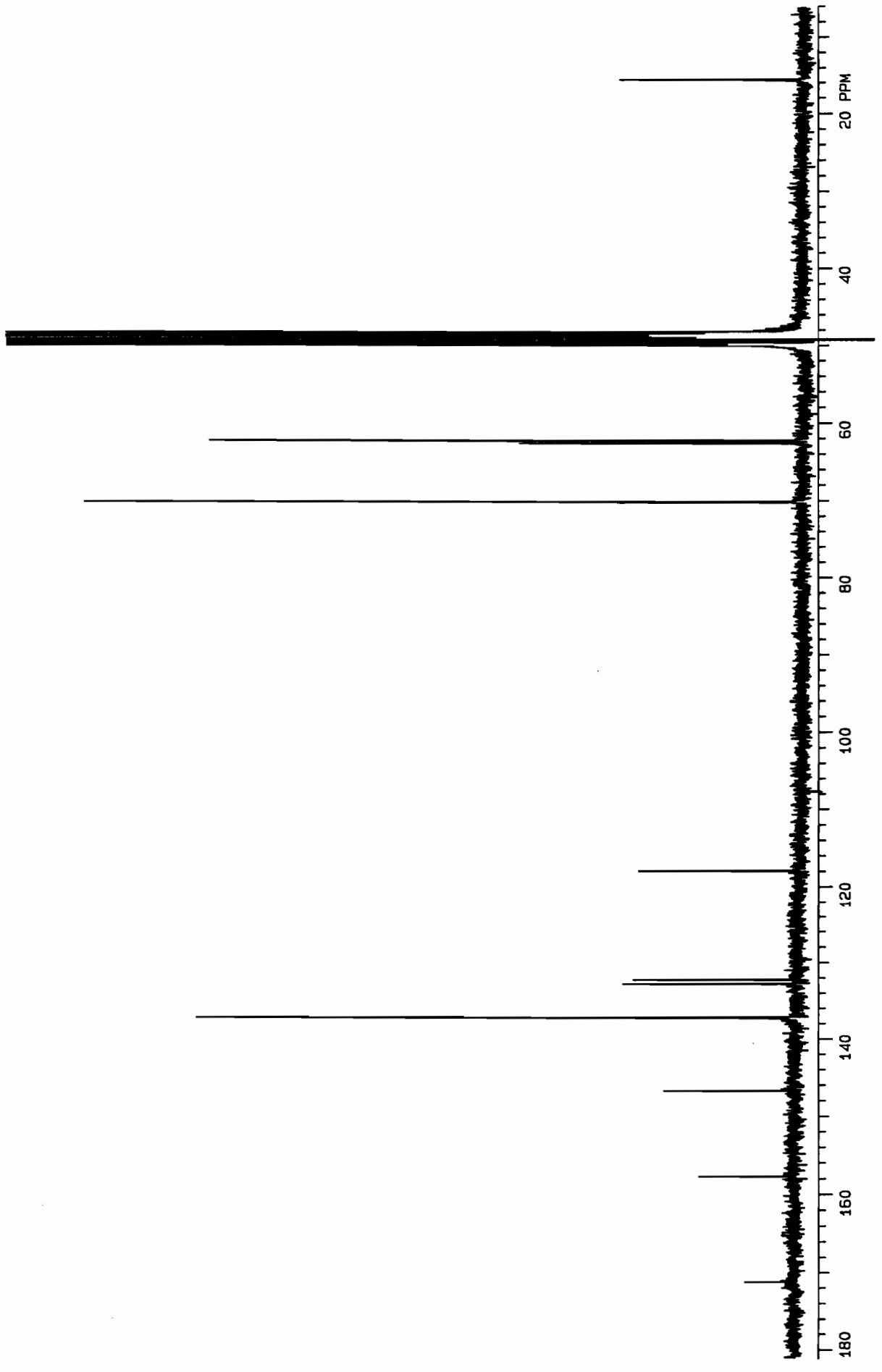


Fig. 42: ^{13}C NMR (300 MHz, CD_3OD) of metabolite 92

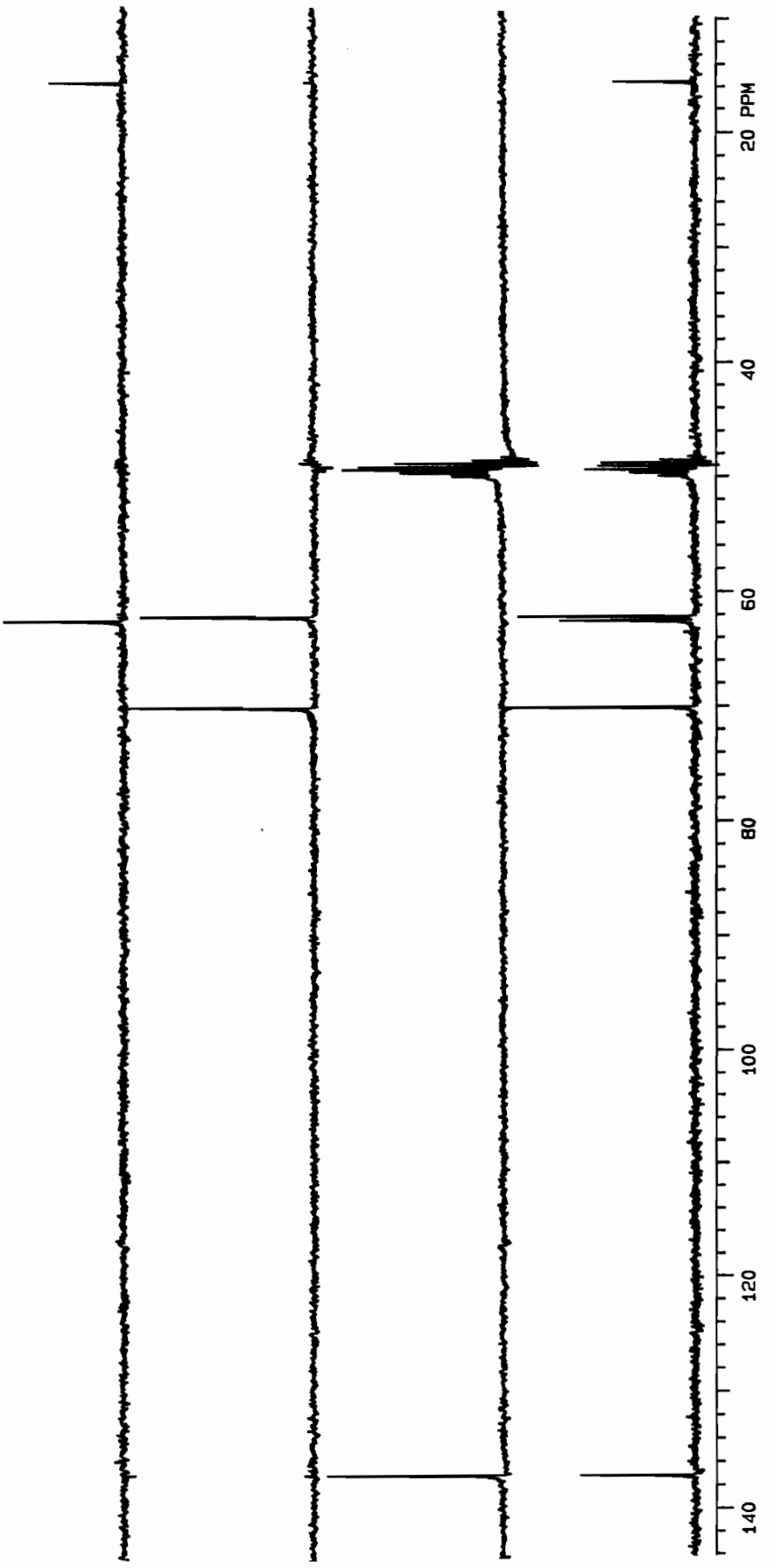
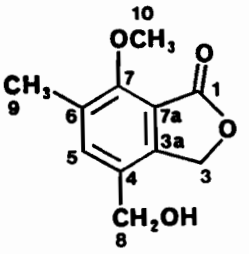


Fig. 43: DEPT NMR (300 MHz, CD_3OD) of metabolite 92

Taking into consideration all of the above data the structure of 4-(hydroxymethyl)-7-methoxy-6-methyl-1(3H)-isobenzofuranone was proposed for metabolite **92**. However, further investigation into this structure was felt nessesary in order to be certain of this assignment.



(92)

The results obtained from the Nuclear Overhauser Enhancement experiments (Tab. 15) on metabolite **92** were consistent with the proposed structure. Strong positive NOE effects were observed between H5--H9 and H8--H5. Saturation of the C-8 protons (4.66 ppm) gave strong enhancement of the H5 signal but only a moderate amount of enhancement of the methyl signal (H9). Very weak effects were found between the methyl and the methoxy groups as well as the C-3 protons and the methoxy. This latter effect could be due to the conformation where the methoxy substituent is away from both the methyl and the neighboring carboxyl and hence, over the plane of the ring and possibly within NOE distance from the C-3 protons.

Table 15: Nuclear Overhauser Enhancement in the ^{1}H NMR (300 MHz, acetone-d_6) spectrum of metabolite 92.

Saturated Signal (δ)	Observed Enhancement (δ)
2.28 (9-CH_3)	3.98 (10-OCH_3, ~2%), 7.48 (H5, ~6%)
3.98 (10-OCH_3)	2.28 (9-CH_3, <2%), 7.48 (H5, <2%)
4.68 (2xH8)	7.48 (H5, ~12%), 5.33 (2xH3, ~3%)
5.33 (2xH3)	4.68 (2xH8, <2%), 3.98 (10-OCH_3, <2%)
7.48 (H5)	2.28 (9-CH_3, ~5%), 4.68 (2xH8, ~2%).

The coupled ^{1}H-^{13}C NMR spectrum of metabolite **92** did not show any long-range coupling between the C-3 protons and the lactone carbonyl. However, even with metabolite **91** the ^{3}J value observed between C-1 and H3 was very small. The values of ^{2}J and ^{3}J coupling constants are greatly affected by the conformation of a molecule and the dihedral angles involved, hence, some differences between the two compounds were to be expected. The splitting patterns of the C-3a and the C-5 carbons were more complex in this case than for metabolite **91** due to extra coupling with the C-8 protons. In the fully coupled spectrum C-3a appeared as a multiplet and C-5 as a doublet of multiplets (Fig. 44c). Selective decoupling of the two C-8 protons led to a change of the C-3a signal to a doublet ($^3J_{C\text{-}H5}$=8.4 Hz) and the C-5 signal to a doublet of quartets (Fig. 44b), analogous to that observed with metabolite **91**. The C-4 signal was also affected (became much sharper) indicating a small ^{2}J coupling with the C-8 protons. Decoupling of H5 had no effect on C-4 but changed the C-3a signal into a very narrow multiplet, or a triplet with a very small coupling constant (Fig. 44a).

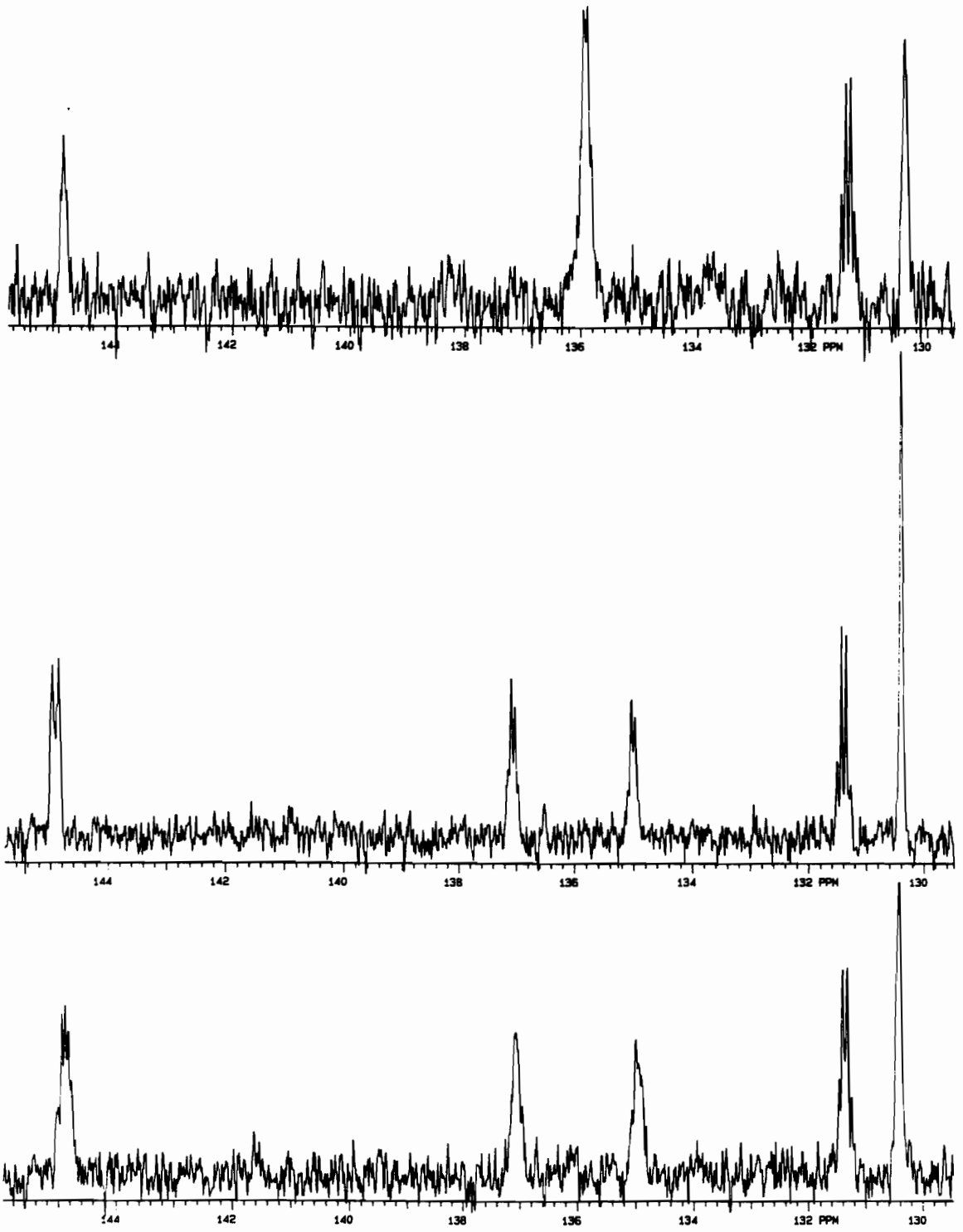
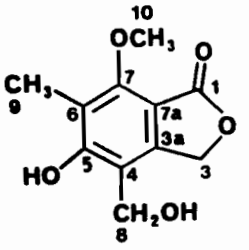


Fig. 44: ^{1}H-^{13}C (300 MHz, CD_3OD) coupled and selectively decoupled signals of metabolite 92.

a) H5 decoupled, b) H8 decoupled, c) fully coupled.

There were a number of other carbon signals which appeared coupled. A triplet ($^1J = 155$ Hz) was observed for C-3 and a triplet of doublets ($^1J = 139$ Hz, $^3J = 12$ Hz) for C-8. The latter signal changed to a doublet ($^3J = 12$ Hz) when the C-8 protons were decoupled and to a triplet ($^1J = 139$ Hz) when H5 was decoupled. The remaining of the data was very similar to that obtained for metabolite **91** and in agreement with the proposed structure of metabolite **92** (Tab. 16).

Finally, the ^{13}C NMR chemical shifts of the literature compound 5-hydroxy-4-(hydroxymethyl)-7-methoxy-6-methyl-1(3H)-isobenzofuranone (**98**), a metabolite of the fungus *Aspergillus duricaulis*,[253] were used in order to calculate the "theoretical" chemical shifts for metabolite **92**. The results obtained using the substituent differences from Table 13, were within ~2 ppm of the experimental values (Tab. 16).



(98)

253. H. Achenbach, A. Muhlenfeld and G. U. Brillinger, *Liebigs Ann. Chem.*, 1596 (1985).

Table 16: ^{13}C NMR (300 MHz, acetone-d_6, - 52°C) of metabolite 92

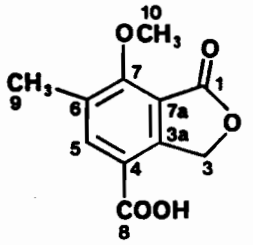
C	decoupled δ	calculated δ	coupled	H8 decoupling	H5 decoupling
1	171.2	~ 170	s	s	s
7	157.7	155.4	m	m	m
3a	146.7	144.6	m	d; $^3J_{C\text{-}H5}$ = 8.4 Hz	s(br)
5	137.2	134.8	d, m; ^1J = 156 Hz	d, q; $^3J_{C\text{-}H9}$ = 5 Hz	m
6	132.7	132.1	q; $^2J_{C\text{-}H9}$ = 6 Hz	q	q
4	131.2	128.1	s	s	s
7a	117.8	116.3	s	s	s
3	70.1	68.4	t; ^1J = 155 Hz	t	t
10	62.5	62.2	q; ^1J = 145 Hz	q	q
8	62.1	59.8	t, d; ^1J = 139 Hz	d; $^3J_{C\text{-}H8}$ = 12 H_2	t
9	15.4	---	d, q; ^1J = 128 Hz, $^3J_{C\text{-}H5}$ = 5 Hz	d, q	q

4. 5. 4 Identification of the metabolite 4-carboxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (93)

Compound **93** was one of the three metabolites of *P. convolvulus* to be isolated from the more polar components of crude **Pc 5** (Fig. 28). As it was mentioned previously, partitioning of **Pc 5** *via* a reverse phase flash column, followed by HPLC chromatography of fractions 20 and 21 gave pure metabolite **93** as an amorphous white solid.

A methyl group at 2.34 ppm, a methoxy at 4.15 ppm and a single aromatic proton at 8.13 ppm were once again present in the ^{1}H NMR spectrum. An additional signal at 5.49 ppm (s, 2H) was the only major difference between this compound and metabolite **91**.

The ^{13}C NMR spectra of the new compound (**93**) was nearly identical to that of **91** with the exception of one carbon. When the spectra was recorded at room temperature, a sharp carbonyl signal at 168.4 ppm (lactone) and a very broad carbonyl signal at 166.4 ppm (carboxylic acid) were observed. An additional broad quaternary signal at 120.5 ppm was assigned to a carbon directly attached to the carboxylic acid. It should be noted that low temperature (-52° C) had the same sharpening effect on these two ^{13}C signals as it did with those of compound **91**. The methoxy and methyl signals appeared at 62.6 and 15.6 ppm respectively and the aromatic =CH- carbon at 138.8 ppm. A low field signal at 161.2 ppm was, once again, assigned to the carbon carrying the methoxy substituent. The remaining three quaternary carbons at 150.7, 132.2 and 117.6 ppm corresponded closely to the carbons C-3a, C-6 and C-7a, respectively, of metabolite **91**. The only major difference observed between the two compounds was in the chemical shift and substitution of the C-3 carbon. In metabolite **91** this carbon appeared at 97.4 ppm (-O-CH-O-) where in **93** it was a $-CH_2-$ carbon at 70.9 ppm (as indicated by DEPT NMR). Such a structural change would also explain the differences observed in the ^{1}H NMR spectrum (a $-O-C\underline{H}_2-$ peak at 5.49 ppm in the spectrum of metabolite **93** in the place of a single -O-C<u>H</u>-O- proton at 6.91 ppm in that of **91**). Hence, the chemical structure of 4-carboxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone was proposed for metabolite **93**.

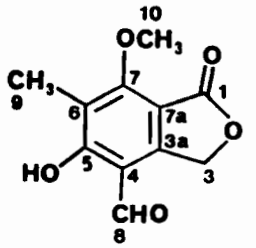


(93)

The IR data confirmed the presence of a carboxylic acid (OH at 3381, 3226 and C=O at 1728 cm^{-1}) and a conjugated five-member ring lactone (C=O at 1773, C=C at 1613 cm^{-1}). High resolution, chemical ionization (NH_3), mass spectrometry gave a molecular ion of mass 223.0621 ($M^+ + 1$) supporting the elemental composition of $C_{11}H_{10}O_5$ (calculated mass for $M^+ + 1$ is 223.0606).

^1H-^{13}C fully coupled and selectively decoupled NMR experiments were also carried out and all of the data obtained was in complete agreement with the proposed structure **93** (Tab. 17). The assignment of each carbon was confirmed on the basis of its chemical shift and splitting patern. The ^3J couplings noted between H5--C-9, H9--C-5, H5--C-3a and a very small ^2J coupling between H3--C-3a confurmed that the proposed ring substitution was correct.

^{13}C chemical shifts of the literature compound 4-formyl-5-hydroxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (**99**)[253] were used to calculate "theoretical" shifts for metabolite **93**. The results obtained were in close agreement with the actual values (Tab. 17).



(99)

Table 17: ^{13}C NMR (300 MHz, acetone-d_6, - 52°C) of metabolite 93.

C	decoupled δ	calculated δ	H-C coupled	H3 decoupling	H5 decoupling
1	168.4	---	s(br)	s(sh)‡	s(br)
8*	166.4*	---	s(br)	s(br)	s(sh)‡
7	161.2	162.0	m	m	m
3a	150.7	153.1	m	d, $^3J_{C\text{-}H5}$ = 7.8 Hz	s(br)
5	138.8	140.3	d, q; ^1J = 162 Hz	d, q	q, $^3J_{C\text{-}H9}$ < 5 Hz
6	132.2	131.8	q $^2J_{C\text{-}H10}$ = 6 Hz	q	q
4*	120.5*	118.0	s	s	s
7a	117.6	116.7	s	s	s
3	70.9	~ 68	t ^1J = 159 Hz	s	t
10	62.6	62.2	q ^1J = 146 Hz	q	q
9	15.6	---	d, q; ^1J = 128 Hz, $^3J_{C\text{-}H5}$ = ~ 4.5 Hz	d, q	q

* broad signals at room temperature

‡ sharpening of signal indicates the removal of coupling having a very small J value.

4. 5. 5 Identification of the compound 3-(4-methoxy-3-methyl-α-pyron-6-yl)-2-methyl-2-butenoic acid (94)

Metabolite 94 was the second compound to be isolated from the HPLC chromatography of fractions 20-21 (reverse phase column of crude Pc 5). Its ^{1}H NMR spectrum (Fig. 45) showed two methyl groups at 1.87 and 2.42 ppm, a methoxy at 4.04 ppm and two single proton peaks at 6.62 and 6.91 ppm. Its poor solubility in nonprotic solvents did not permit exchangable protons (if any) to be observed, neither was any coupling noted between the observed signals.

Nuclear Overhauser Enhancement experiments (Tab. 18) indicated the close proximity between the proton at 6.91 ppm and both the methyl at 2.42 ppm and the methoxy group (4.04 ppm), however, there was no NOE effect observed between the methoxy and that methyl. The other methyl group at 1.87 ppm and the proton at 6.62 ppm did not participate in any NOE interaction.

Table 18: Nuclear Overhauser Enhancement in the ^{1}H NMR Spectrum of metabolite 94

Saturated signals (δ)	Observed enhancement
2.42 (-CH_3)	6.91 (H, ~10%)
4.04 (-OCH_3)	6.91 (H, ~12%)
6.91 (H)	4.04 (-OCH_3, 4%), 2.42 (-CH_3, ~3%)

The room temperature ^{13}C NMR spectrum of this compound (Fig. 46) showed a total of eleven carbons as in all previous cases. There were three downfield quaternary carbons which could tentatively be assigned to a carboxylic acid (broad signal at 169.4 ppm), a lactone (167.9 ppm) and an aromatic or olefinic carbon to which the methoxy group was attached (166.4 ppm). The methoxy group resonance was at 57.5 ppm and the two methyls at 13.6 and 8.8 ppm. The APT NMR spectrum

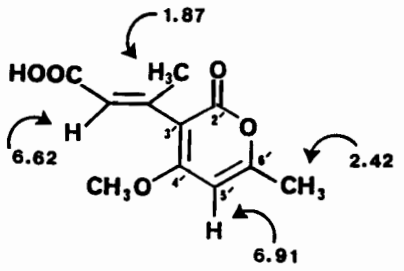
indicated the presence of three quaternary carbons at 159.2, 143.2 and 105.3 ppm, all of which had a shift difference of at least 10 ppm from the possibly equivalent carbons (C-3a, C-6, C-7a, respectively) of the previously identified metabolites. In addition, there were two tertiary carbons at 98.4 and 121.8 ppm, of which the latter was broad in the normal ^{13}C NMR and nonobservable in the APT spectra.

The coupled ^{1}H-^{13}C NMR spectrum of the compound showed doublets at 121.8 (^{1}J=165 Hz) and 98.4 (^{1}J=170 Hz) ppm, a quartet for the methoxy group at 57.5 ppm (^{1}J=147 Hz), a multiplet for the 8.8 ppm methyl and a quartet (^{1}J=129 Hz) for the one at 13.6 ppm. All other signals appeared as singlets.

The IR showed the presence of a hydroxyl group (3628, 3618 cm^{-1}), double bonds (1654, 1635 cm^{-1}) and two carbonyls, however, their absorption frequencies (1717 and 1707 cm^{-1}) were dramatically different from those of the previous compounds. Carbonyl frequencies of ~1700-1720 cm^{-1} are usually characteristic of conjugated carboxylic acids and α-pyrones (structure **96 C**) such as those produced by the *Alternaria* fungi (compounds **34-37**) and by *Phomopsis* (compounds **87** and **88**).

The high resolution, chemical ionization (NH_3), mass spectrum of metabolite **94** showed an intense molecular ion at 225.0739 (M^++1), suggesting a molecular composition of $C_{11}H_{12}O_5$ (calculated mass of M^++1 is 225.0763). At this point the general sructure of an α-pyrone was proposed but the substitution pattern was not completely clear.

The possible structure **94a** was first investigated. Its substitution pattern and most likely conformation was in reasonable agreement with both the ^{1}H NMR data and the NOE effects observed. In such a case, it was assumed that the methyl group at 2.42 ppm was the C-6' CH_3, the proton at 6.91 ppm was H5' and there would be no long-range coupling involved. Its ^{13}C chemical shifts were compared to those of literature compounds, and to those estimated from calculations using empirical parameters. The data obtained did not show any major discrepancies but neither did it provide adequate proof for the structure.



(94a)

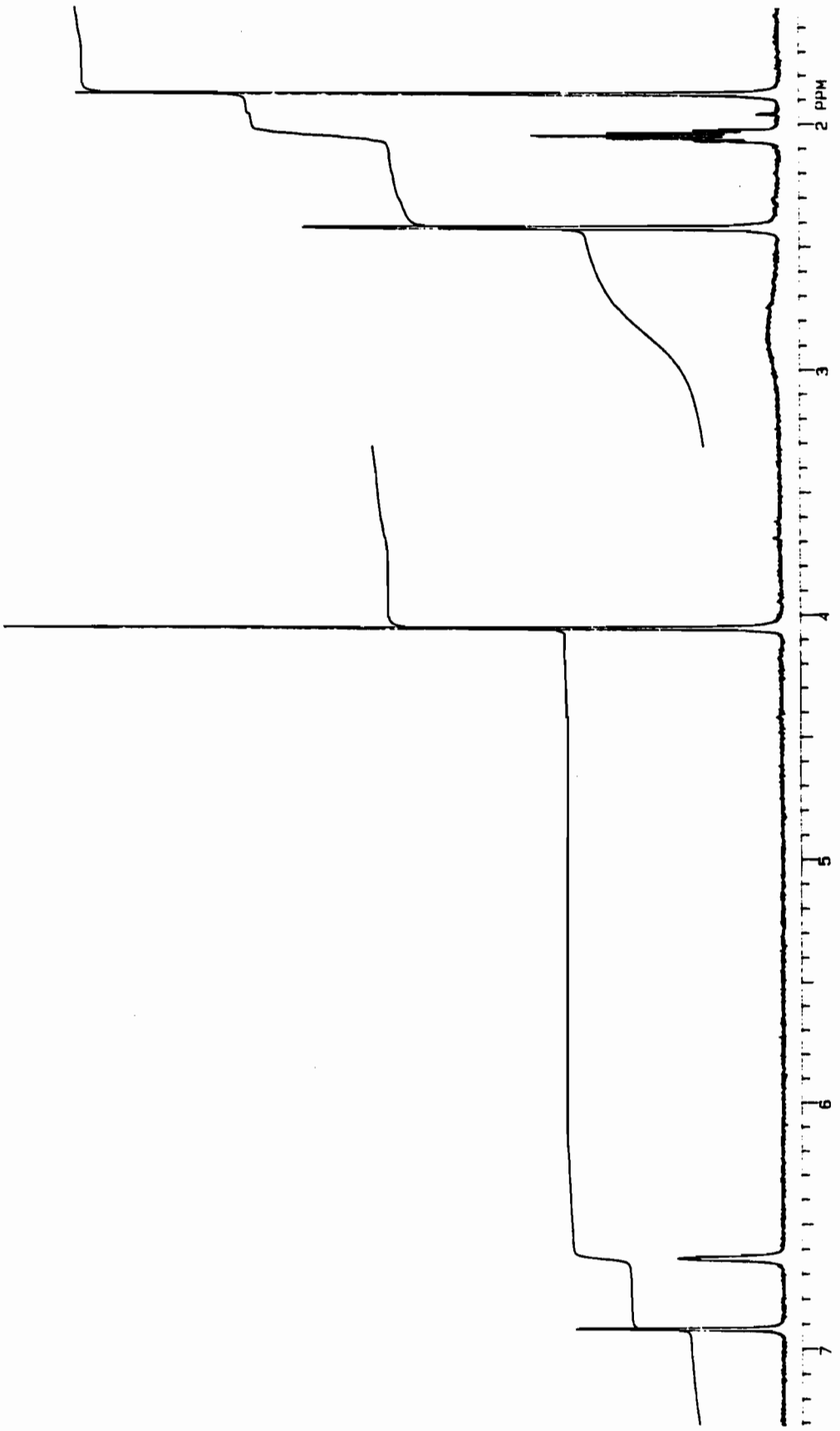


Fig. 45: ^{1}H NMR (200 MHz, acetone-d_6) of metabolite 94

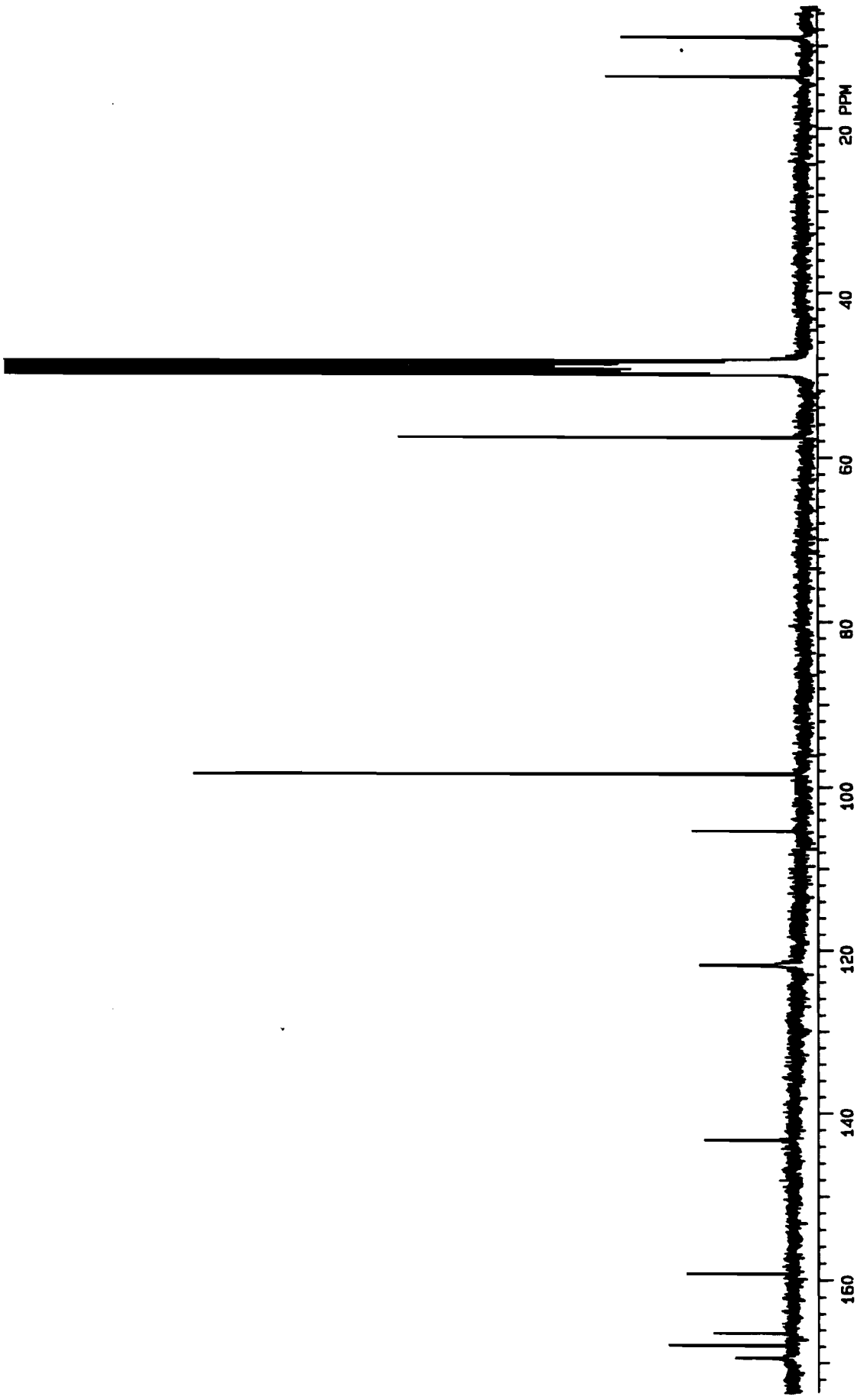
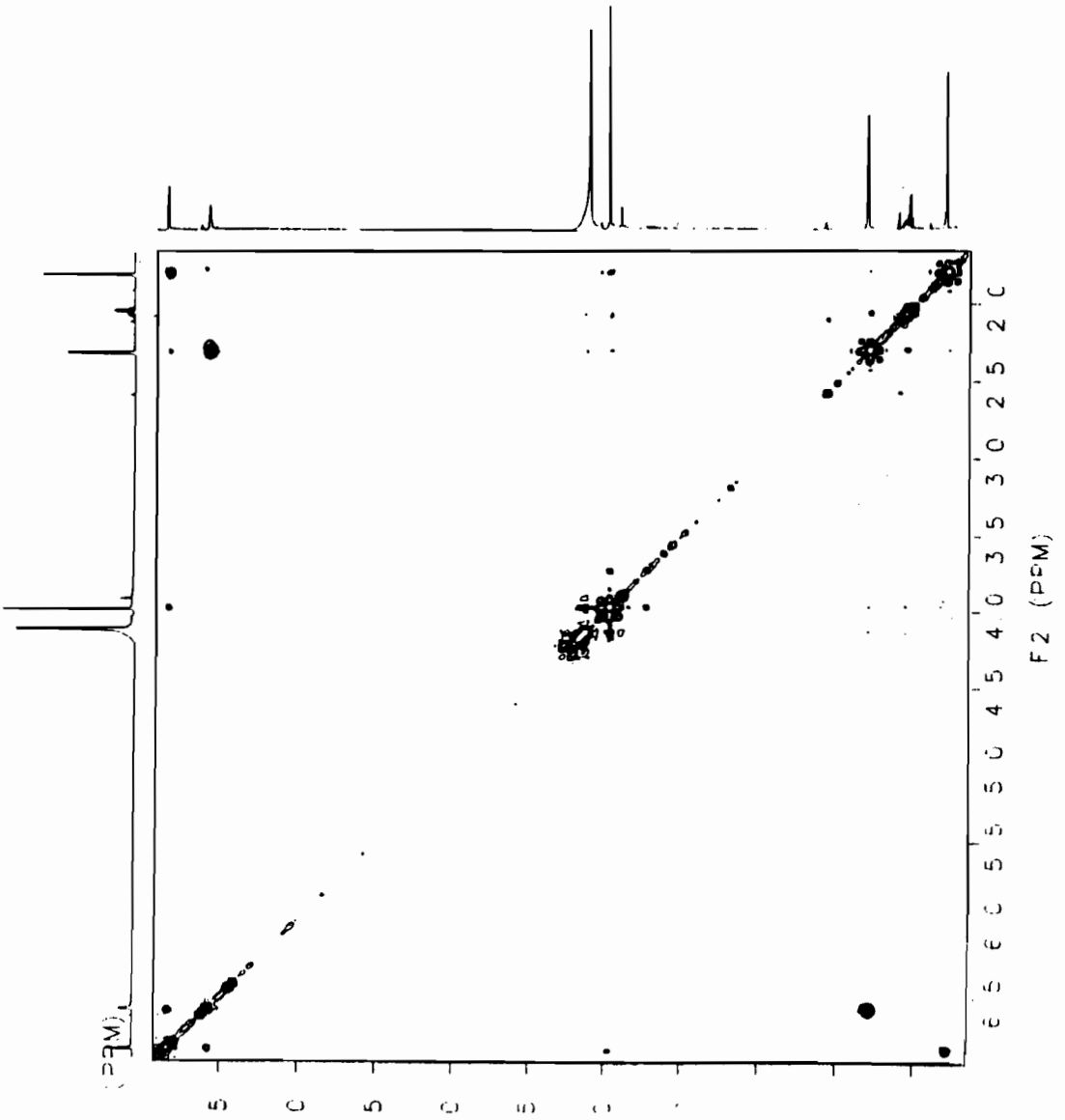


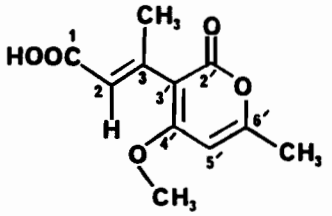
Fig. 46: ^{13}C NMR ((300 MHz, CD_3OD) of metabolite 94

The ^1H NMR which was originally recorded in acetone-d_6 did not show any coupling between signals, although the proton at 6.62 ppm and the methyl at 2.42 ppm appeared broad (Fig. 45). The addition of a few drops of D_2O led to the change of these two signals into doublets (J = 1.3 Hz). COSY NMR (Fig. 47) in the same solvent system confirmed coupling between these two signals (H at 6.62 ppm to methyl at 2.42 ppm) and in addition it showed strong coupling between the proton at 6.91 ppm and the methyl at 1.87 ppm. Weak coupling was observed between the two single protons (6.62 to 6.91 ppm) and between the proton at 6.91 ppm and the methoxy at 4.04 ppm.

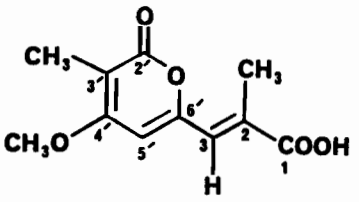
Fig. 47: COSY NMR (300 MHz, acetone-d_6/D_2O) of metabolite 94



The HETCOR NMR spectrum (300 MHz) of metabolite **94** at room temperature, over a period of 24 hours, failed to show a resonance for the -CH- carbon at 121.8 ppm. However, coupling between the carbon at 98.4 and the proton at 6.91 ppm was clearly present indicating that the 121.8 ppm signal should be connected to the 6.62 ppm proton. ^{1}H-^{13}C correlations were also observed for the methoxy (57.4 ppm to 4.04 ppm), and the two methyls (13.6 ppm to 1.87 ppm and 8.8 ppm to 2.42 ppm). These results, in conjuction with the NOE and COSY data, could not support the structure **94a**. Coupling between H5' and the C-3 CH_3 (methyl at 1.87 ppm) or between H5' and H2 would be highly unlikely in that case, hence, the alternate structure **94b** was proposed.

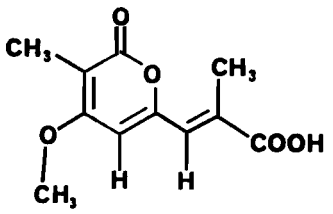


(94a)

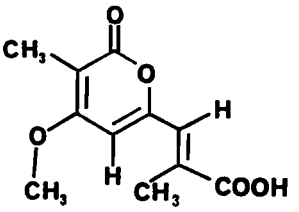


(94b)

The structure of **94b** was supported by all data thus far obtained. Coupling between H5' and the C-3' CH_3 could reasonably be expected as well as between H5' and H3. The latter proton (H3) would also be coupled to C-2 CH_3. In considering, however, the NOE effects observed, the energy involved for the two most likely conformers of this molecule (**94-I** and **94-II**) were examined. Initially it was assumed that the molecule would be nearly planar in order to permit conjugation of the two carbonyls and the double bonds. It was then thought that conformer I would be more stable than II due to the lack of steric hindrance between H5' and the C-2 CH_3. If that was the case the absence of any NOE effect between H5' and CH_3 could shed some doubt as to the validity of the proposed structure.

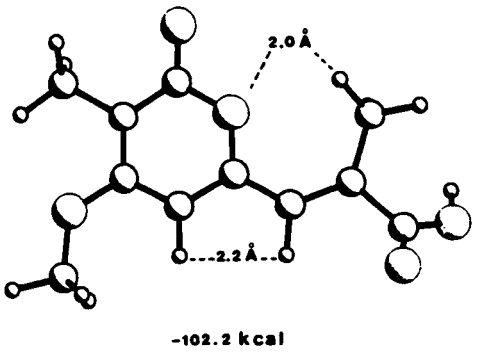


(94-I)

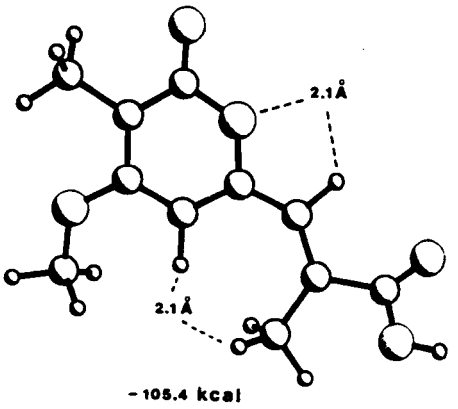


(94-II)

Molecular modeling calculations for both of the above structures gave an energy difference of 3.2 kcal in favor of 94-II which would imply an equilibrium ratio of about 1/99 for these two comformers. These results provided a good explanation for the presence of a positive NOE effect between H5' and the C-2 CH_3, as well as the absence of one between H5' and H3. The energies of several other conformers were also calculated and they were all found to have an energy higher than that of conformer 94-II (-105.4 kcal). However, the existance of these other minimum energies indicated limitations in the modeling program used and the possibility that even the -105.4 kcal value may represent a local minimum and not the absolute minimum energy of the compound.



(94-I)



(94-II)

4. 6. Biological testing of the compounds produced by P. convolvulus

The ideal assay for determining the toxicity of a compound to plant tissues would be a large scale screening process of the type utilized by major herbicide industries. This process involves pre- and postemergence applications of the test compound on dozens of crop and weed plants in the well controlled environment of a green house.

In natural product research, such an approach is not feasible due to the minute amounts of metabolites usually isolated. Although a number of bioassay techniques requiring small quantities of material are available, most of them are inadequate in testing all aspects of phytotoxic activity. To further complicate testing, phytotoxicity manifests itself in a variety of different ways. The symptoms can include inhibition of seed germination, seedling growth, photosynthesis, chloroplast development, leakage of electrolites, wilting and others.

External factors may also influence the toxicity of a test compound. For example, the plant species, the physiological growth stage, the age of the test tissue, the environmental conditions, and even the existance of a synergistic compound are all factors which have been shown to affect the results of a bioassay.[254] The necrotic effects of fusaric acid on tomato plants, for instance, are highest when plants are treated at noon and the symtoms appear more pronounced on the mature leaves than on the young ones . Even the growth medium of the test plants may be decisive in the outcome of a bioassay; nitrogen deficient plants show a higher resistance to fusaric acid injury than plants grown in a nitrogen rich soil.[254]

Progress in the isolation of novel, biologically active metabolites from microorganisms is often limited by the capacity to observed specific physiological changes on the test tissues induced by very small and initially crude samples. The sensitivity and specificity of the bioassays is very important since it dictates which compounds out of a large crude mixture of metabolites will be actively studied, purified and characterized.

At the initial stages of this project a number of different bioassays were considered, and tested for their ability to indicate the presence of phytotoxic metabolites in the crude extracts (**Pc 1** and **Pc 2**) of *P. convolvulus*. The use of the aquatic small plant *Lemna* was found to be a suitable

254. S. Naef-Roth, in *Phytotoxins in Plant Diseases*, Eds. R.K.S. Wood, A. Ballio and A. Graniti, Academic Press, New York, 49 (1972).

system for measuring inhibition of growth and chlorophyll synthesis. However, since the principle objective was to isolate a host-specific phytotoxin for field bindweed, bioassays using leaf-cuttings of this weed were also used.

4. 6. 1 Bioassays of P. convolvulus metabolites on Lemna plants and field bindweed leaf-cuttings

Lemna, commonly called duckweed, is a small aquatic macrophyte which consists of a leaf-like front and a single root. Its floating fronts appear as a rosette of three to four leaves and they propagate vegetatively. The usefulness of *Lemna* plants in phytotoxicity bioassays has been demonstrated.[255] The *Lemna* species *L. paucicostata* was chosen for testing the *P. convolvulus* metabolites and standardized growth conditions suitable for biochemical experimentation were followed according to literature procedures.[256]

The *Lemna* plants were grown in sterile nutrient solutions of approximatly 100 mL over a maximum period of seven days. Beyond that period, the growth rate of the plants was diminished and after a ten day period, chlorotic lessions (yellow spots) were observed indicating the depletion of nutrients. All tested metabolites (or metabolite mixtures) were dissolved in absolute ethanol, aliquotes of which (50-5 μL) were added to *Lemna* nutrient broth to make the final test samples.

The toxicity of ethanol to *L. paucicostata* and field bindweed tissue was initially evaluated. In leaf-puncture bioassays of bindweed, toxicity was not observed at concentrations of ethanol up to 2% in water. With *L. paucicostata* plants, growth inhibition and chlorosis were observed when the concentrations of ethanol was greater than 1% (v/v) of the total nutrient medium (Tab. 19). Hence, most bioassays were performed with test solutions containing less than 1% ethanol in either *Lemna* nutrient medium or doubly distilled water. In few cases where higher than 1% concentration of ethanol had to be used in order to solubilize the test samples a control sample of equal ethanol content was always used. Since in all cases the concentration of ethanol in the control was the same as that of the most concentrated test sample, any observation of phytotoxicity was compared to the control before it was considered to be reliable. The concentrations at which each of the pure metabolites was tested varied, depending on the solubility of the compound in an aqueous system and the availability of sample. Since the quantities of the isolated metabolites were in the range of 0.5-10

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255. F.A. Einhellig, G.R. Leather and L.L. Hobbs, *J. Chem. Ecol.* 11, 65 (1985).

256. A.H. Datko, S.H. Mudd and J. Giovanelli, *Plant Physiol.* 65, 906 (1980).

mg, their biological testing was carried out only once. A fairly large number of *Lemna* fronds (25-30) and bindweed leaves (5-6) was used in each test in order to increase the reliability of the results obtained. In all cases, the observations made with the *Lemna* bioassays were consistent with the results obtained by the leaf-puncture bioassays.

Table 19: Toxicity of ethanol to Lemna plants

% Ethanol in 10 mL *Lemna* medium	% Growth after incubation period of:		
	24 hrs	**72 hrs**	**120 hrs**
0.0	20	67	127
0.5	15	67	96
1.0	15	70	100
2.0	14	46	55*
4.0	0	5*	18*

* **chrorosis observed**

Small samples (~1 mg) of each fraction obtained from reverse phase flash column chromatography of the crude **Pc 5** were dissolve in 50 μl of absolute ethanol and suspended into 2 mL[257] of *Lemna* growth medium or 2 mL water in the case of the bindweed leaf-puncture bioassays. An equivalent 2.5% solution of ethanol was used as the control sample in each set of tests.

After a period of less than 24 hours the *Lemna* leaves exposed to fractions 6, 7 and 8 were strongly effected by chlorosis. Minor toxicity symptoms were also observed with fraction 9, whereas all other samples appeared unaffected (Fig. 48). Due to the very short incubation period, the effects

257. The concentration of ethanol used (2.5%) does not significantly effect *Lemna* plants for the first 3 days of incubation. Relatively high concentrations of ethanol had to be used in order to solubilize the test samples, however, the results were recorded after only 24 hours.

on the growth rate were not considered in this assay. A longer incubation period was not nessesary except perhaps to reveal the presence of weakly active fractions. However, the relatively high concentration of ethanol that had to be used, would have interfered with any long-term observations.

Fig. 48: Bioassay of fractions from the reverse phase flash column of crude Pc 5

From left to right:

top row:	**control / fractions 1-4 / fraction 6**
middle row:	**fraction 7 / fraction 8 / fraction 9**
bottom row:	**fraction 10 / fraction 11 / fraction 12**

The phytotoxicity of the above fractions to field bindweed leaves was also evaluated. Leaf cuttings (5-8) which were either punctured with the tip of a fine needle, cut along the middle vein of the leaf or just cut at the steam (no injury to the leaf tissue), were placed on a filter paper and soaked with the test solution in a petridish. Fractions 6 to 8 showed browning and wilting after only a few hours. Two days later all of the leaves were extensively damaged irrespective of their original injuries (Fig. 49).

Fig. 49: Phytotoxic effects of the crude metabolite mixture fractions 6 to 8 (from P. convolvulus) to field bindweed after 2 days.

Biological testing of ergosterol and its peroxide was limited by the very poor solubility of the compounds in aqueous solvents. In the *Lemna* assay, a suspension of 0.25 mg/ml of each compound in a 0.5% ethanol in *Lemna* medium showed no effect with ergosterol and only a 10% inhibition of growth with the peroxide. Similarly, there was no observable toxicity to bindweed with ergosterol and only a very small amount of browning was observed on the injured leaf tissue with the peroxide in a suspension of 0.2mg/ml of water.

Ergosterol peroxide has been isolated previously from other fungi which feed on plant tissues, such as the fungi *Alternaria dianthicola*,[258] *Fusarium moniliforme*[259] and *Guingnardia laricina*.[260] Its weak phytotoxic effects were reported in association with the latter microorganism, *G. laricina*, which is the causal agent of shoot blight in coniferous larch trees. Ergosterol peroxide was isolated from its mycelium and shown to be one of its phytotoxic metabolites using lettuce seedling bioassays.

The *Lemna* bioassays of the three phthalide metabolites of *P. convolvulus* were carried out only at those concentrations where a true solution could be obtained using a concentration of ethanol lower or equal to 1% of the total volume of the test sample. Five dilutions of each metabolite were tested and in each case a 1% ethanol solution in *Lemna* medium was used as the control.

Metabolite **93** was found to be the most potent phytotoxin, causing total inhibition of growth and 100% chlorosis of the *Lemna* tissues within 12 hours at concentrations of 5.9×10^{-4} M and within 24 hours at concentrations of 3.5×10^{-4} M. Observation of this bioassay beyond 48 hours was not carried out, since all of the plants in two of the dilutions (5.9x10-4 and 3.5x10-4 M) had completely died during the first 24 hours and the remaining dilutions had approximately the same growth rate as the control.

Metabolites **91** and **92** were also found to inhibit the growth of *Lemna*, however, **92** was found to be considerably less active than **91** (Fig. 50). Neither of these two compounds was as phytotoxic as metabolite **93**.

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

259. E.P. Serebryakov, A.V. Simolin, V.F. Kucherov and B.V. Rosynov, *Tetrahedron* **26**, 5215 (1970).

260. N. Otomo, H. Sato and S. Sakamura, *Agric. Biol. Chem.* **47**, 1115 (1983).

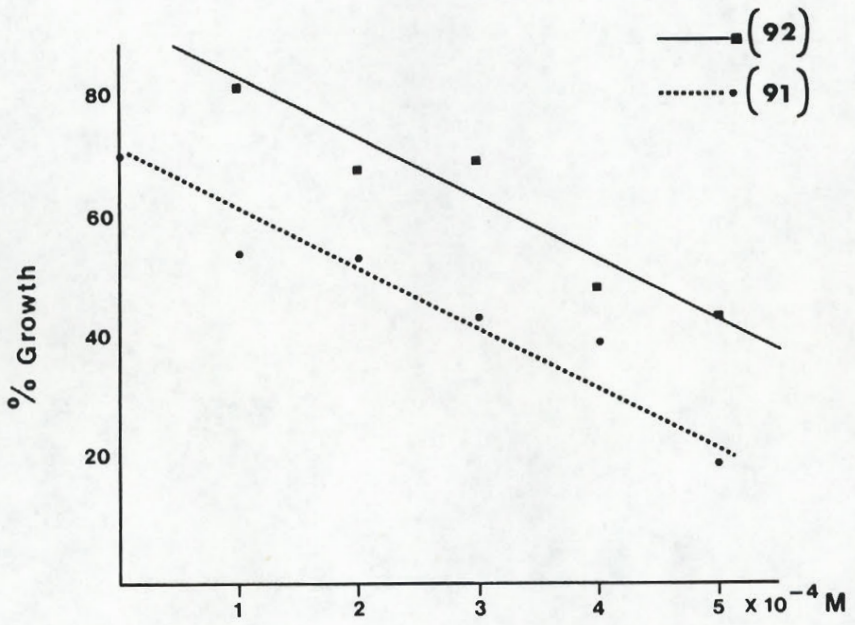


Fig. 50: Growth of Lemna plants exposed to metabolites 91 and 92 for a period of 75 hours.

The phytotoxic effects of all three metabolites on field bindweed were evaluated using leaf-cuttings from plants 3-4 weeks old, using only the top two leaves of the young shoots. For each assay an average of 5-6 leaves were cut, with a scalpel under water, at the base of the stem and placed on a moist piece of filter paper (0.5 mL H_2O) in a petridish. The phytotoxic metabolite samples, dissolved in absolute ethanol, were diluted to a total volume of 2 mL using doubly distilled water. The most concentrated sample of each assay had an ethanol content of 1%, hence, the control solution was also an 1% ethanol solution in water. All leaves were rinsed with the test solutions and incubated under a grow-lamp for a period of one week. Toxic symptoms of wilting and browning were observed with metabolite **93** after four hours at concentrations of 3.5×10^{-4}M (Fig. 51). Twelve hours later, these same symptoms appeared on the leaves exposed to metabolite **91** (conc. 5×10^{-4}M) but they were not seen at all with metabolite **92**, even after several days.

Fig. 51: Phytotoxic effects of metabolite 93 on the leaf tissue of field bindweed.

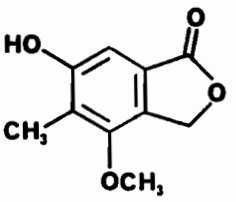
It is interesting to note that the extent of browning of the bindweed leaves did not seem to progress very much after the first 24 hours. A possible explanation for this phenomenon could be the death of cells around the base of the leaf and the injured areas which would effectively stop absorption and, hence, prevent the further uptake of the toxins. In order to partly test this hypothesis several young bindweed shoots (~2" long) were placed in a solution of the less potent phytotoxic metabolite **91**, at a concentration of $\sim 2 \times 10^{-3}$ M. It was assumed that metabolite **91** will have a less pronounced and immediate effect on the injured cells, permiting its own absorption and delivery throughout the cutting's tissue. A solution of 1% ethanol in water was used as the control and both sets of plants were observed and compared over a period of 5-6 days. After 40-48 hours the plants exposed to the toxin displayed obvious signs of wilting, although they showed some recovery 20-24 hours later.

At the end of the observation period (~5-6 days later) the systemic absorption and toxicity of metabolite **91** was very clear, as the leaves of the treated plants were almost completely wilted where the plants in the control solution did not show any such symptoms (Fig. 52).

Fig. 52: Wilting of field bindweed caused by the phytotoxic metabolite 91

A number of phytopathogenic fungi are known to produce phthalide metabolites having a variety of biological activities. For example, more than 19 highly substituted phthalides were isolated by Achenbach[248,253] from the fungus *Aspergillus duricaulis*. Some of them were found to exhibit antibiotic activity including the previously mentioned compounds **97** and **98**. Biological testing of all these natural products, and few of their derivatives, indicated that the antibiotic activity may be connected to the presence of a 4-formyl group on the phthalide skeleton. Metabolites having a 4-hydroxymethyl substituent, such as compund **98**, were found to be inactive. It is rather interesting that the phytotoxic effects of metabolites **91**, **92** and **93** seem to indicate an analogous structure-activity relationship.

The compound silvaticol (**100**) is one of several metabolites of *Aspergillus silvaticus* having a phthalide structure. It is a weak antifungal antibiotic which inhibits the growth of *Helminthosporium maydis*.[251b]



Silvaticol (100)

Talaromyces flavus is yet another microorganism which has been shown to produce phthalide metabolites. Aqueous extracts of this fungus have been shown to inhibit chlamidiospore production in *Verticillium dahliae*, the causative fungus of eggplant wilt. Ayer and Racok[261] have isolated two phthalide metabolites (**101** and **102**) from its metabolite mixture and have investigated their biosynthetic significance.

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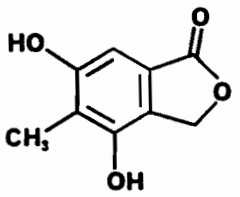
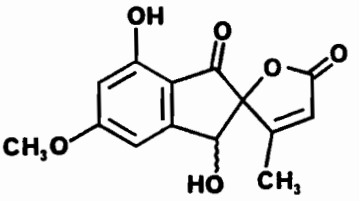
261. W.A. Ayer and J.S. Racok, 3rd Chemical Congress of North America, abstract No 438 Organic (1988).



(101)



(102)

Finally, the biological properties of the metabolite 3-(4-methoxy-3-methyl-α-pyron-6-yl)-2-methyl-2-butenoic acid (94) were examined. The *Lemna* bioassay was carried out at concentrations ranging from $0.9x10^{-4}$ M to $7x10^{-4}$ M. Inhibition of growth proportional to increasing concentration of metabolite was observed, however, the overall activity was relatively weak. The phytotoxic effects observed with bindweed leaf-cuttings were also very minor.

α-Pyrones are an important class of fungal metabolites which exhibit a wide range of biological activities. Some phytotoxic metabolites of the *Alternaria* species were already described in the introduction of this thesis. The metabolite pestalotin is a 5,6-dihydro-α-pyrone (103) and the active principle of the phytotoxic fungus *Pestalotia cryptomeriaecola*.[262] The insect deterrent compounds of the phomopsolide structure (87, I and II) and nectriapyrone (88) represent examples from *Phomopsis*. The structural similarities of these natural products are worth noting, since they may imply a common biosynthetic origin in fungal metabolism. For example, in all of the methoxy α-pyrone metabolites the methoxy group is at the 4-position.[263] In the case of the *Alternaria* metabolites (33-37) an ether linkage is found at the same position. In many of these compounds, an alkyl side chain is attached to the C-6' carbon of the pyrone often having a double bond conjugated with those of the α-pyrone. Hence, the general structure 104 appears to be common to many fungal metabolites of the α-pyrone class.

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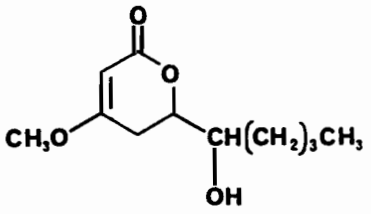
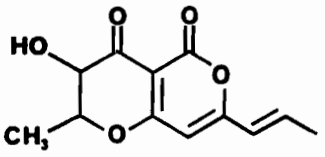
262. Y. Kimura, K. Katagiri and S. Tamura, *Tetrahedron Lett.*, 3137 (1971).

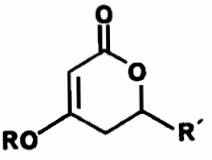
263 a) M.S.R. Nair and S.T. Carey, *Tetrahedron Lett.*, 1655 (1975).
b) M. Tanabe and H. Seto, *J. Am. Chem. Soc.* 92, 2157 (1970).
c) G.A. Ellestad, W.J. McGahren and M.P. Kunstmann, *J. Org. Chem.* 37, 2045 (1972).
d) L.J. Mulheirn, R.B. Beechey and D.P. Leworthy, *J. Chem. Soc., Chem. Commun.*, 874 (1974).



Pestalotin (103)



Radicinin (34)



(104)

The biosynthesis of one of these metabolites, radicinin (34) from *Stenphylium radicinum*, has been investigated using ^{13}C NMR techniques.[264] Fermentation of the fungus in the presence of sodium acetate-1-^{13}C or sodium acetate-2-^{13}C led to incorporation of the label in alternating carbons, suggesting the polyacetate origin for the compound (Fig. 53).

264. M. Tanabe, H. Seto and L. Johnson, *J. Am. Chem. Soc.* **92**, 2157 (1970).

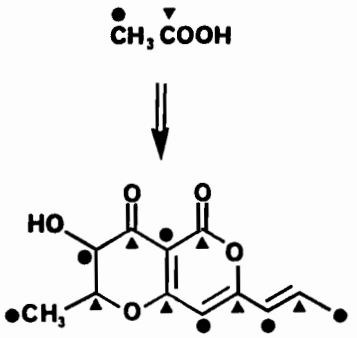


Fig. 53: The polyacetate origin of radicini.

In conclusion, the nonsteroidal natural products isolated from *Phomopsis convolvulus* are, to the best of our knowledge, novel compounds. However, they possess structural features common to other biologically active fungal metabolites. The physiological effects observed on field bindweed when infected with *P. convolvulus* were shown to be chemically mediated. The biological activity of metabolites **91** and **93** and to a lesser extent **90**, **92** and **94** clearly suggests that they play an important role in the development of the phytotoxic symptoms. It is therefore conceivable that these compounds or their synthetic analogues could provide the agriculture industry with an effective herbicide for the control of field bindweed.

CHAPTER 5

ATTEMPTS TOWARDS THE SYNTHESIS OF A SULFUR ANALOGUE OF ERGOSTEROL PEROXIDE

5. 1. Introduction

In search of a herbicidal compound for the control of the perennial weed *Convolvulus arvensis* (field bindweed), the metabolites of the host-specific pathogen *Phomopsis convolulus* were investigated (Chapter 4). Two of the isolated metabolites were the steroids ergosta-5,7,22-trien-3-ol (ergosterol, **89**) and ergosta-6,22-dien-5α,8α-epidioxy-3-ol (ergosterol peroxide, **90**). Ergosterol peroxide was found to be weakly phytotoxic, to both *Lemna* plants and bindweed leaf-cuttings, while no biological activity was observed with ergosterol. Hence, it seemed rational to assume that the peroxide bridge of the first compound was responsible for its phytotoxicity, and efforts were initiated toward the synthesis of an analogue possessing a more stable or perhaps biologically more active linkage at that position.

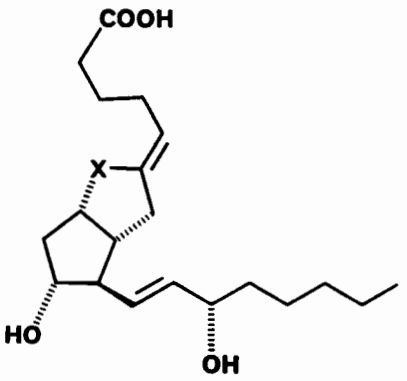
In recent years, sulfur analogues of biomolecules have been shown to often mimic or even enhance the physiological effects of the natural products. Among the best known examples are those of prostaglandin derivatives.[265] The synthetic analogue of prostacyclin (**105a**, PGI_2), 6,9-thiaprostacyclin[1a,b] (**105b**) exhibits comparable potency to the natural prostacyclin in inhibiting platelet aggregation. However, unlike the natural compound, it does not lose its biological activity in neutral saline solution.[266] Similarly, the endodisulfide analogue (**106b**) of the endoperoxide PGH_2 (**106a**) has also been synthesized and shown to strongly mimic the effects of thromboxane A_2, causing contraction of rabbit aorta strips and irreversible platlet aggregation.[267] Several other sulfur

265. a) K.C. Nicolaou, W.E. Barnette, G.P. Gasic and R.L. Magolda, *J. Am. Chem. Soc.* **99**, 7736 (1977).
b) M. Shibasaki and S. Ikegami, *Tetrahedron Lett.*, 559 (1978).
c) P.G. Baraldi, A. Barco, S. Benetti, C.A. Gandolfi, G.P. Pollini, E. Polo and D. Simoni, *Gazz. Chim. Ital.* **114**, 177 (1984).

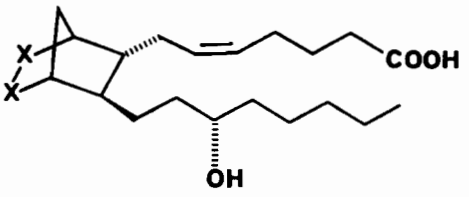
266. K.C. Nicolaou, W.E. Barnette and R.L. Magolda, *J. Am. Chem. Soc.* **103**, 3486 (1981), and references therein.

267. a) S.S. Ghosh, J.C. Martin and J. Fried, *J. Org. Chem.* **52**, 862 (1987), and references therein.
b) H. Miyake, S. Iguchi, H. Itoh and M. Hayashi, *J. Am. Chem. Soc.* **99**, 3536 (1977).

derivatives of biologically important metabolites have been investigated including analogues of clavulanic acid,[268] the leukotriene B_4,[269] monoamine oxidase inhibitors,[270] and vitamin B_6 derivatives.[271]



a: X=O,
b: X=S
(105)



a: X=O
b: X=S
(106)

Although not numerous, sulfur derivatives of steroid compounds have also been reported in the literature. For instance, compounds having one or two sulfur atoms inserted in to the A ring of the steroid skeleton (**107**), have been found to possess androgenic activity.[272] The C-19 sulfur derivative 19-methylthio-4-androstene-3,17-dione (**108**),[273] was shown to inhibit the enzyme

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268. J.L. Douglas, A. Martel, G. Caron, M. Menard, L. Silveira and J. Clardy, *Can. J. Chem* **62**, 3382 (1984)

269. Y. Guindon and D. Delorme, *Can. J. Chem.* **65**, 1438 (1987).

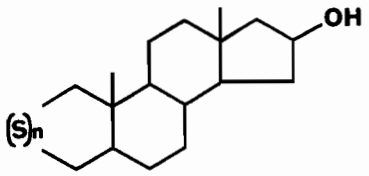
270. T.R. Bosin, R.P. Maickel, A. Dinner, A. Snell and E. Campaigne, *J. Heterocyclic Chem.* **9**, 1265 (1972).

271. M. Iwata and H. Kuzuhara, *Bull. Chem. Soc. Jpn.* **58**, 2502 (1985).

272. G. Zanati and M. E. Wolff, *J. Med. Chem.* **15**, 368 (1972).

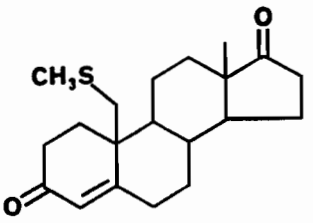
273. J.N. Wright, M.R. Calder and M. Akhtar, *J. Chem. Soc., Chem. Commun.*, 1733 (1985).

aromatase (from human placenta) which is responsible for the conversion of androgens to oestrogens. Clinically, aromatase inhibitors are potentially useful drugs as contraceptives or for the treatment of oestrogen-dependent diseases such as breast cancer.[274]



n = 1, 2

(107)



(108)

Hence, the synthesis of ergosta-6,22-dien-5α,8α-epidisulfide-3-ol (109), the sulfur analogue of ergosterol peroxide (90), was attempted *via* the Diels-Alder addition of diatomic sulfur to the C-5, C-7 diene of ergosterol.

5. 2. Efforts towards the synthesis of ergosta-6,22-dien-5α,8α-epidisulfide-3-ol (109)

Ergosterol peroxide (90) can easily be synthesized *via* a Diels Alder addition of $^1O_2$ to the ergosterol diene.[237b] Hence, it was considered appropriate to attempt the same reaction with diatomic sulfur (likely $^1S_2$) in an effort to synthesize its sulfur analogue compound 109.

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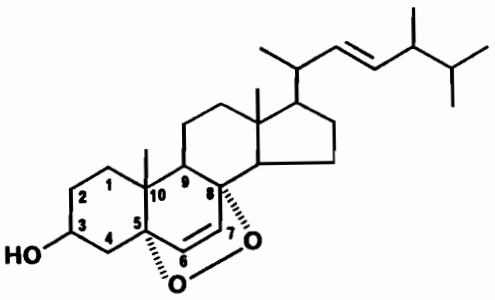
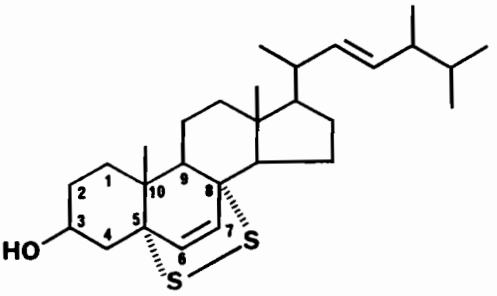
274. A.M. Brodie, W.M. Garrett, J.R. Hendrickson, C.H. Tsai-Morris and J.G. Williams, *J. Steroid Biochem.* 19, 53 (1983).



(90)



(109)

A number of synthetically useful techniques for the generation of S_2 have been reported in the literature by Steliou[275] Schmidt[276] and Harpp.[277] In 1984 Steliou first reported that in the presence of triphenyldibromophosphorane, organometallic trisulfides of general structure **110**, liberate diatomic sulfur which can be trapped by a diene. The reaction mechanism proposed (Fig. 54) involved a six-member-ring intermediate (**111**) or the formation of a four-member ring phosphine sulfide (**112**) as in the case of singlet oxygen.

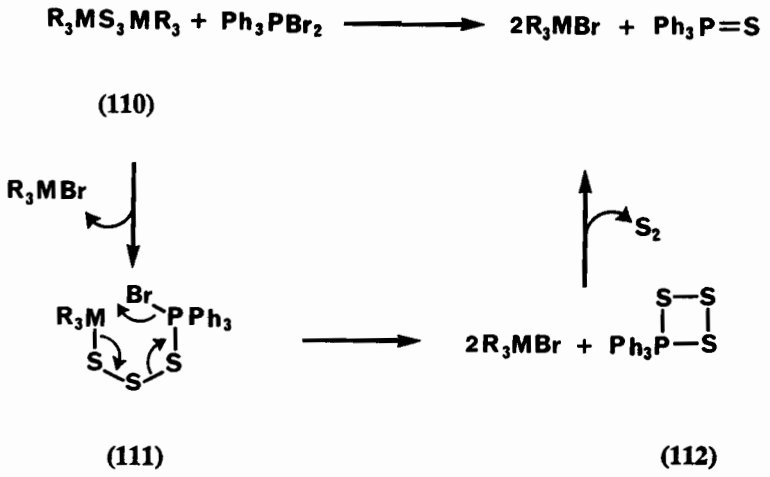


Fig. 54: Generation of S_2 from organometallic trisulfides (M=Si or Ge).

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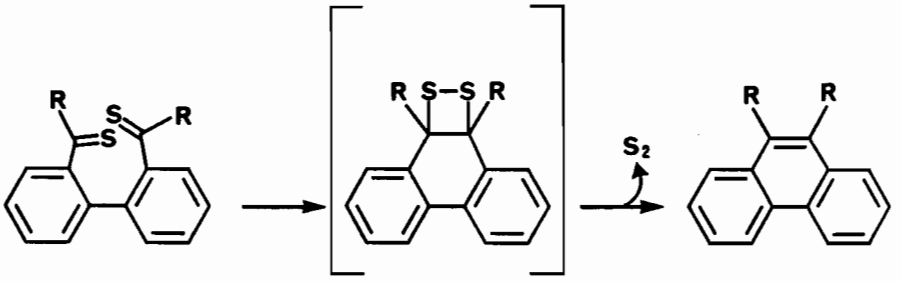
275. a) K. Steliou, Y. Gareau and D.N. Harpp, *J. Am. Chem. Soc.* **106**, 799 (1984).
b) K. Steliou, P. Salama, D. Brodeur and Y. Gareau, *J. Am. Chem. Soc.* **109**, 926 (1987).

276. M. Schmidt and U. Gorl, *Angew Chem. Int. Ed. Eng.* **26**, 887 (1987).

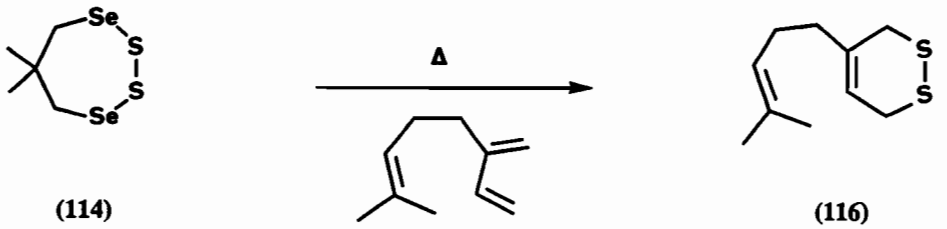
277. D.N. Harpp and J.G. MacDonald, *J. Org. Chem.* **53**, 3812 (1988).

Three years later, the thermal rearrangment of the aromatic thioketone (113) was shown by Steliou[275b] to generate singlet sulfur, which he was able to trap using a number of dienes in high yields. However, attempts to utilize this reagent with ergosterol as the diene, have been thus far unsuccessful.[278]



(113)

At the same time, the thermal decomposition of the organometalic disulfides 5,5-dimethyl-1,2-dithia-3,7-diselenacycloheptane (114) was reported by Schmidt[276] as a source of S_2. This reagent was successfully employed in the conversion of myrcene (115) to 4-(4-methyl-3-pentenyl)-3,6-dihydro-1,2-dithiin (116), an antibacterial natural product found in beer.[279]



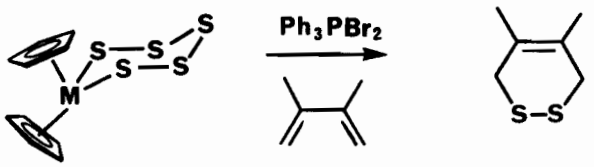
(114) (116)

(115)

278. P.L. Folkins and D.N. Harpp, unpublished results.

279. J.A. Elvidge and S.P. Jones, *J. Chem. Soc. Perkin I*, 1089 (1982).

More recently, Harpp[277] reported the generation of diatomic sulfur from organometallic pentasulfides of titanium and zirconium complexes (**117**). These reagents are analogous to the organometallic trisulfides (**110**) in that they undergo S_2 extrusion in the presence of triphenyldibromophosphorane (Fig. 55).



(**117**)

Fig. 55: Generation of S_2 from organometallic pentasulfides.

Several attempts to induce a Diels Alder reaction between S_2 and the C-5, C-7 ergosterol diene, using Ph_3PBr_2 and bis(*n*-cyclopentadienyl)titana(IV)cyclohexasulfane (**117a**)[280] led to the formation of triphenylphosphine sulfide, elemental sulfur (S_8) and the C-6 and C-7 mercaptans (**118**, **119** respectively) in low yields. The C-7 mercapto ergosterol (ergosta-5,7,22-trien-7-mercapto-3-ol, **119**) was the main product of the reaction and its ^1H NMR spectrum showed loss of the C-7 proton and a downfield shift of both the 18-CH_3 and 19-CH_3 as expected. The ^{13}C NMR spectrum showed a signal at the characteristic shift of quaternary sp^2 carbons attached to sulfur (140.74 ppm), and the expected downfield shift of C-5 (150.85 ppm) and upfield shift of C-8 (123.14 ppm). The ^1H NMR spectrum of the minor product C-6 mercapto ergosterol (ergosta-5,7,22-trien-6-mercapto-3-ol, **118**) showed greater downfield shifts of the C-3 proton (3.90 ppm *vs* 3.63 ppm in compound **118** and ergosterol) and the 19-CH_3 as expected. All other spectral data was consistent with the structural assignments of these two products.

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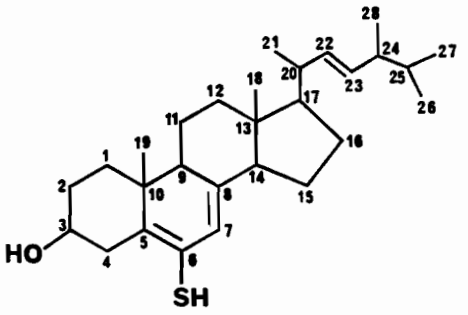
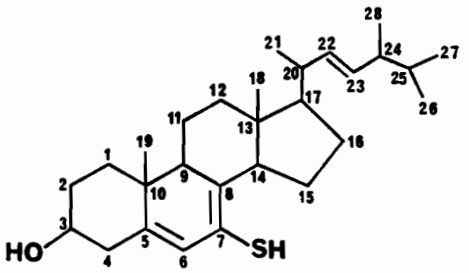
280. a) This reagent was a generous gift from C.R. Williams and D.N. Harpp.
b) The reagent was first prepared by J.M. McCall and A. Shaver, *J. Organomet. Chem.* **C37**, 193 (1980).



(118)



(119)

Although the addition of sulfur to the double bonds was evident, it was not clear why the Diels Alder reaction did not proceed or whether diatomic sulfur was in fact formed during this reaction. The mechanism followed was equally uncertain and it could possibly involve a sulfur transfer intermediate analogous to that proposed by Steliou[275,281] (Fig. 56), or the insertion of diatomic sulfur directly from the titanium pentasulfide reagent (Fig. 57). Both mechanisms appear reasonable and can be used to rationalize the formation of either compound **118** or **119**. In both cases, in the presence of elemental sulfur, reasonable mechanisms can be proposed for the conversion of the hydrodisulfide anions to their respective thiols.

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281. K. Steliou, Y. Gareau, P. Salama and G. Milot, manuscript in preparation.

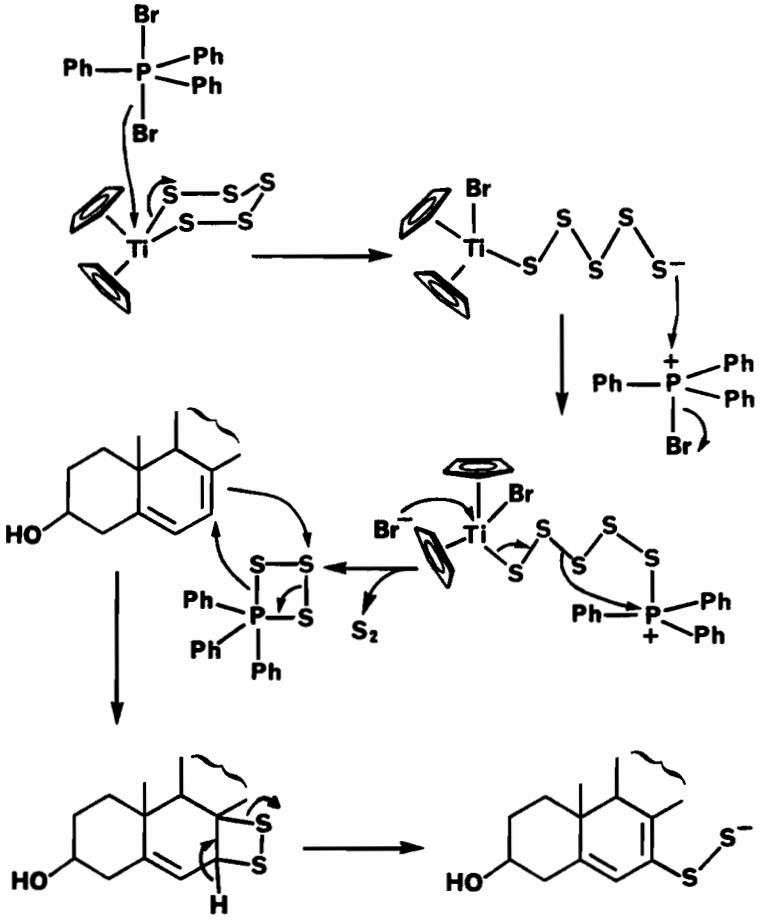

Fig. 56: Proposed mechanism (I) for the formation of C-6 mercapto ergosterol (118).

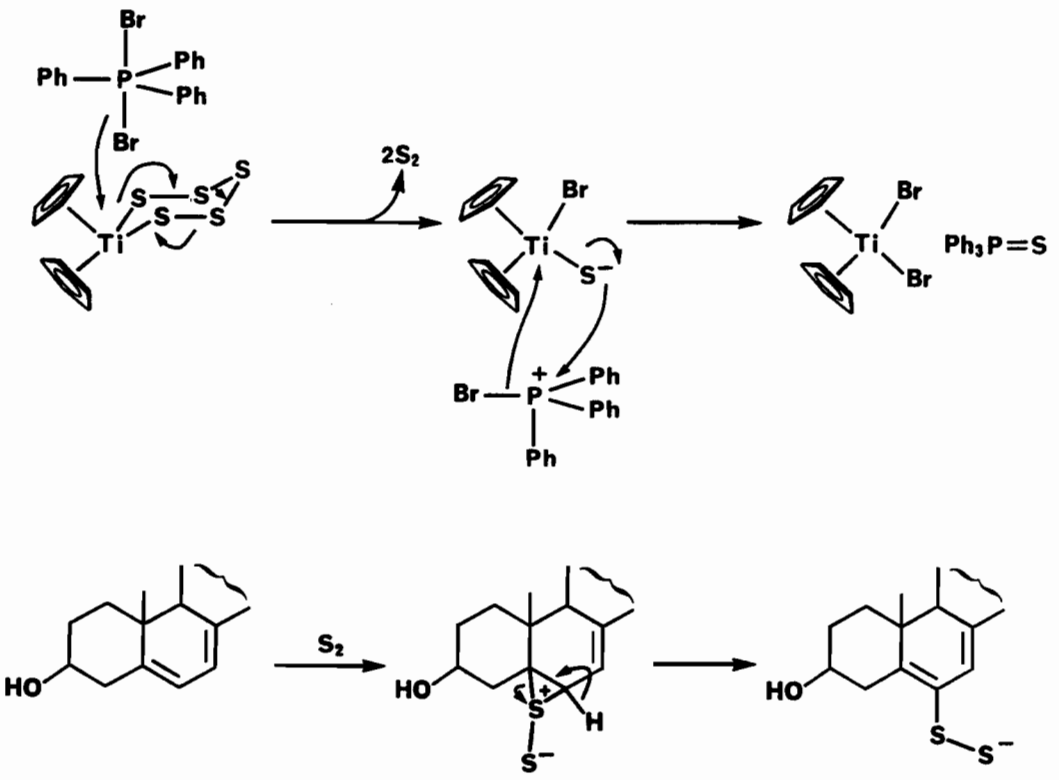

Fig. 57: Proposed mechanism (II) for the formation of C-6 mercapto ergosterol (118).

At this point, the absence of any detectable Diels Alder product was perceived as an indication that the above reaction did not involve diatomic sulfur but possibly elemental sulfur (S_8), reacting with the olefinic carbons in an ionic or radical type of mechanism.[282] In such a case, it was speculated that the same two compounds (**118, 119**) would form upon reaction of ergosterol with elemental sulfur. However, ergosterol failed to react with S_8 in refluxing toluene over a period of several hours. The possibility that "activated" sulfur was the true reagent responsible for the formation of compounds **118** and **119** was subsequently investigated.

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282. M.G. Voronkov, N.S. Vyazankin, E.N. Deryagina, A.S. Nakhmanovich and V.A. Usov, in *Reactions of Sulfur with Organic Compounds*, Ed. J.S. Pizey, Plenum Publishing Corporation, New York, N.Y., (1987).

Liquid ammonia and triethylamine are widely used as activating agents in the reactions of elemental sulfur with organic compounds.[283] The reaction of ergosterol and elemental sulfur in the presence of triethylamine gave a large mixture of products most of which showed evidence of decomposition to unidentified aromatic compounds. Decomposition through the loss of elemental sulfur (S_8) during flash column chromatography was also observed. The formation of aromatic compounds produced by dehydration and dehydrogenation of six-member ring secondary alcohols[284] has been observed before in similar reactions of elemental sulfur at even milder experimental conditions.

The main product isolated from the above reaction showed a downfield shift of the H6 and H7 olefinic protons and loss of the long range coupling observed in the starting material. In fact, the ^1H NMR spectrum of this compound (Fig. 58) was quite misleading, in that it resembled the spectrum of ergosterol peroxide very closely (Fig. 35). However, the APT NMR spectrum clearly showed that it was not the desired disulfide **109**, since the anticipated loss of two quaternary sp^2 carbons, C-5 and C-8, and the simultaneous appearence of two quaternary C-S signals had not taken place. Instead, one additional quaternary sp^2 carbon was present in the APT NMR spectrum while in the DEPT NMR spectrum there was one less $-CH_2$ signal (believed to be that of C-4) and one -CH signal from the aliphatic region had been replaced by one in the olefinic region (quaternary sp^2 carbon), as compared to ergosterol. In addition, the C-3 OH had been oxidized to a ketone as was confirmed by both its ^{13}C NMR and IR data. Chemical ionization mass spectrometry gave an intense molecular ion at a mass of 393 suggesting an elemental composition of $C_{28}H_{40}O$. Finally, given that all other spectral data was similar to ergosterol the structures of ergosta-4,6,8(14),22-tetraen-3-one (**120**) or ergosta-4,6,8(9),22-tetraen-3-one (**121**) were proposed. Compound **120** has been reported before, as a derivative of a steroid metabolite from *Aspergillus niger*.[285]

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283. a) Chapter 2 in *Reactions of Sulfur with Organic Compounds* (previous reference), and references therein.
b) R. Mayer, *Z. Chem.* **13**, 321 (1973).

284. a) Chapter 6, in *Reactions of Sulfur with Organic Compounds*, and references therein.
b) L. Ruzicka, *Angew. Chem.* **51**, 5 (1938).

285. D.H.R. Barton and T. Bruun, *J. Chem. Soc.*, 2728 (1951).

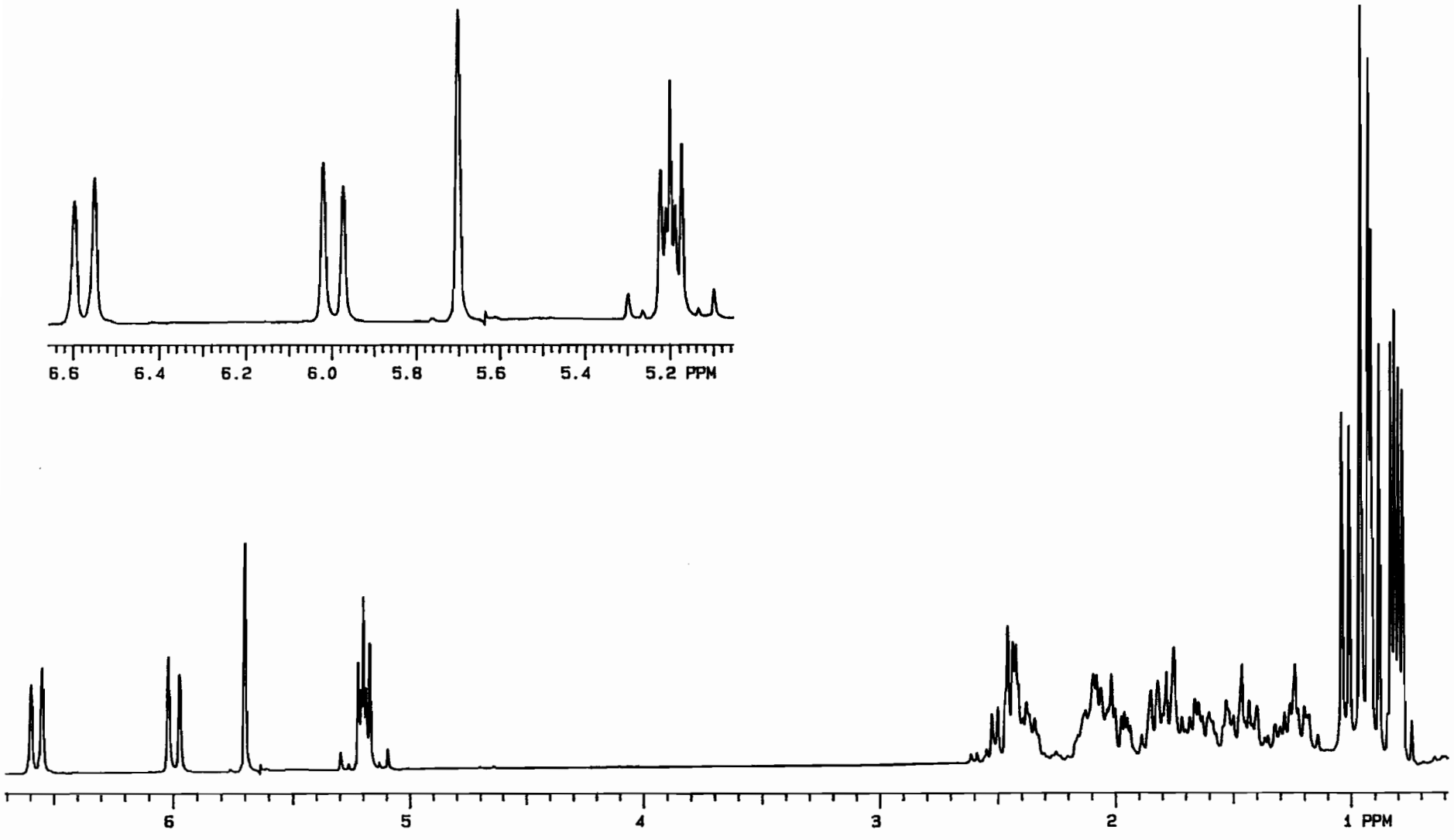
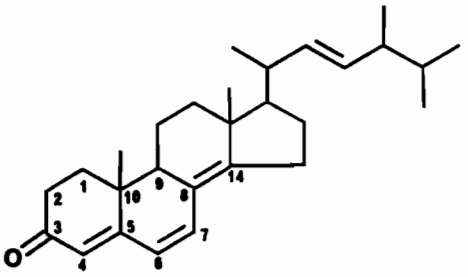
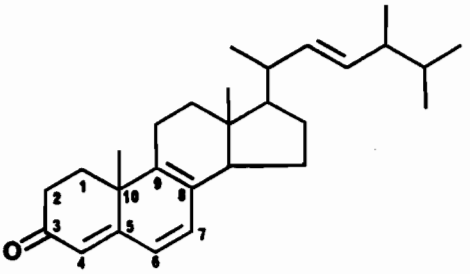


Fig. 58: ^{1}H NMR (200 MHz, $CDCl_3$) of compound 120.



(120)



(121)

The mechanism which is believed to lead to the formation of such products involves first the oxidation of the C-3 OH, by participation of the sulfur, followed by the temporary nucleophilic addition of the sulfur to the C-4 carbon and elimination of H9 or H14 to give a conjugated carbonyl (Fig. 59). The exact structure of this compound was shown to be that of **120** through X-ray crystallography (Fig. 60).

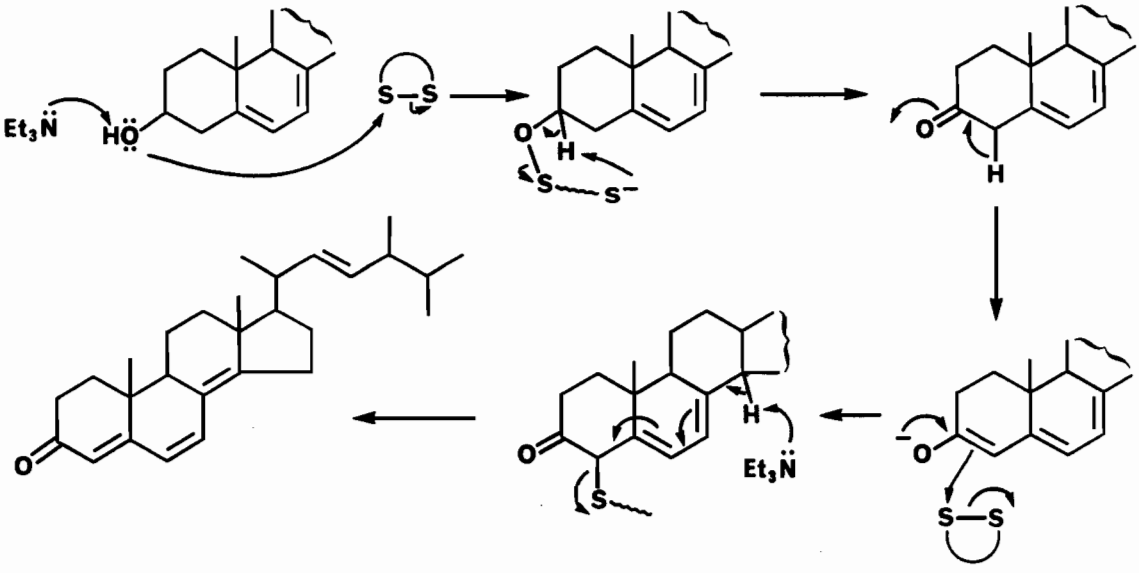


Fig. 59: Proposed mechanism for the formation of compounds 120.

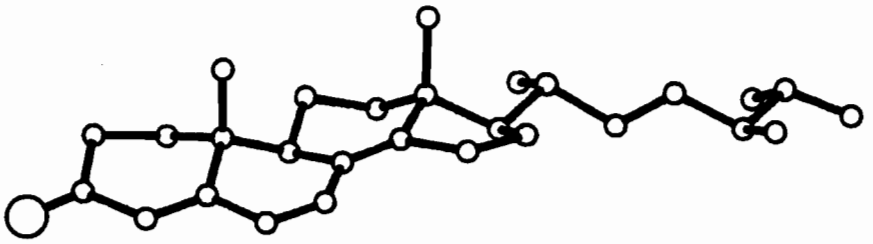


Fig. 60: X-Ray structure of compound 120.

Attempts to minimize side products of the above reaction by first converting the C-3 OH to its acetate were also unsuccessful, leading to an even greater amount of unidentifiable products. Hence, it would seem that the reaction of ergosterol with bis(*n*-cyclopentadienyl)-titana(IV)cyclohexasulfane (**119a**) did involve diatomic sulfur insertion into the double bonds, although, the Diels-Alder product was not favored under these conditions.

A thorough investigation of the reactions of diatomic sulfur with ergosterol was beyond the scope of this thesis, however, efforts towards the synthesis of the desired disulfide **109** are being continued.[278]

CONTRIBUTIONS TO KNOWLEDGE

The biologically active metabolites of *Pisolithus tinctorius*, *Scuillus cavipes* and *Phomopsis convolvulus* were investigated. These microorganisms are of special interest and value to forestry and agriculture, hence, their metabolites and synthetic analogues are of potential commercial value.

The ectomycorrhizal fungus *Pisolithus tinctorius*, which lives symbiotically on the roots of forest trees, is well known for its ability to stimulate the growth of its host plants. Investigation of the fungus, as part of this thesis, led to the isolation of metabolites from its growth media having antifungal activity. The production of such compounds strongly indicates that the microorganism also provides protection to its host trees against disease causing pathogens.

The antifungal metabolites isolated were assigned the chemical stuctures of *p*-hydroxybenzoylformic acid (**37**) and (R)-(-)-*p*-hydroxymandelic acid (**38**), and they were found to effectively inhibit spore germination and cause hyphal lysis to several phytopathogenic and dermatopathogenic fungi. The absolute stereochemistry of the chiral metabolite **38** was determined through stereospecific synthesis of both the (R) and (S) enantiomer of **38**, and by means of NMR techniques using chiral solvating agents.

The structure-activity relationship of these natural products was investigated using a number of related compounds. It was shown that the commercially available compounds benzoylformic acid and both the (R)-(-) and (S)-(+)-mandelic acids, also exhibit antifungal activity. It is rather interesting that the (S) enantiomers of the α-hydroxy acids, mandelic acid and *p*-hydroxymandelic acid, were more potent antibiotics than their respective (R) enantiomers. The metal salts of both natural products were found to be void of any biological activity, suggesting that their mode of action may involve the chelation of metal ions which are essential for the normal growth of the test organisms. This notion was further supported by the differences in the ^{13}C NMR data between the free acids and the metal salts of each compound.

The microorganism *Suillus cavipes* is another ectomycorrhizal fungus commonly found on the roots of forest trees. Its ability to induce dichotomous branching and increased root development in hypocotyl cuttings of conifers was investigated. Although the isolation of a biologically active metabolite was unsuccesful, the physiological phenomena induced by this fungus were attributed to a metabolite which induces ethylene biosynthesis.

Finally, the fungus *Phomopsis convolvulus*, a host-specific pathogen of the weed *Convolvulus arvensis* (field bindweed), was investigated. It was shown that the symptoms of the disease caused by *P. convolvulus* are at least in part chemically mediated and caused by a number of phytotoxic metabolites. The isolation scheme for these compounds, as well as bioassays for the assessment of

their phytotoxicity, were developed.

Two known steroids, ergosterol (89) and ergosterol peroxide (90) were isolated, the latter of which was found to be weakly phytotoxic. In hope of producing a compound with strong herbicidal activity, efforts were initiated towards the synthesis of the disulfide analogue (109) of ergosterol peroxide. The addition of diatomic sulfur *via* a Diels Alder reaction was attempted following literature procedures. However, none of the reagents used led to the formation of the desired product.

An additional four novel natural products were also isolated from the active crude of *P. convolvulus*. The phthalides, 4-carboxy-3-hydroxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (91) and 4-carboxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (93), were found to be strongly phytotoxic to both *Lemna* and field bindweed. Weaker biological activity was observed with the phthalide, 4-(hydroxymethyl)-7-methoxy-6-methyl-1(3H)-isobenzofuranone (92) and the α-pyrone, 3-(4-methoxy-3-methyl-α-pyron-6-yl)-2-methyl-2-butenoic acid (94). The need for an effective herbicide for the control of field bindweed cannot be overstated, hence, the isolation of phytotoxic metabolites from its host specific pathogen, *P. convolvulus*, is not only of academic interest but potentially of great value to agriculture.

CHAPTER 6

EXPERIMENTAL

6. 1 General Methods

Reagents and Chemicals:

Sodium *p*-hydroxybenzoylformate, benzoylformic acid, (R) and (S) mandelic acid and racemic *p*-hydroxymandelic acid were obtained from Aldrich Chemical Co (Milwaukee, W.). The compounds 2-chloroethylphosphonic acid (ethephon) and 2-amino-4-(2'-aminoethoxy)-trans-3 butenoic acid [L-α-(2-aminoethoxyvinyl)-glycine, AVG] were purchased from SIGMA Chemical Company (St. Louis, Mo.). *n*-Octadecyltrichlorosilane was purchased fron Fluka Chemie AG. Doubly distilled water and HPLC grade methanol were filtered through a 0.45μ filter membrane (Millipore Corp., Bedford, MA) before using them for HPLC. All chromatographic solvents were fractionally distilled prior to use with the exception of acetic acid.

Chromatography:

Silica gel chromatography was performed on Merck Kieselgel 60 (230-400 mesh, #9385) using flash chromatography.[119] Reverse phase flash column chromatography was carried out on silica gel (Merck Kieselgel 60, 230-400 mesh, #9385) reacted with *n*-octadecyltrichlorosilane. Cellulose thin-layer chromatography (TLC) was performed on Eastman cellulose (Eastman Kodak Co., #13254) plates (0.16 mm thickness), while CF 11 Whatman powder (American Chemicals LTD. Montreal, Que.) was used for column chromatography. Paper chromatography was performed on Whatman paper sheets (3mm Chr). Desalting of metabolites was carried out on an ion exchange column (~2 cm x 100 cm) packed with pre-swollen and washed Dowex 50W-X8 (H^+ form, 20-50 mesh) resin (J.T. Baker Chemical Co., Phillipsburg, N.J.). Ion exchange chromatography was performed using a column (~2 cm x 100 cm) packed with pre-swollen and washed IRC-50 resin purchased from BDH Inc.(Montreal, Que.).

HPLC:

Analysis and purifications were carried out on a Waters instrument (pump model 501, variable wavelength detector model 450, U6K injector). Two reverse phase C18 columns were used; Whatman Partisil 5 ODS 3 (10 cm x 9.4 mm ID, 5 μm particles, Chromatographic Specialties Inc.) and CSC-S ODS2 (25 cm x 9.4 mm ID, 5 μm particles, Chromatography Science Company Inc., Montreal, Que.).

Spectra:

Ultraviolet spectra were recorded on a Hewlett Packard 8451A DIODE ARRAY Spectrophotometer. Nuclear Magnetic Resonance spectra were obtained at 20-22°C (unless otherwise indicated) using Varian XL-200, XL-300 and Bruker 500 MHz instruments. ^{1}H and ^{13}C-NMR chemical shifts are quoted in ppm and are referenced to the internal deuterated solvent downfield from tetramethylsilane (TMS). The samples used for D_2O-^{1}H NMR were first dissolved in ~1mL of 99.8% D_2O and lyophilized two times before their data was recorded in 99.96% D_2O. All mass spectra were performed at the Biomedical Mass Spectrometry Unit, McGill University, unless otherwise indicated. The low resolution chemical ionization (NH_3) spectra were obtained using a HP 5980A spectrometer. FAB and high resolution chemical ionization spectra (NH_3) were obtained using a ZAB 2F HS instrument.

6. 2 Chapter 2

6. 2. 1 General extraction and isolation of antifungal metabolites from P. tinctorius

Erlenmeyer flasks (250 mL) containing 100 mL of liquid Melin Norkans Medium (MMN)[98,110a,286] were inoculated with *Pisolithus tinctorius* disks (4x6 mm^2), cut from the edge of a colony growing on MMN agar plates. The solutions were buffered to pH 5.5 with 0.1M sodium citrate/citric acid. The flasks were incubated at 25°C on a rotary shaker at 100 rpm for approximately 50 days.

The liquid cultures of *P. tinctorius* were then collected and filtered through several layers of

286. D.H. Marx and W.C. Bryan, *For. Sci.* **21**, 245 (1975).

cheese-cloth to remove the mycelia. The reddish-brown liquid obtained was reduced in volume (~1/5) under high vacuum at 40°C. The remaining water was removed by freeze-drying to give a solid, the yields of which varied from 2.5 to 4.3 grams per liter of original culture.

Soxhlet extraction of the above solid with USP diethyl ether (500mL/day/15g of solid) over a period of 2-3 days gave a light orange oil upon evaporation of the solvent. A small amount of glucose often crystallized out of the ether solution, which was removed by filtation. The yields of biologically active material ranged from 7 to 60 mg per gram of solid (average=33 mg/g) but could be increased if the extraction was allowed to continue for up to 7 days. A longer period of extraction, however, led to the isolation of a much darker crude, whose subsequent purification was much more difficult.

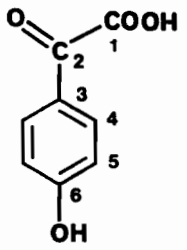
Further purification was carried out on a tightly packed column (using air pressure) of Whatman Cf11 cellulose powder, applied as a slurry in ethanol. Once the crude toxin was applied to the top of the column, a solvent mixture of 95% *n*-butanol (saturated with water)-5% glacial acetic acid was allowed to filter through by gravity. Cellulose TLC was used to follow the elution of active metabolites (compound **37**, Rf=~0.28 and compound **38**, Rf=~0.33 in *iso*-propanol/0.5M NH_4HCO_3). The majority of the middle fractions were combined and rechromatographed.

Paper chromatography (Whatman 0.33mm) with a solvent mixture of *iso*-propanol/0.5M NH_4HCO_3 (4/1) for a total length of ~40 cm (12-15 hours) separated the mixture into five bands at Rf values 0.05-0.1 (yellow), 0.22-0.32, 0.32-0.4, 0.4-0.56 (fluorescent) and at 0.55-0.65. All bands were cut, dried, eluted with doubly distilled water and freeze-dried. The active material (band at Rf 0.22-0.32) was obtained as a light brown solid containing both metabolites **37** and **38**, average yield 30-35 μg/mg of crude.

A second purification through descending paper chromatography, eluted with 95% *n*-butanol (saturated with water)-5% acetic acid for ~15 hours, led to the separation of the two biologically active metabolites. Metabolite **37** was found at Rf 0.2-0.6 and **38** at Rf 0.6-0.7. The visualization of these bands was difficult and it could only be done in a completely dark room under uv light. The strips of paper impregnated with the antifungal compounds were cut and dried in a desiccator, under high vacuum. Elution of the papers with doubly distilled water followed by freeze-drying led to the isolation of *p*-hydroxybenzoylformic acid (**37**) and (R)-(-)-*p*-hydroxymandelic acid (**38**).

The final purification was carried out on a reverse phase C18 HPLC column (CSC-S ODS2, 25cm x 9.4mm ID, 5 μm particles) using a solvent mixture of 91.7% H_2O, 7.3% CH_3OH, 0.9% CH_3COOH at a flow rate of 2.0 mL/min. *p*-Hydroxybenzoylformic acid eluted after 5-6 minutes while (R)-(-)-*p*-hydroxymandelic acid had a retention time of 8-9 minutes.

p-Hydroxybenzoylformic acid (37):



IR (KBr): 3150-3380, 1735, 1654, 1608, 1586, 1443, 1400, 1252, 1173, 979, 855, 621 cm^{-1}.

UV: (0.1M HCl, nm), max 296, min 226 and 206; (0.1M NaOH, nm), max 334, min 240.

^{1}H NMR (500 MHz, DMSO) δ: 6.88 (dd, J=8 and 0.9 Hz, 2H, H4), 7.75 (dd, J=8 and 0.9 Hz, 2H, H5), 10.74 (s, 1H, -COOH) ppm.

^{13}C NMR free acid (300 MHz, DMSO+D_2O) δ: 116.0 (C-5), 123.3 (C-3), 132.3 (C-4), 163.8 (C-6), 166.8 (C-1), 187.0 (C-2) ppm.

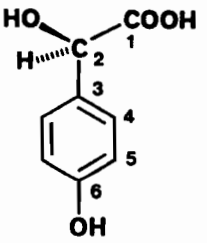
^{13}C NMR sodium salt (300 MHz, DMSO) δ: 115.2 (C-5), 125.7 (C-3), 131.5 (C-4), 162.2 (C-6), 171 (br, C-1) 195 (br, C-2) ppm.

^{252}Cf-plasma desorption MS:

electrosprayed sample[122c] m/z: 211.2 $(M^{2-}+H^+ +2Na^+)^+$, 165.1 $(M^{2-}+H^+)$

tridodecylmethylammonium chloride salt[122a,b] m/z: 1774.7 $[(TDMA^+)_3(M^{2-})]^+$, 866.7 $[(TDMA^+)(M^{2-}+H^+)_2]^-$, 165.1 $(M^{2-}+H^+)$, 121.1 $(M^{2-}+H^+-CO_2)$.

(R)-(-)-p-Hydroxymandelic acid (38):



UV: (0.1M HCl, nm) max 228, min 206 and 274; (0.1M NaOH, nm) max 248, min 292.

^{1}H NMR (300 MHz, D_2O) δ: 5.0 (s, 1H, H2), 6.7 (d, J=8.2 Hz, 2H, H5), 7.1 (d, J=8.2 Hz, 2H, H4) ppm.

^{13}C NMR (300 MHz, DMSO+D_2O) δ: 74.4 (C-2), 117.3 (C-5), 130.4 (C-4), 132.3 (C-3), 157.5 (C-6), 178 (C-1) ppm.

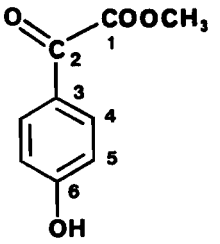
^{252}Cf-plasma desorption MS:

electrosprayed sample[122c] m/z: 213.2 $(M^{2-}+H^+ +2Na^+)^+$, 167.1 $(M^{2-}+H^+)^-$;

tridodecylmethylammonium chloride salt[122a,b] m/z: 167.1 $(M^{2-}+H^+)^-$, 869.9 $[(TDMA^+)(M^{2-}+H^+)_2]^-$, 1776.6 $[(TDMA^+)_3\ M^{2-}]$, 121.1 $(M^{2-}-HCO_2H)^-$.

6. 2. 2 Synthetic derivatives and analogues of metabolites 37 and 38

Synthesis of methyl p-hydroxybenzoylformate (47):



p-Hydroxybenzoylformic acid (~5 mg) and catalytic amounts of *p*-toluenesulfonic acid were dissolved in 10 mL of methanol and refluxed overnight. Evaporation to dryness of the reaction mixture followed by flash chromatography on silica gel, with CH_2Cl_2, gave quantitative amounts of the methyl ester as a clear oil. TLC (EtOAc) Rf=0.56, (on silica gel compound has a bright yellow color).

^{1}H NMR (200 MHz, $CDCl_3$) δ: 3.9 (s, 3H, $-CH_3$), 6.3 (s, 1H, C-6 OH), 6.9 (d J=9.0 Hz, 2H, H5), 7.9 (d J=9.0 Hz, 2H, H4) ppm.

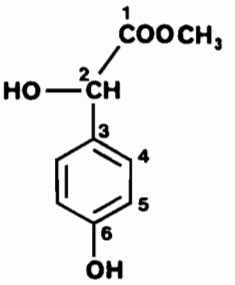
^{13}C NMR (300 MHz, $CDCl_3$) δ: 52.9 ($-CH_3$), 116.1 (2xC-5), 124.9 (C-3), 133.3 (2xC-4), 162.8 (C-6), 164.6 (C-1), 185.0 (C-2) ppm.

MS [C.I.(NH_3), direct inlet, ~105°C], m/z (% relative intensity, assignment): 181 (M^++1), 198 ($M+NH_4^+$), 121 ($HO\text{-}C_6H_4\text{-}CO^+$).

Synthesis of methyl p-hydroxymandelate (52):

A racemic mixture of *p*-hydroxymandelic acid (~1 g) was refluxed overnight in methanol (50 mL) in the presence of catalytic amounts of *p*-toluenesulfonic acid (~0.5 mg). Evaporation of the solvent gave a reddish oil containing the desired methyl ester (**52**) in ~50% yield, the minor product methyl 2-methoxy-*p*-hydroxymandelate (**53**, a red oil) in ~5% yield and unreacted starting material. The mixture was separated by flash column chromatography using EtOAc/CH_2Cl_2 (1:1) as the eluting solvent.

(R/S)-methyl-*p*-hydroxymandelate (52):

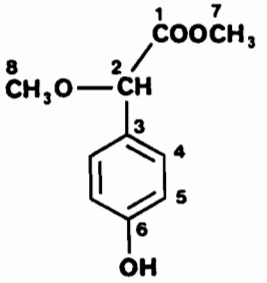


TLC: EtOAc/CH_2Cl_2 (1:1), Rf=0.38.

^{1}H NMR (200 MHz, $CDCl_3$) δ: 3.36 (d J=6.5 Hz, 1H, C-2 OH), 3.78 (s, 3H, -CH_3), 4.9 (bs, 1H, -C-6 OH), 5.13 (d J=6.5 Hz, 1H, H2), 6.82 (d J=8.7 Hz, 2H, H5), 7.29 (d J=8.7 Hz, 2H, H4) ppm.

M.S. [C.I.(NH_3), direct inlet, ~105°C], m/z (% relative intensity, assignment): 200 (10.6, $M^+ + NH_3$), 182 (13,M^+), 165 (100, M^+-OH), 123 (20.4, M^+-COOMe).

(R/S) methyl 2-methoxy-*p*-hydroxymandelate (53):



TLC: EtOAc/CH_2Cl_2, Rf=0.52.

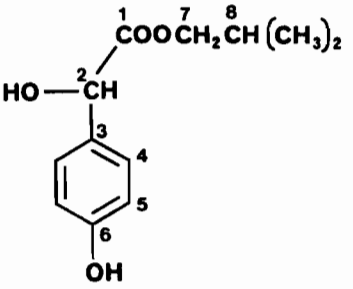
^{1}H NMR (200 MHz, $CDCl_3$) δ: 3.36 (s, 3H, C-8 -CH_3), 3.70 (s, 3H, C-7 -CH_3), 4,69 (s, 1H, H2), 4.84 (s, 1H, C-6 OH), 6.80 (d J=8.3 Hz, 2H, H5), 7.29 (d J=8.3 Hz, 2H, H4) ppm.

M.S. [C.I. (NH_3), direct inlet, ~70°C], m/z (% relative intensity, assignment): 214 (4.7, $M^+ + NH_3$), 182 (18.0, $M^+ + NH_3$-MeOH or M^++1-Me), 165 (100.0, M^+-OMe), 137 (61.9, M^+-COOMe).

Synthesis of racemic isobutyl p-hydroxymandelate (54):

A racemic mixture of *p*-hydroxymandelic acid (**38**, 1.02g) was refluxed in isobutyl alcohol (75 mL) in the presence of catalytic amounts of *p*-toluenesulfonic acid (~0.5 mg) for 12 hours. Evaporation of the solvent gave a reddish oil which was a mixture of two products and some starting material. After flash column chromatography with Pet. Ether/EtOAc (3:1) compound **54** was isolated as the major product (40% yield after chromatography and recrystallization) and its 2-isobutyloxy derivative (**55**) as a minor side product (6% yield).

(R/S) isobutyl *p*-hydroxymandelate (54):



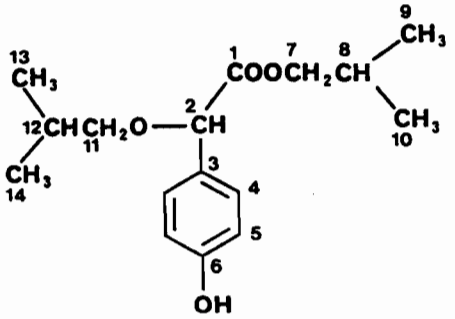
TLC: Pet. Ether/ EtOAc (3:1), Rf=0.18.

^{1}H NMR (200 MHz, $CDCL_3$) δ: 0.80 (2d J=7.6 Hz, 6H, 2xCH_3), 1.86 (m, 1H, H8), 3.44 (d J=5.4 Hz, 1H, C-2 OH), 3.93 (m, 2H, H7), 4.94 (s, 1H, C-6 OH), 5.11 (d J=5.4 Hz, 1H, H2), 6.80 (d J=8.5 Hz, 2H, H5), 7.26 (d J=8.5 Hz, 2H, H4) ppm.

^{13}C NMR (300 MHz, $CDCl_3$) δ: 18.8 (C-9, C-10), 27.7 (C-8), 72.1 and 72.5 (C-7 and C-2), 115.5 (2C-4), 128.0 (2C-5), 130.5 (C-3), 155.9 (C-6), 174.0 (C-1) ppm.

M.S. [C.I. (NH_3), direct inlet, ~60°C], m/z (% relative intensity, assignment): 242 (0.8, $M^+ + NH_3$), 224 (11.8, M^+), 207 (100, M^+-OH), 123 (M^+-$CO_2CH_2CHMe_2$).

(R/S) isobutyl 2-isobutyloxy-*p*-hydroxymandelate (55):



TLC: Pet. Ether/ EtOAc (3:1), Rf=0.53.

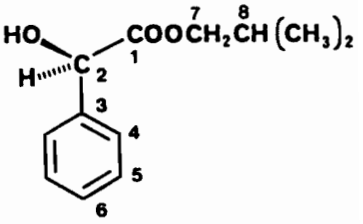
^{1}H NMR (200 MHz, $CDCl_3$) δ: 0.81 (d J=6.7 Hz, 6H, C-9 and C-10 2xCH_3), 0.89 (dd J=6.7 and 2.2 Hz, 6H, C-13 and C-14 2xCH_3), ~1.85 (m, 1H, H8), ~1.95 (m, 1H, H12), 3.23 (m, 2H, H11), 3.80 (m, 2H, H7), 4.77 (s, 1H, H2), 5.70 (s, 1H, C-6 OH), 6.78 (d J=8.7 Hz, 2H, H5), 7.28 (d J=8.7 Hz, 2H, H4) ppm. A number of selective decoupling experiments had to be carried out in order to correctly assign all proton signals as indicated.

^{13}C NMR (300 MHz, $CDCl_3$) δ: 18.9 (C-9 and C-10), 19.3 (C-13, C-14), 27.7 (C-12), 28.4 (C-8), 71.3 (C-11), 76.4 (C-7), 80.9 (C-2), 115.6 (2xC-5), 128.3 (C-3), 128.6 (2xC-4), 156.5 (C-6), 172.3 (C-1) ppm.

Synthesis of optically pure (R)-(-)-isobutyl mandelate (57a):

R-(-)-mandelic acid (**56a**, 1.0 g) was refluxed in isobutyl alcohol (~75 mL) in the presence of catalytic amounts of *p*-toluenesulfonic acid (~0.5 mg) for 12 hours. Evaporation of the solvent followed by flash column chromatography with a solvent gradient of Pet. Ether/ CH_2Cl_2 (1:1) to CH_2Cl_2 gave the (R)-(-)-isobutyl mandelate (**57a**) as a clear liquid (which solidified under vacuum, m.p.=35-36°C) in quantitative yields. Using the same procedure the (S)-(+)-isobutyl mandelate (**57b**) was prepared from the (S)-(+)-mandelic acid (**56b**).

<u>(R)-(-)-isobutyl mandelate ester (57a):</u>



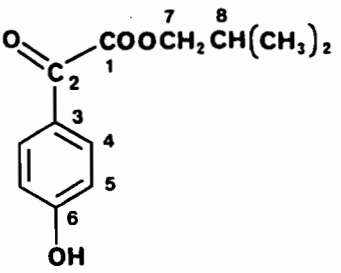
TLC: Pet. Ether/ EtOAc (6:1), Rf=0.3-0.45.
^{1}H NMR (200 MHz, $CDCl_3$) δ: 0.80 (2d J=6.7 Hz, 6H, 2xCH_3), 1.86 (m, 1H, H8), 3.53 (d J=4.8 Hz, 1H, C-2 OH), 3.93 (m, 2H, H7), 5.17 (d J=4.8 Hz, 1H, H2), 7.32 to 7.44 (m, 5H, aromatic) ppm.

Synthesis of isobutyl p-hydroxybenzoylformate (58):

Commercially available sodium salt of *p*-hydroxybenzoylformic acid (**37**, 0.85 g) was refluxed in isobutyl alcohol (~40 mL) in the presence of catalytic amounts of *p*-toluenesulfonic acid (~0.5 mg) for 24 hours. Evaporation of the solvent gave a purple-brown oil which after flash column chromatography, with CH_2Cl_2/ EtOAc (10:0.5), was found to contain approximately an equal mixture of the desired product **58** (0.2 g, 20% yield), its decarboxylated analogue *p*-hydroxybenzaldehyde **63** and starting material.

The reaction was repeated with the protonated form of the starting material in which case **58** was formed in nearly quantitative yields. The free acid was obtained by passing the salt through an ion exchange column (Dowex 50W-X8, H^+, 20-50 Mesh) and removing the water on a lyophilizer.

isobutyl-p-hydroxybenzoylformate (58):

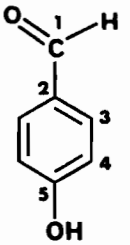


TLC: CH_2Cl_2/ EtOAc (10:0.5), Rf=0.40.

^{1}H NMR (200 MHz, $CDCl_3$) δ: 0.97 (d, J=6.7 Hz, 6H, 2xCH_3), 2.08 (m, 1H, H8), 4.15 (d, J=6.7 Hz, 2H, H7), 6.95 (d, J=9.0 Hz, 2H, H5), ~7.3 (br s, 1H, C-6 OH) and 7.93 (d, J=9.0 Hz, 2H, H4) ppm.

^{13}C NMR (300 MHz, $CDCl_3$) δ: 19.0 (C-9 and C-10), 27.7 (C-8), 72.4 (C-7), 116.2 (2C-4), 125.0 (C-3), 133.2 (2C-5), 162.8 (C-6), 164.7 (C-1) and 185.6 (C-2) ppm.

***p*-hydroxybenzaldehyde (63):**



TLC: CH_2Cl_2/ EtOAc (10:0.5), Rf=0.23.

^{1}H NMR (200 MHz, MeOD-d_4 + $CDCl_3$) δ: 4.8 (s br, 1H, Ar-OH), 6.95 and 7.78 (2d, J=8.3 Hz, 4H, Ar) and 9.78 (s, 1H, -COH) ppm.

^{13}C NMR (300 MHz, MeOD-d_4 + $CDCl_3$) δ: 115.1 (2xC-4), 128.1 (C-2), 131.7 (2xC-3), 163.1 (C-5) and 191.1 (C-1) ppm.

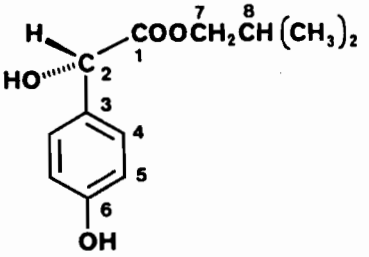
Synthesis of optically active (S)-(+)-isobutyl-p-hydroxymandelate ester (54b):

Isobutyl-*p*-hydroxybenzoylformate (**58**, 603 mg, 2.72 mmol), dissolved in dry THF (15 mL) was added dropwise to a (R)-(+)-BINAL-H[287] reagent solution (4 equivalents) at -78° C over a

287. (R)-(+)-BINAL-H and (S)-(-)-BINAL-H reagent were prepared following the procedure developed by Noyori (reference **147**). These reagents were used to synthesize the (S) and (R) isobutyl-*p*-hydroxymandelate esters respectively.

period of 10-15 minutes. The reaction was stirred at -78°C for 3.5 hours and quenched with 3 equivalents of glacial acetic acid (~0.5 mL, the excess hydride was estimated to be ~3 equivalents). EtOAc (100 mL) and H_2O (50 mL) were added, the mixture was filtered through celite and allowed to separate at room temperature. The EtOAc layer was removed, the aqueous layer was further adjusted to pH 6-7 and re-extracted with EtOAc (3x50 mL). Flash column chromatography of the organic layer with Pet. Ether/ EtOAc (3:1) afforded the (S) enantiomer of compound **54** (**54b**, 90 mg, 15% yield after chromatography) in ~60% enantiomeric purity (as determined by ^{1}H NMR), the side product alcohol **64** (40 mg, 10% yield), binapthol and unreacted starting material.

(S)-(+) isobutyl *p*-hydroxymandelate (54b):

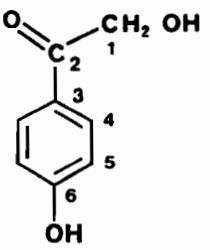


TLC: Pet. Ether/ EtOAc (3:1), Rf=0.18.

$[\alpha]_D$= +28.7

^{1}H NMR and ^{13}C NMR were identical to the racemic **54** reported previously. However in the presence of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol (1.5-2 eq) the proton spectra (300 MHz) clearly showed two sets of peaks for the C-2 proton; the (S) enantiomer upfield from the (R) with integration of S/R = ~4/1 (~60% ee).

2-(4-hydroxyphenyl)-2-oxoethanol (64):



TLC: Pet. Ether/ EtOAc (3:1), Rf=0.08.

^{1}H NMR (200 MHz, acetone-d_6) δ: 4.79 (s, 2H, CH_2), 6.95 and 7.87 (dd, 4H, Ar) ppm, exchangable protons were not seen.

^{13}C NMR (300 MHz, acetone-d_6) δ: 65.6 (C-1), 116.3 (2C-4), 127.1 (C-3), 131.1 (2C-5), 163.9 (C-6) and 198.6 (C-2) ppm.

APT (D2=4 msec), **NMR** (300 MHz, acetone-d_6) δ: 127.1, 163.9 and 198.6 ppm.

DEPT NMR (300 MHz, acetone-d_6) δ: 1CH_2 (65.6 ppm), 2CH (116.3, 131.1 ppm).

Hydrolysis of (R)-(-)-isobutyl-p-hydroxymandelate ester (54a) to (R)-(-)-p-hydroxymandelic acid (38a):

To a solution of ester **54a** (60 mg, 0.27 mmol) in dioxane (3 mL), 2 equivalents of NaOH were added (1M solution, 535 μL). The reaction was stirred at room temperature for 15 hours, at which point 10 mL of H_2O were added and the pH adjusted to ~6.5 with 0.1N HCl. The aqueous layer was extracted with EtOAc (3x15 mL) in order to remove unreacted starting material. The aqueous layer was evaporated to dryness under high vacuum at 40°C, re-dissolved in H_2O (~5 mL) and passed through an ion exchange resin (Dowex 50W-X8, H^+, 20-50 Mesh) in order to remove Na^+. The free acid **38a** was isolated in 90% yield (41 mg). Its optical rotation, $[\alpha]_D$= -10.2°, was compared to that of the natural product (**38a**), $[\alpha]_D$= -2.7°. Although, it was concluded that metabolite **38a** must have the (R) absolute configuration, the values for the optical activity obtained were inaccurate; in solution, compound **38** is acidic enough to catalyze its own racemization.

6. 2. 3 Bioassays:

Almost all of the bioassays were performed by Harry Kope, Department of Forestry, Laval University. However, the experimental procedures were outlined in a collaborative effort.

All phytopathogenic and dermatogenic fungi were cultured on Potato Dextrose Agar (Qué-Bact, Quélab, Montreal,Qué). From the edge of each actively growing colony a 5 mm^2 plug was cut and placed into a well of a Multiwell tissue culture plate (Bectan Dickson Labware, Oxnard, CA) to which 300 μL of Potato Dextrose Broth (Difco Laboratories, Detroit, Mich), diluted by 50%, were added. The fungi were incubated at 25°C for 24 hours in order to assure good growth. The nutrient broth was then removed from all wells and the test compounds, dissolved in 300 μL of sterile distilled water, were added. The cultures were incubated once again for a five day period, after which the Growth Inhibition Concentration (GIC 50) was determined. Control cultures were incubated in pure sterile water.

The ability of each compound to cause hyphal lysis was assessed using pregerminated spores of the test organisms. The active compounds caused lysis of newly formed hyphae within four hours of their addition.

6. 3 Chapter 3

6. 3. 1 Culture growth of Suillus cavipes

Discs (7 mm^2) were excised from the edge of a three week old actively growing *Suillus cavipes* colony and fragmented to a slurry in a Waring blender with a small amount of MMN liquid medium.[184b] Erlenmeyer flasks (250 mL) containing an additional 100 mL of sterile MMN liquid medium at pH 5.5, were inoculated using this slurry; 25 mL MMN and 8 discs of mycelium were used to inoculate each flask. The cultures were grown at room temperature on a rotary shaker for 6-7 weeks.

6. 3. 2 Soxhlet extraction

The culture media of *S. cavipes* was filtered through several layers of cheese-cloth to remove the mycelium. The clear yellow liquid was reduced in volume (~1/5) under high vacuum at 40°C. The remaining water was removed by freeze-drying to give a solid (~1.5 gr/L).

Soxhlet extraction of the solid material with diethyl ether (100 mL/5 gr solid) over a period of 12 hours led to the isolation of a biologically inactive crude. Further extraction with methylene chloride (100 mL/5gm of solid) for an additional 12 hours gave a mixture of metabolites which appeared to be almost identical, on cellulose TLC, to that found in the ether extract. The methylene chloride crude did not exhibit any biological activity either, as was expected.

However, biological testing of the solid remaining in the soxhlet apparatus showed enhanced root development on *Larix laricina* hypocotyl cuttings, at concentrations of 2.0 mg/mL and 1.5 mg/mL. Hence, the soxhlet extraction was continued with acetone (100 mL/5gr of solid) for an additional 36 hours. Biological testing of both the acetone extract and the remaining solid failed to show any of the expected root promoting effect on hypocotyl cuttings, suggesting that the biologically active metabolite(s) were unstable at high temperatures.

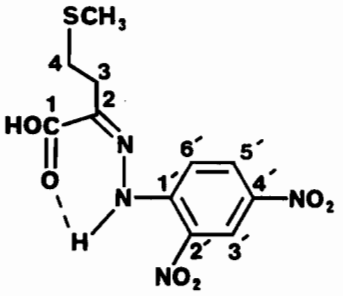
6. 3. 3 Attempts towards the isolation of 2,4-dinitrophenylhydrazone derivatives of methional or 4-methylthio-2-oxobutanoic acid from S. cavipes

2,4-Dinitrophenylhydrazine reagent:

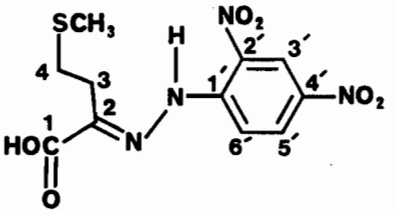
This reagent was prepared by dissolving 400 mg of 2,4-dinitrophenylhydrazine in 100 mL of a 2M H_2SO_4 solution (0.4% w/v).

Synthesis of the 2,4-dinitrophenylhydrazone derivative of 4-methylthio-2-oxobutanoate:

Commercial 4-methylthio-2-oxobutanoic acid (20 mg) was added to 25 mL of 2,4-dinitrophenylhydrazine reagent. After 30 minutes of stirring at room temperature the orange precipitate formed was collected by centrifugation. ^{1}H NMR of the isolated compound indicated the presence of an isomeric mixture (E and Z) of 2,4-dinitrophenylhydrazone-4-methylthio-2-oxobutanoate.[209]



(E)

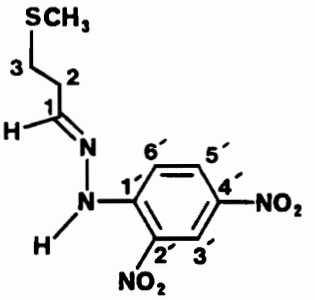


(Z)

TLC: CH_2Cl_2/CH_3OH (7:3), Rf=0.46 and 0.58.

^{1}H NMR (200MHz, $CDCl_3$) δ: 2.1 and 2.2 (2s, 6H, 2x-SCH_3), 2.8-3.1 (m, 8H, 2xH3 and 2xH4), 8.1 (2d, 2H, 2xH6'), 8.4-8.5 (2dd, 2H, 2xH5'), 9.2 (2d, 2H, 2xH3'), 11.1 and 11.2 (s, 2H, 2x NH) ppm.

Synthesis of the 2,4-dinitrophenylhydrazone derivative of methional



3-(Methylthio)proprionaldehyde (methional, 900 mg) was reacted with 30 mL of 2,4-dinitrophenylhydrazine reagent at room temperature for 30 min. The orange precipitate formed was collected by centrifugation and identified to be the desired product by TLC and ^{1}H NMR. Only one set of signals was observed on ^{1}H NMR, suggesting that only one isomer was formed.[209]

TLC: CH_2Cl_2, Rf=0.24.

^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.18 (s, 3H, -SCH_3), 2.80 (m, 4H, H2 and H3), 7.58 (t, 1H, H1), 7.95 (d, 1H, H6'), 8.32 (dd, 1H, H5'), 9.14 (d, 1H, H3') and 11.10 (s, 1H, NH) ppm.

Isolation of 2,4-dinitrophenylhydrazone derivatives from the culture media of S. cavipes

Suillus cavipes culture medium (20 mL) was mixed with 20 mL of 2,4-dinitrophenylhydrazine reagent and stirred at room temperature for 30 minutes. The suspension formed was centrifuged in order to remove a brightly colored precipitate. A large mixture of 2,4-dinitrophenylhydrazone derivatives was found in this precipitate. However, after flash column chromatography and isolation of several components none matched the TLC and ^{1}H NMR characteristics of the methional or 4-methylthio-2-oxobutanoic acid derivatives.

6. 3. 4. Plant tissue culture growth for biological testing

Biological assays for this part of the thesis were outlined in collaboration with J. Andre Fortin and Ann Stein, Department of Forestry, Laval University. All experiments were carried out by A. Stein.[184b]

The roots of *L. laricina* seedlings (30 days old) were severed 3 mm above the transition zone between root and hypocotyl. The bases of the cuttings were inserted into test tubes containing 15 mL MMN agar medium supplemented with trace elements and the compounds or crudes to be tested. The pH of all media was adjusted to 5.5 just prior to sterilization. The seedlings were incubated in growth chambers under optimum conditions of light and humidity. The overall root development and morphology was examined after a minimum incubation period of four weeks.

6. 4 CHAPTER 4

6. 4. 1 General extraction of active metabolites from P. convolvulus

Stock cultures of *P. convolvulus* (conidia) were maintained at 4°C in slant tubes containing potato carrot agar, covered with mineral oil. An aqueous suspension of conidia was used to inoculate potato dextrose agar plates which were then incubated at room temperature for a period of 2-4 weeks. The new conidia were isolated by washing the surface of the agar plates with a small volume (5-10 ml/ plate) of sterile water. Large scale cultures were subsequently initiated by inoculating moist barley grains in Erlenmeyer flasks (150 x 250 mL flasks, 20 grams of grain plus 30 ml of H_2O in each) under aseptic conditions. The cultures were stored at room temperature, with only occasional shaking. After an incubation period of four weeks, 100 ml of H_2O were added to each flask and they were placed overnight on a rotary shaker. Filtration through several layers of cheese cloth and centrifugation at 5,000 rpm for 8 minutes led to the removal of barley grains, separation of new conidia and isolation of a biologically active aqueous mixture of metabolites (**Pc 1**).

The volume of **Pc 1** (~15 L) was reduced (~3 L) under high vacuum at 30°C and then freeze-dried to obtain ~27 g of a very fine powder. This powder was extracted twice with methanol (2x1.5 L), the first time at 40°C for three hours and the second time at room temperature overnight. The methanolic solution (**Pc 2**) was filtered through several layers of cheese cloth in order to remove the bulk of the undissolved matterial, which did not exhibit any biological activity. The remaining solid was removed by centrifugation, since the powder was too fine to be filtered.

The methanolic mixture of metabolites was evaporated to dryness and redissolved in ~100 mL of water to obtain a cloudy suspension which was acidified to pH 2.5-3 with 0.1 M HCl. Ethyl acetate extraction (3 x 150 mL, plus overnight with 300 mL) led to the isolation of a biologically active crude (**Pc 3**), where the remaining aqueous mixture was found to be void of biological activity. The yields of the combined ethyl acetate extracts varied greatly (0.25-7.5 mg/gram of barley) giving an average of 2.5 mg of the light brown gum (**Pc 4**) per gram of infected barley grains.

The active crude **Pc 3** was suspended in CH_2Cl_2 (100 mL) and extracted with a saturated solution of $NaHCO_3$ (3 x 100 mL) to partition the mixture into crudes **Pc 4** and **Pc 5**. The organic layer (**Pc 4**) contained mostly steroidal metabolites exhibiting very weak phytotoxicity while the aqueous layer (**Pc 5**) was strongly phytotoxic and it contained a large mixture of compounds. The aqueous layer (**Pc 5**) was subsequently acidified to pH ~2.5-3 with 0.1 M HCl, reduced in volume under high vacuum at 30°C and extracted with ethyl acetate (3 x 100 mL) in order to recover the phytotoxic mixture of metabolites in **Pc 5**.

6. 4. 2 Isolation of ergosterol (89) and ergosterol peroxide (90)

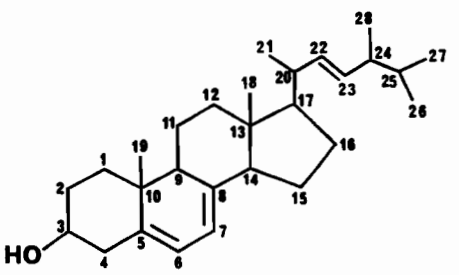
Flash column chromatography[119] of crude **Pc 4** using a solvent mixture of petroleum ether-ethyl acetate (1:1) led to the isolation of ergosterol (**89**, Rf=0.55) and ergosterol peroxide (**90**, Rf=0.28). Both of these compounds were also isolated from the dry *P. convolvulus* mycelia through soxhlet extraction with methylene chloride or acetone. Further purification was required for both of these metabolites.

Pure white crystalline ergosterol was obtained after a second flash column, eluted with (5:1) petroleum ether/ethyl acetate (Rf=0.16) followed by recrystallization from ethanol. The pure compound was kept in the dark under vacuum since it is fairly unstable to light and air.

The crude ergosterol peroxide was purified on preparative thin layer silica gel plates, developed twice with a mixture of petroleum ether-ethyl acetate (1:1). Since the compound is not uv active, the TLC plates were examined over a light table. The high density band visualized at Rf 0.4-0.5 corresponded to ergosterol peroxide. The silica gel was extracted with ethyl acetate and pure ergosterol peroxide was crystallized from methylene chloride or ethanol.

The presence of both of these steroids in the crude fungal extracts was always detected by the strong red color produced when the TLCs were dipped into a 5% vanillin-sulfuric acid solution.

<u>ergosterol (89):</u>



TLC: pet. ether/ EtOAc (1:1), Rf=0.55,

CH_2Cl_2, Rf=0.21.

M.p.: 163-164° C (lit. value 165° C[237b]).

IR ($CDCl_3$): 3650 (OH), 2960, 2872 (CH), 1601 (C=H) cm^{-1}.

UV (CH_3CH_2OH, nm): max 274, 284, min 294 (shd).

^1H NMR (300 MHz, $CDCl_3$) δ: 0.61 (s, 3H, 18-CH_3), 0.79-0.88 (2d, J=6.8 Hz, 6H, 26-CH_3 and 27-CH_3), 0.90 (d, J=6.8 Hz, 3H, 28-CH_3), 0.93 (s, 3H, 19-CH_3), 1.02 (d, J=6.6 Hz, 3H, 21-CH_3), 1.22-2.03 (m, 19H), 2.26-2.27 (tm, 1H, H4a), 2.41-2.49 (ddd, 1H, H4b), 3.60-3.66 (m, 1H, H3), 5.16-5.20 (2dd, 2x1H, H22 and H23), 5.35-5.38 (m, 1H, H7) and 5.54-5.56 (dd, 1H, H6) ppm.

^{13}C NMR: data in Table 10.

DEPT NMR (300 MHz, $CDCl_3$):

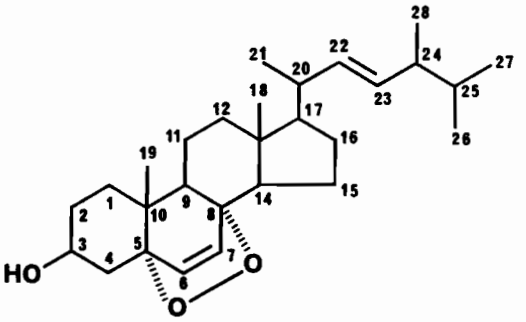
Type of carbon	Chemical shift (δ)
6 -CH_3	12.1, 16.3, 17.6, 19.7, 20.0, 21.1 ppm.
7 -CH_2	21.2, 23.0, 28.3, 32.1, 38.4, 39.2, 40.9 ppm.
11 -CH	33.1, 40.4, 42.9, 46.3, 54.6, 55.8, 70.5, 116.4, 119.7, 132.1, 135.6 ppm.

APT (D2=4 msec) **NMR** (300 MHz, $CDCl_3$) δ: 37.1, 42.9, 139.8, 141.4 ppm.

COSY NMR: spectrum in Figure 33. **HETCOR NMR:** spectrum in Figure 34.

M.S. [C.I. (NH_3), direct inlet, 220°C], m/z (% relative intensity, assignment): 414 (2, $M^+ + NH_3$), 397 (40, $M^+ + 1$), 396 (7, M^+), 379 (100, 397-H_2O).

ergosterol peroxide (90):



TLC: Pet. ether/EtOAc, Rf=0.28

m.p.: 176-177°C (lit. value 178°C[237b])

^{1}H NMR (200 MHz, $CDCl_3$) d: 0.82 (s, 3H, 18-CH_3), 0.80-0.85 (2d, 6H, 26-CH_3 and 27-CH_3), 0.89 (s, 3H, 19-CH_3), 0.91 (d, 3H, 28-CH_3), 1.00 (s, 3H, 21-CH_3), 1.2-2.2 (m, 21H, steroid sckeleton), ~4 (m br, 1H, H3), 5.15-5.21 (2dd, 2x1H, H22 and H23), 6.25 (d J=8.5 Hz, 1H, H6), 6.51 (d J=8.5Hz, 1H, H7) ppm.

DEPT NMR (300 MHz, $CDCl_3$):

Type of carbons	Chemical shifts (δ)
6 -CH_3	13.0, 17.7, 18.3, 19.8, 20.1, 21.0 ppm
7 -CH_2	20.8, 23.5, 28.8, 30.2, 34.8, 37.0, 39.4 ppm.
11 -CH	33.2, 39.9, 42.9, 51.1, 51.8, 56.3, 66.5, 130.7, 132.2, 135.2, 135.4 ppm.

APT (D2=4 msec) **NMR** (300 MHz,$CDCl_3$) δ: 37.1 (C-10), 44.7 (C-13), 79.5 (C-5), 82.2 (C-8) ppm.

MS [high resolution, C.I. (i-But), direct inlet, 220°C]: m/z calculated ($M^+ + 1$) ion 429.33685, found 429.33687

6. 4. 3 Isolation of phytotoxic metabolites from crude Pc 5 of P. convolvulus

6. 4. 3a Preparation of C_{18} reverse-phase silica gel:[244]

Silica gel (40 grams, Merck Kieselgel 60, 230-400 mesh) was added to 300 mL dry CCl_4 (redistilled from P_2O_5) in a septum-capped round bottom flask under a nitrogen atmosphere. *n*-Octadecyltrichlorosilane (4 mL) was added and the suspention was stirred at room temperature for two hours.

The product was filtered into a dry seinterglass funnel and washed free of unreacted silane with dry CCl_4 (3x100 mL). Any residual chloride substituents were converted to methoxy groups by washing the silica with dry methanol (2x100mL, redistillied over Mg metal). The product was then quickly washed with with dry CH_2Cl_2 (2x100 mL, redistilled from P_2O_5) and allowed to briefly dry under vacuum.

A fresh 300 mL volume of dry CCl_4 was added to the bonded silica along with 4 mL of trimethylchlorosilane. The mixture was stirred at room temperature for an additional two hours, then filtered and washed with dry CH_2Cl_2 (3x100 mL). The bonded silica was dried at 40°C overnight and under high vacuum for a day.

6. 4. 3b Reverse phase flash column chromatography of phytotoxic crude Pc 5:

A reverse phase silica gel column (12 mm x 32 cm) was packed as a methanolic slurry (~12 grams C_{18} bonded silica) and then very slowly equilibrated with H_2O.

The crude **Pc 5** (~1.3 grams) was dissolved in a mixture of H_2O/ CH_3OH/ CH_2Cl_2, and absorbed onto a small amount of C_{18} bonded silica gel (1-2 grams) by evaporating off the organic solvents. The aqueous slurry obtained (5-10 mL) was added to the top of the reverse phase column and eluted with the gradient of solvents described in Table 11 at a flow rate of ~1"/min. All fractions (20 mL each) were tested for biological activity. Strong phytotoxicity was observed with fractions 6, 7 and 8 which were further purified by HPLC.

A new sample of **Pc 5** (~1.2 grams) was chromatographed using a reverse phase column as described above. Smaller fractions (8 mL each) were collected and compared with the previously isolated active crudes (fractions 6, 7 and 8) by TLC.

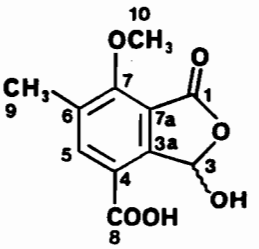
Metabolites **91** and **92** were isolated from fractions 18 and 19 after HPLC chromatography

on a C_{18} reverse phase column, using an eluent mixture of 59.8% H_2O, 40% CH_3OH and 0.2% CH_3COOH at a flow rate of 2 mL/min. The retention time of **91** was 22-23 minutes after the void volume where that of **92** was 16-17 minutes. Metabolite **92** was rechromatographed on the same HPLC column using a solvent mixture of 54.1% H_2O, 45.8% CH_3OH and 0.1% CH_3COOH. At a flow rate of 2 mL/min its elution time was 10-11 minutes after the void volume.

HPLC chromatography of fractions 20 and 21, on the same C_{18} reverse phase column using 54.4% H_2O, 45.4% CH_3OH and 0.2% CH_3COOH as the eluting solvent, led to the isolation of metabolites **93**, **94** and **96**. At a flow of 2 mL/min, the retention times were 20-22 minutes for **94**, 25-26 minutes for **96** and 27-29 minutes for **93** (from the void volume). Compound **96** was chromatographed for a second time under the same conditions in order to achieve complete separation from **93**.

6. 4. 4 Identification of phytotoxic metabolites from crude Pc 5

4-carboxy-3-hydroxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (91):



TLC: CH_2Cl_2/EtOAc/CH_3COOH (5:5:0.2), Rf=0.24.

IR (CH_3CN): 3381, 3226 (OH), 1773 (C=O, lactone), 1728 (C=O, carboxylic acid), 1613 (C=C) cm^{-1}.

UV (CH_3CH_2OH, nm): max 220, 250 (shd), min 300.

^{1}H NMR (200 MHz, acetone-d_6) δ: 2.35 (s, 3H, 9-CH_3), 4.14 (s, 3H, 10-OCH_3), 6.91 (s, 1H, H3) and 8.09 (s, 1H, H5) ppm.

NOE: data given in Table 12.

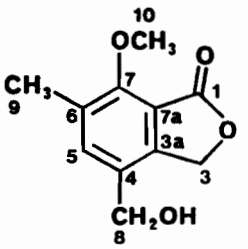
^{13}C NMR (300 MHz, acetone-d_6, T=-46.5°C) δ: 15.7 (C-9), 62.6 (C-10), 97.4 (C-3), 118.3 (C-7a), 121.9 (C-4), 134.3 (C-6), 139.3 (C-5), 149.4 (C-3a), 160.5 (C-7), 165.7 (C-8) and 166.4 (C-1) ppm.

Coupled ^{1}H-^{13}C NMR: data given in Table 14.

APT (D2=4 msec) **NMR** (300 MHz, acetone-d_6, T=-46.5°C) δ: 118.3, 121.9, 134.3, 149.4, 160.5, 165.7 and 166.4 ppm (97.4, 139.3 ppm tertiary carbons).

MS [C.I. (NH_3), direct inlet, 260°C]: m/z (% relative intensity, assignment): 256 (100, $M^+ + NH_3$), 239 (53, $M^+ + 1$), 227 (56, $M^+ + NH_3$-CHO), 209 (15, M^+-CHO).

4-(Hydroxymethyl)-7-methoxy-6-methyl-1(3H)-isobenzofuranone (92):



TLC: CH_2Cl_2/EtOAc/CH_3COOH (5:5:0.2), Rf=0.38.

IR (CH_3CN): 3630, 3540 (OH), 1762 (C=O, lactone), 1600 (C=C) cm^{-1}.

UV (CH_3CH_2OH, nm): max 212, 238 (shd), min 300.

^1H NMR (300 MHz, $CDCl_3$, T=-51.5°C) δ: 2.26 (s, 3H, 9-CH_3), 2.6-2.7 (br, 1H, C-8 OH)[288], 3.90 (s, 3H, 10-OCH_3), 4.66 (d, J=6 Hz, 2H, H8), 5.22 (s, 2H, H3) and 7.31 (s, 1H, H5)[289] ppm.

NOE: data given in Table 15

^{13}C NMR (300 MHz, CD_3OD) δ: 15.4 (C-9), 62.2 (C-8), 62.5 (C-10), 70.1 (C-3), 117.9 (C-7a), 132.2 (C-4), 132.7 (C-6), 137.2 (C-5), 146.7 (C-3a), 157.7 (C-7) and 171.2 (C-1) ppm.

Coupled ^1H-^{13}C NMR: data given in Table 16.

DEPT NMR (CD_3OD, 300 MHz):

Type of carbon	Chemical shift (δ)
2 -CH_3	15.4, 62.5 ppm.
2 -CH_2	62.2, 70.1 ppm.
1 -CH	137.2 ppm.

MS [high resolution, C.I.(NH_3), direct inlet, 250°C]: m/z for (M^++1) ion: calculated 209.0814, found 209.0814.

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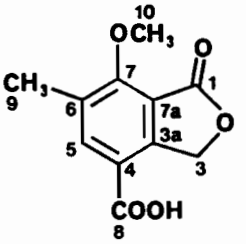
288. D_2O exchange causes the disappearence of this signal, as expected, and the collapsing of the doublet at 4.66 ppm to a singlet.

289. Decoupling of H5 led to the change of the OH signal from a broad multiplet to a broad triplet ($J_{H8\text{-}OH}$ = ~6Hz), indicating the presence of long range coupling between H5 and OH.

4-carboxy-7-methoxy-6-methyl-1(3H)-isobenzofuranone (93):



TLC: $CH_2Cl_2/EtOAc/CH_3COOH$ (5:5:0.2), Rf=0.45.

IR (CH_3CN): 3629, 3542 (OH), 1764 (C=O, lactone), 1725 (sh), 1717 (C=O, carboxylic acid), 1630 (C=C) cm^{-1}.

UV (CH_3CH_2OH, nm): max 216, 246 (shd), min 300.

^1H NMR (200 MHz, acetone-d_6) δ: 2.34 (s, 3H, 9-CH_3), 4.15 (s, 3H, 10-OCH_3), 5.49 (s, 2H, H3) and 8.13 (s, 1H, H5) ppm.

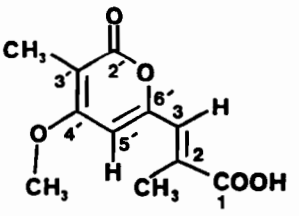
^{13}C NMR (acetone-d_6, 300 MHz, T=-46.5°C) δ: 15.6 (C-9), 62.6 (C-10), 70.9 (C-3), 117.6 (C-7a), 120.5 (C-4), 132.2 (C-6), 138.8 (C-5), 150.7 (C-3a), 161.2 (C-7), 166.4 (C-8) and 168.4 (C-1) ppm.

Coupled ^1H-^{13}C NMR: data given in Table 17.

APT (D2=4 msec) **NMR** (acetone-d_6, 300MHz, T=-46.5°C) δ: 117.6, 120.5, 132.6, 150.7, 161.2, 166.4 and 168.4 ppm (138.8 ppm tertiary carbon).

MS [high resolution, C.I.(NH_3), direct inlet, 250°C]: m/z for (M^++1) ion: calculated 223.0606, found 223.0621.

3-(4-methoxy-3-methyl-α-pyron-6-yl)-2-methyl-2-butenoic acid (94):



TLC: $CH_2Cl_2/EtOAc/CH_3COOH$ (5:5:0.2), Rf=0.33.

IR (CH_3CN): 3628, 3618 (OH), 1717 (C=O, α-pyrone), 1707, 1702 (sh) (C=O, carboxylic acid) cm^{-1}.

UV (CH_3CH_2OH, nm): max 238, min 340.

^1H NMR (200 MHz, acetone-d_6) δ: 1.87 (s, 3H, C-3'-CH_3), 2.42 (s, 3H, C-2-CH_3), 4.04 (s, 3H, C-4'-OCH_3), 6.62 (s, 1H, H3) and 6.91 (s, 1H, H5') ppm.

NOE NMR: data given in Table 18.

^{13}C NMR (300 MHz, CD_3OD) δ: 8.8 (C-3'-$\underline{C}H_3$), 13.6 (C-2-$\underline{C}H_3$), 57.5 (C-4'-O$\underline{C}H_3$), 98.4 (C-5'), 105.3 (C-3'), 121.8 (C-3), 143.2 (C-2), 159.2 (C-6'), 166.4 (C-4'), 167.9 (C-2') and 169.4 (br, C-1) ppm.
Coupled ^{1}H-^{13}C NMR (400 MHz, CD_3OD) δ: 8.8 (m, C-3'-$\underline{C}H_3$), 13.6 (q, J = 129 Hz, C-2-$\underline{C}H_3$), 57.5 (q, J = 147 Hz, C-4'-OCH_3), 98.4 (d, J = 170 Hz, C-5') and 121.8 (d, J = 165 Hz, C-3) ppm.
APT (D2 = 4 msec) NMR (300 MHz, CD_3OD) δ: 105.3, 143.2, 159.9, 166.4, 167.9 and 169.4, (98.4, 121.8 ppm, tertiary carbons).
MS [high resolution, C.I. (NH_3), direct inlet, 250°C]: m/z calculated for the ($M^+ + 1$) ion 225.0763, found 225.0739.

Unknown metabolite (96):

The small quantity of available sample (~0.2-0.5 mg) of metabolite **96** did not permit total structure determination, however, the NMR and mass spectra of the compound showed structural similarities to metabolites **91**, **92** and **93**.

^{1}H NMR (200 MHz, acetone-d_6) δ: 1.26 (t, J = 7.1 Hz, 3H), 2.30 (s, 3H, -CH3), 3.84 (t, J = ~1, 1H), 3.88 (dd, J = ~1.6 Hz, 1H), 4.00 (s, 3H, -OCH3), 4.65-4.85 (2d, J = ~9.5, 2H), 6.47 (s, 1H) and 7.61 (s, 1H) ppm.
COSY NMR (300 MHz, acetone-d_6): triplet at 1.26 ppm was coupled to both signals at 3.84, 3.88 ppm, and the two protons at 4.65-4.85 ppm were coupled to each other.
^{13}C NMR (300 MHz, CD_3OD) δ: 15.4, 30.3, 72.8, 102.7, 108.8, 124.1, 137.9, 151.4, 172.9 ppm, the remaining carbons could not be observed.
MS [C.I. (NH_3), direct inlet, ~150°C], m/z (% relative intensity, assignment): 207 (100, $M^+ + 1$), 224 [43, $(M+NH_4)^+$], 413 (8, $2xM^+ + 1$)
MS [high resolution, E.I. (70 eV), direct inlet, 180°C]: calculated m/z for $C_{11}H_9O_4$ (M^+-1) ion 205.05008, found 205.05010.

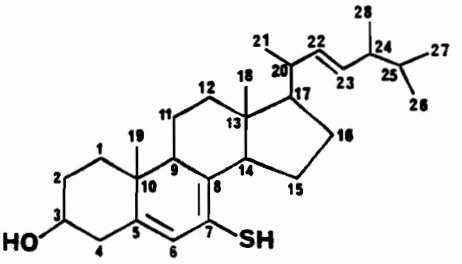
6. 5 Chapter 5

6. 5. 1 Reaction of ergosterol with bis(n-cyclopentadienyl)titana(IV)cyclohexasulfane:

A solution of Br_2 (50 μL, 1.0 mmol) in dry CH_2Cl_2 (5.0 mL) was added dropwise to solution of triphenylphosphine (275 mg, ~1.1 mmol in 5.0 mL dry CH_2Cl_2) at 0° C. The suspension of triphenyldibromophosphorane formed was allowed to warm-up to room temperature and then it was added, drop-wise, to a solution of ergosterol (800 mg, 1.0 mmol) and bis(*n*-cyclopentadienyl)titana(IV)cyclohexasulfane (340 mg, 1.0 mmol) in 15 mL dry CH_2Cl_2. The reaction mixture was stirred at room temperature for four hours, evaporated to dryness and purified by chromatography.

Flash column chromatography[119] of the reaction mixture, using pure CH_2Cl_2 as the eluting solvent, led to separation of the isomeric mixture of thiols **118** and **119** (fractions 25-30) from the remaining of the crude. Further thin layer chromatography was carried out, on preperative silica gel plates developed five times with petroleum ether/ethyl acetate (5:1), in order to achieve partial separation of the two compounds. The major product **119** was isolated in ~15% yield (~250 mg) and the minor product **118** in ~5% yield (~20 mg).

ergosta-5,7,22-trien-7-mercapto-3-ol (119):

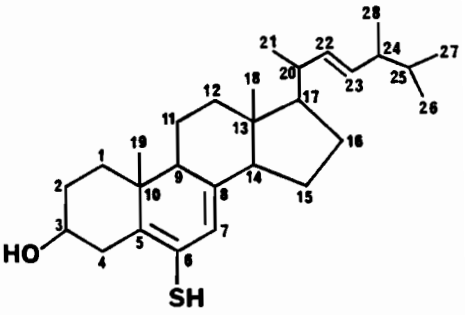


TLC: CH_2Cl_2/EtOAc (2:1), Rf=0.59

^{1}H NMR (200 MHz, $CDCl_3$) δ: 0.80-0.85 (2d, J= ~6.8 Hz, 6H, 26-CH_3 and 27-CH_3), 0.82 (s, 3H, 18-CH_3), 0.92 (d, J=6.8 Hz, 3H, 28-CH_3), 0.98 (s, 3H, 19-CH_3), 1.03 (d, J=6.5 Hz), 1.2-2.4 (m, 21H, steroid skeleton), 3.63 (m, 1H, H3), 5.20-5.25 (m, 2H, H22 and H23) and 5.33 (m, 1H, H6) ppm.

^{13}C NMR (300 MHz, $CDCl_3$) δ: 15.83, 17.67, 18.39, 19.65, 19.98, 21.00, 21.87, 25.31, 26.60, 31.72, 33.10, 35.35, 36.57, 36.69, 36.95, 38.33, 38.91, 40.96, 42.83, 44.82, 57.10, 71.01, 117.79, 123.14, 132.07, 135.52, 140.74 and 150.85 ppm.

MS [C.I. (NH_3), direct inlet, 250°C], m/z (% relative intensity, assignment): 397 (61, M^++1-SH), 270 (17, M^+-SH-125), 125 (100, side chain).



ergosta-5,7,22 trien-6-mercapto-3-ol (118):

TLC: CH_2Cl_2/EtOAc (2:1), Rf=0.59.

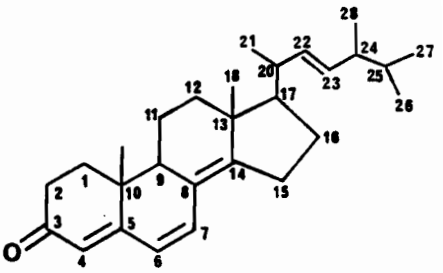
^{1}H NMR (200 MHz, $CDCl_3$) δ: 0.80-0.85 (2d, J= ~6.8 Hz, 6H, 26-CH_3 and 27-CH_3), 0.84 (s, 3H, 18-CH_3), 0.92 (d, J=6.8 Hz, 3H, 28-CH_3), 1.03 (d, J=6.6 Hz, 3H, 21-CH3), 1.11 (s, 3H, 19-CH_3), 1.2-2.3 (m, 21H, steroid skeleton), 3.90 (m, 1H, H3), 5.20-5.25 (m, 2H, H22 and H23) and 5.36 (m, 1H, H7) ppm.

6. 5. 2. Reaction of ergosterol with elemental sulfur

Ergosterol (5.0 g, 12.6 mmol) and S_8 (3.00 g, 11.7 mmol= ~4 eq. of S_2) were refluxed in dry toluene (~200 mL) in the presence of one equivalent of triethylamine (1.5 mL, ~12 mmol). After a period of 24 hours the crude mixture was filtered, washed with water (3x100 mL) and evaporated to dryness.

Flash column chromatography (2x) of the reaction mixture with petroleum ether/ ethyl acetate (4:1), (2nd chromatography was performed on fractions 13-17 of the 1st column) led to the isolation of compound **120** as an orange-yellow solid. Preparative thin layer chromatography on silica gel plates, developed twice with hexane/ethyl acetate (9:1), followed by recrystallization from ethanol gave crystalline compound **120** in approximately 5% yield (30 mg).

ergosta-4,6,8 (14),22-tetraen-3-one (120):



TLC: Pet ether/EtOAc (3:1), Rf=0.45 (fluorescent spot under uv).

M.p.: 112.5-113°C (lit. value 114-115° C[285]).

IR ($CDCl_3$): 1646 (C=O), 1636, 1584 (C=C) cm^{-1}.

UV (CH_3CH_2OH, nm): max 350.

^1H NMR (200 MHz, $CDCl_3$) δ: 0.78-0.83 (2d, J=6.7 Hz, 6H, 26-CH_3 and 27-CH_3), 0.90 (d, J=6.8 Hz, 3H, 28-CH_3), 0.93 (s, 3H, 18-CH_3)[290], 0.96 (s, 3H, 19-CH_3), 1.03 (d, J=6.7 Hz, 3H, 21-CH_3), 1.15-2.55 (m, 18H, steroid skeleton), 5.17-5.22 (m, 2H, H22 and H23), 5.70 (s, 1H, H2), 6.00 (d, J=9.5 Hz, 1H, H6) and 6.57 (d, J=9.5 Hz, 1H, H7) ppm.

^{13}C NMR (300 MHz, $CDCl_3$) δ: 16.62 (C-18),[290] 17.61 (C-28), 2x18.95 (C-19 and C-2), 19.63 (C-26), 19.96 (C-27), 21.19 (C-21), 25.33 (C-11), 27.69 (C-16), 33.05 (C-25), 2x34.09 (C-1 and C-12), 35.55 (C-15), 36.71 (C-10), 39.25 (C-20), 42.83 (C-24), 43.95 (C-13), 44.28 (C-9), 55.66 (C-17), 122.95 (C-4), 124.38 (C-6), 124.42 (C-8), 132.49 (C-23), 133.97 (C-7), 134.96 (C-22), 156.04 (C-14), 164.32 (C-5) and 199.43 (C-3) ppm.

APT (D2=4 msec) **NMR** (300 MHz, $CDCl_3$) δ: 36.75 (C-10), 43.96 (C-13), 124.26 (C-8), 155.89 (C-9), 164.17 (C-5) and 199.20 (C-3) ppm.

DEPT NMR (300 MHz, $CDCl_3$):

Type of carbon	Chemical shift (δ)
6 -CH_3	16.61, 17.61, 18.95, 19.63, 19.96, 21.19 ppm.
6 -CH_2	18.95, 25.33, 27.69, 2x34.09, 35.55 ppm.
10 -CH	33.05, 39.25, 42.83, 44.28, 55.66, 122.95, 124.42, 132.49, 133.97, 134.96 ppm.

MS [C.I. (NH_3), direct inlet, 150°C], m/z (% relative intensity, assignment): 394 (31, $M^+ + 1$), 393 (100, M^+).

290. In both the ^1H and ^{13}C NMR spectra, the assignments for C-18 and C-19 could be reversed.