INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Isolation and characterization of secondary metabolites from Canadian indoor strains of *Penicillium brevicompactum* and *Penicillium chrysogenum*

by

Regina De La Campa, B.Sc.H.

A thesis submitted

to the Faculty of Graduate Studies and Research

in partial fulfillment of

the requirements for the degree of

Master of Science

Department of Chemistry

Carleton University

Ottawa, Ontario

May, 2005

Abstract

An investigation of the chemotypes of building associated strains of *Penicillium brevicompactum* and *Penicillium chrysogenum* isolated from Canadian homes is presented here. Recently there has been an increased concern about the adverse health effects associated with fungal growth in buildings. *P. brevicompactum* is one of the most common inhabitants of the sapwood of conifer lumber in North America *P. chrysogenum* is a ubiquitous fungus, among the most common eukaryotic life forms on earth. It occupies a wide range of habitats including soils, decaying vegetation and foods. Distribution appears to be universal, throughout all biologically accessible regions and climates. Both species are often reported in indoor environments.

It was established that the *P. brevicompactum* strains produced roquefortine C, mycophenolic acid or meleagrin but only the wood strains produced significant amounts of asperphenamate and brevianamide A. Most of the *P. chrysogenum* strains produced penicillin G, roquefortine C, meleagrin. Only the building related strains accumulated xanthocillin x. The production of penicillin G from strains growing on water-damaged building materials has not previously been reported in the literature.

Brevianamide A, mycophenolic acid and roquefortine C were identified, isolated and purified by means of several chromatographic methods. These metabolites were submitted for studies of the toxigenic potential of building-associated fungi and to investigate their biological activity. For the toxicology studies the documentation of the purity of these compounds is of outmost importance. HPLC coupled with MS and/or NMR offered effective ways of obtaining purity information about the samples.

Acknowledgements

I would like to thank NSERC for providing funding for this project.

The completion of this project would not have been possible were it not for the kind and generous support of many people. To begin, I owe my heartfelt gratitude to my supervisor, teacher and mentor Dr. J. David Miller for his guidance throughout these past years and for facilitating my growth not only as a scientist but also as an individual. Thank you for believing in me from the beginning. Your generosity, patience and invaluable direction will never be forgotten.

I would also like to thank Dr. Evaloni Puniani for sharing her knowledge on the isolation, purification and identification of natural products. Your experience and ideas proved most valuable especially during frustrating days. It was a delight to have you as part of our lab even if it was for only a short time.

Thank you to Dr. John ApSimon for taking the time to review and comment on my progress and for reminding me that science and life don't have to always be taken so seriously. Thanks to Dr. Barbara Blackwell, Agriculture and Agri-Food Canada, for your help with deciphering NMR spectra and for always being there to provide excellent advice.

Furthermore, I would also like to thank all the students in our research team for their support and for keeping the lab an entertaining place to work. Your stories and experiences kept me laughing and enjoying the daily routine. Thank you Alice for your help in analysing the penicillin samples and for the Indonesian food. Don, I thank you for collecting the isolates and for teaching me how to take care of them. I have enjoyed working and discussing science and sports with you. In particular however, I would like

to thank my colleague Mark Sumarah for his friendship (even if only in Ontario) and encouragement from day one. Thank you for your sunny disposition, the treats and your scientific advice. I could not have done this without you, D.

Finally, I would like to dedicate this thesis to my parents Angel y Toni, to my sister Paulina, to my brother Johnny and to my love, soul mate and husband Mark. Thank you for your support and words of wisdom. Without you I would not have made it this far! Gracias por todo, los extrano.

List of Figures

List of Tables and Schemes

1. INTRODUCTION

1.1 Fungi

Fungal intervention in human affairs is evident everywhere as in the biochemical ability of the saprobic fungi in the production of beer, wine, bread, cheeses, soy sauce, some antibiotics and immunosuppressants, organic acids, and many other useful chemicals. Fungi are even being used to convert plant waste into high-protein animal feed. Some of the parasitic forms are now being employed to attack insects, weeds and other fungi that threaten our welfare[25].

Fungi are eukaryotic, heterotrophic, osmotrophic, they develop a diffuse branched tubular body (radiating hyphae making up mycelium or colonies) and reproduce by means of spores. They provide a critical part of nature's continuous rebirth as they recycle dead organic matter into useful nutrients. Around 100000 described species of fungi are divided into four groups based largely on the characteristics of their reproductive structures.

Indoors, three fungal groups are important. The Ascomycetes, the Basidiomycetes and to a lesser extent the Zygomycetes[25]. The ascomycota are fast-growing, primary colonizers of substrates containing accessible carbon sources like sugar or starch. This group includes the molds, which are specialized parasites of animals, plants and even other fungi.

1.2 Fungal growth in indoor environments

Indoor air can be contaminated by pollutants of chemical origin, as well as by those of biological origin such as dust mites, animal allergens and mold. Microorganisms, including mold, have been identified as the primary source of indoor air contamination in at least 35-50% of indoor air quality studies conducted over the last decade by the

National Institute for Occupational Safety and Health (NIOSH)[53]. This is partly due to the fact that indoor air quality investigations have recently moved away from strictly chemical contaminant studies to a more interdisciplinary approach. This new approach combines the evaluation of physical, chemical and microbiological constituents of indoor environments. As a consequence, there has been an increased concern about the adverse health effects associated with fungal growth in buildings. This has been especially true in cold weather countries like Canada where people spend the majority of their time indoors.

Fungi can colonize dead and decaying organic matter on materials such as textiles, leather, wood, paper and even damp inorganic materials if nutrients containing dust or soil particles are available. Species can grow and reproduce under a variety of conditions, as a result fungi are found in a wide range of habitats. Fungal growth on building materials occurs as a result of high water activity (a_w) and suitable temperatures[63]. In addition, under certain metabolic conditions, fungi produce secondary metabolites, low-molecular weight compounds that can initiate toxic or allergic responses in humans. Secondary metabolite production in buildings has been shown to occur when the a_w at the surface of the construction material exceeds 0.9, although it has been suggested that significant toxin production does not begin until the a_w reaches 0.95[62]. The development of an indoor mold problem might involve a series of water intrusion events that allow large quantities of fungal biomass to accumulate and ultimately result in secondary metabolite formation. This is then followed by a period of drying that promotes the dispersion of spores and fungal fragments throughout the building[62]. The primary mode of human exposure to these compounds is via inhalation of spores and

mycelium fragments. Studies have shown that living in a damp house is associated with increased rates of disease, the belief is that this is due to exposure to biological contaminants[1]. The correlation between building dampness, mold growth and symptoms such as allergies, airway problems, fatigue, skin irritation and asthma has been demonstrated over the past several years, although causality has not yet been established[63].

There are characters of mold biochemistry that are of special interest in terms of human health. These include biologically active cell wall components (1, 3 β-D glucan), allergens, polyclonal cell activators and secondary metabolites to name a few. Disease occurs when there is structural or functional harm resulting in signs and symptoms that can either be infectious, non-infectious or can be a combination of both. Infectious diseases occur when the fungus grows and replicates in viable body tissues, or sterile fluids. Non-infectious disease does not involve the growth and replication of the fungus in the body. Indoor mold related disease is typically either allergic, toxic or a combination of these[38,19]. Allergic disease includes the activation of asthma, allergic rhinitis, allergic alveolitis and allergic sinusitis. Skin allergic disease is based on an increase in immune expression when the immune system is challenged by an allergen[59].

In a collaborative study, Nielsen *et al.* (1999) investigated the microfungal contamination in damp buildings and the risk it posed to construction materials in Denmark[63]. They reported that the microfungal genera most frequently encountered in building materials were *Penicillium* (68%), *Aspergillus* (56%), *Chaetomium* (22%), *Ulocladium,* (21%), *Stachybotrys* (19%) and *Cladosporium* (15%). *Penicillium chrysogenum, Penicillium brevicompactum, Aspergillus versicolor,* and *Stachybotrys*

chartarum were the most frequently occurring species[63]. In 1988, Miller *et al.* identified and quantified fungal products present in 50 Canadian homes. They identified 42 different fungal species where *Penicillium* was the most common genus in both air and dust samples, followed by *Cladosporium* and *Alternaria*. *Aspergillus fumigatus* and *Stachybotrys atra* were rarely present[61]. In 1997, a Canadian study on indoor mold exposure reported twenty-five of the most common fungal species isolated from homes in Wallaceburg, Ontario. *P. brevicompactum* was identified as the most common species and *P. chrysogenum* was one of the top 15 species present[58]. *P. chrysogenum*, *P. brevicompactum* and their secondary metabolites will be the focus of this study.

1.3 *Penicillium* identification, morphology and ecology

The genus *Penicillium* is characterised by the production of conidia in chains from verticils of phialides. Phialides may be directly supported on a stipe or on one, two or rarely three compact stages of supporting cells: metulae and rami, on occasion with ramuli in between. Species of *Penicillium* are recognized by their dense brush-like spore-bearing structures. Conidia are usually shades of dull green; philiades have short straight necks and smooth walls and are characteristically produced on a stipe or a metulae over a period of time but not simultaneously[74]. Branching is an important feature for identifying *Penicillium* species. Some are unbranched and simply bear a cluster of phialides at the top of the stipe. Others may have a cluster of branches, each bearing a cluster of phialides. A third type has branches bearing a second order of branches, bearing in turn a cluster of phialides. These three types of spore bearing systems are called monoverticillate, biverticillate and terverticillate respectively (Figure 1.1).

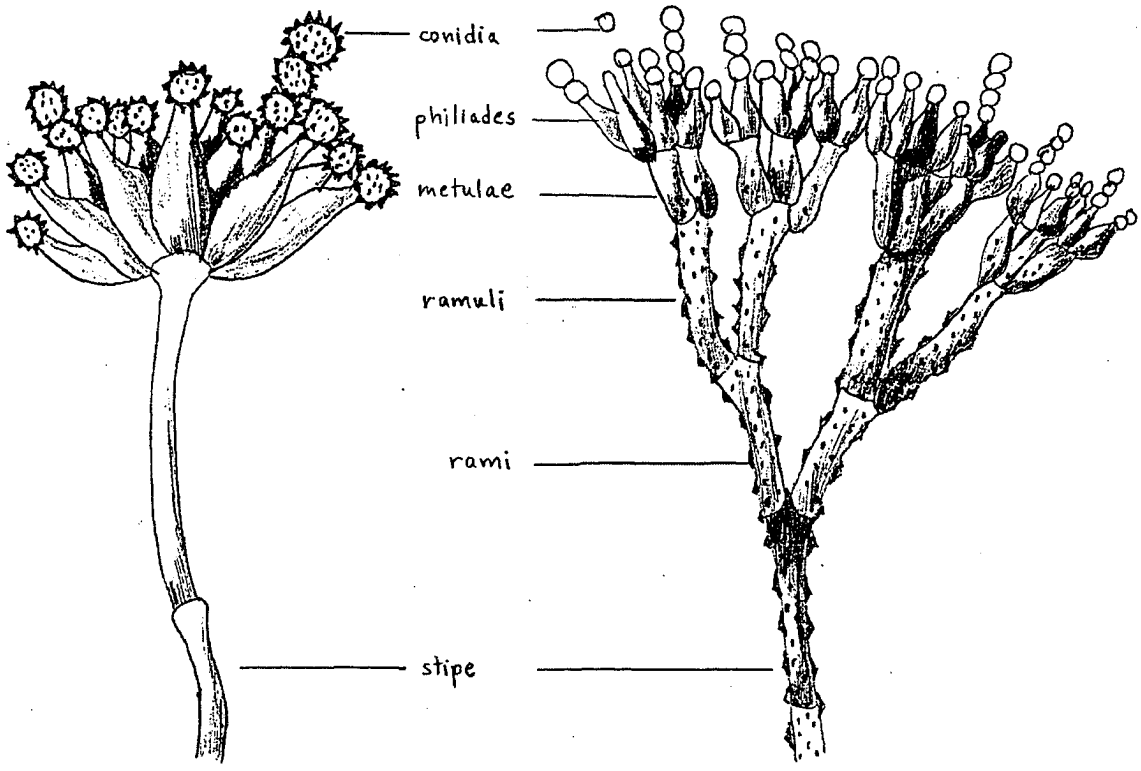


Figure 1.1 Morphological structures of *Penicillium* (adapted from Pitt, 2000)[74].

Penicillium species are associated with two ascomycetous teleomorph genera (sexual stages), *Eupenicillium* and *Talaromyces* which are phylogenetically and taxonomically distinct (Figure 1.2).

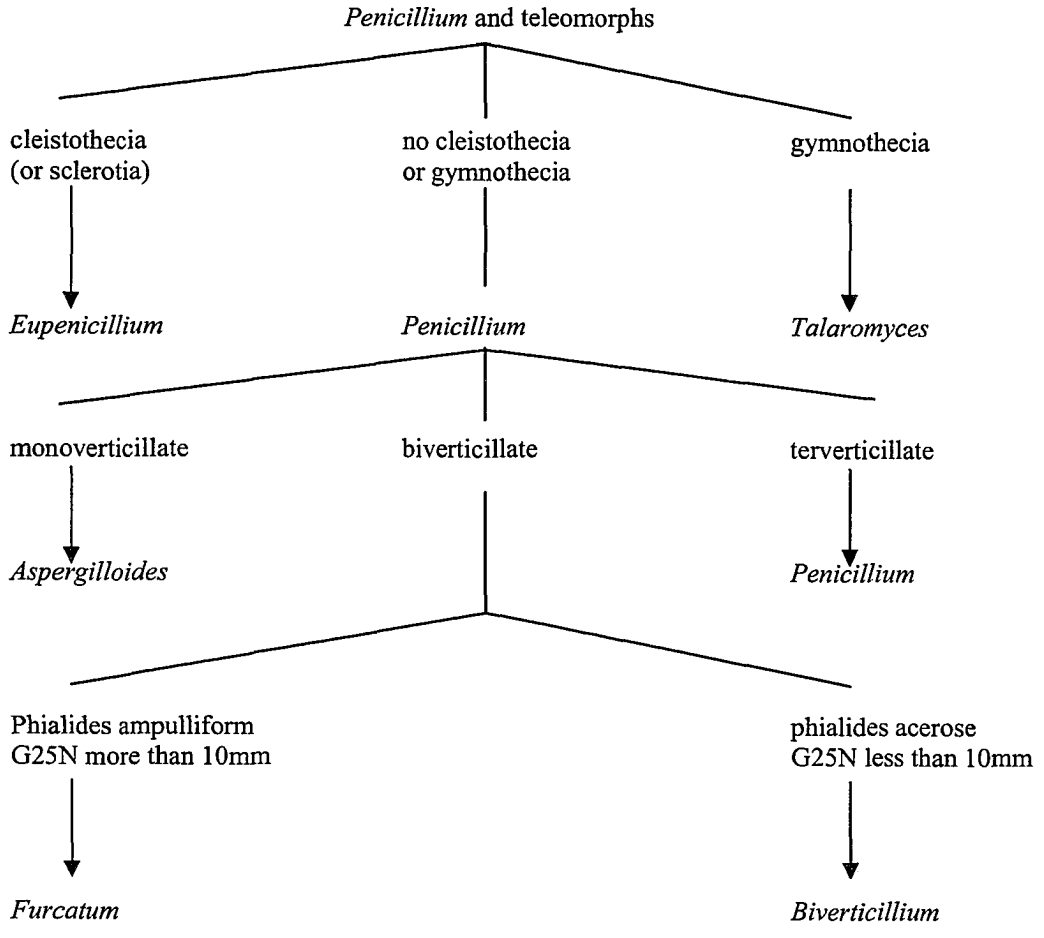


Figure 1.2 Schematic key to subgenera of *Penicillium* and its teleomorphs[74].

Penicillium brevicompactum produces distinctive features such as compact penicilli, often broad and long. Its metulae are short and broad, often apically inflated, fanning out so that the outermost philiades point in almost diametrically opposite directions. It is a widespread fungus, occupying habitats in soil and decaying vegetation in many geographical areas. It has been frequently isolated from foods, cereals, textiles, paints and building materials (Figure 1.3)[74].

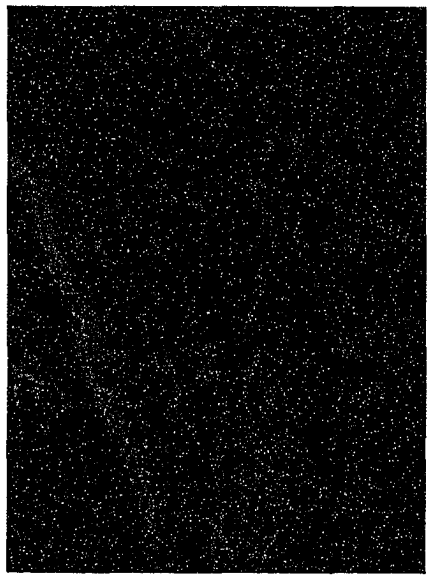


Figure 1.3 *P. brevicompactum* (with permission from Centraalbureau voor Schimmelcultures)

Penicillium chrysogenum is also a distinct species. Its colonies grow rapidly on malt extract agar at 25°C and on Czapek yeast extract agar they produce a luxuriant growth coloured by blue-green conidia, and by yellow exudate, soluble pigment and reverse. Microscopically, penicilli are terverticillate, smooth walled and rather delicate by comparison with most other species in this subgenus. *P. chrysogenum* is a ubiquitous fungus, among the most common eukaryotic life forms on earth. It occupies a wide range of habitats including soils, decaying vegetation and foods. Distribution appears to be universal, throughout all biologically accessible regions and climates (Figure 1.4)[74].

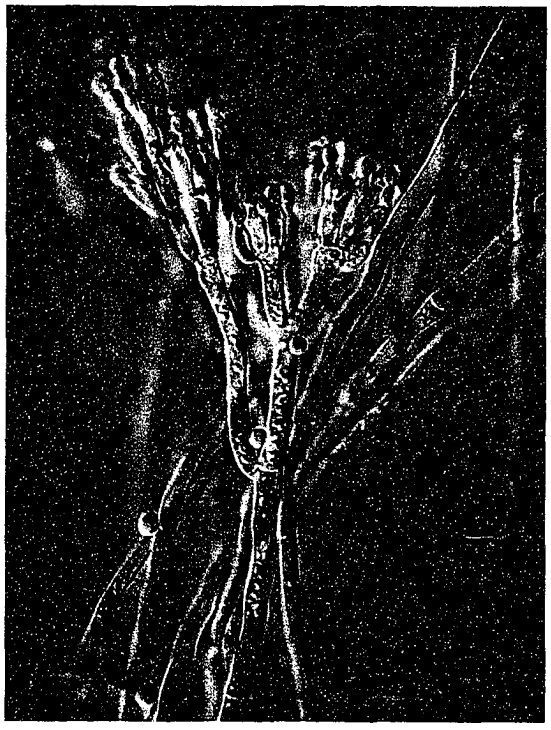


Figure 1.4 *P. chrysogenum* (with permission from Centraalbureau voor Schimmelcultures).

1.4 Taxonomy

The concept of a species is based on the belief that species can be delimited and described through the combining use of the outward directed features of the organism together with its relation to the environment and its inheritance[91]. This model serves to define and identify species efficiently, to discover new species, to discover useful features across species and to discover products or pathways of biotechnological interest. The dominant method of species identification in fungi has been the morphological species recognition method. With few exceptions, fungi are diagnosed by morphological characters or by other phenotypic characters, e.g. growth at different temperatures, growth at different water activities or the presence of pigments[11,90,97].

Taxonomic mycologists have over the last decade however, focused on the elaboration of molecular phylogenies and more recently on the development of the

phylogenetic species concepts. The phylogenetic species concept is a method that defines the boundaries of a species by determining where the congruence stops between phylogenies derived from different genes for the same set of sample organisms[90]. This is often used because once progeny evolutionary species have formed from an ancestor, changes in gene sequences occur and can be recognized before changes in mating behaviour or morphology[97]. However, it contains limited information about current ecological behaviour and morphology.

The species in the subgenus *Penicillium* have proven difficult to classify by taxonomy experts based on morphology characters alone. The taxonomic treatment of the asymmetric terverticillate Penicillia has been very different in the literature up until recent years[78, 83, 72, 42, 75, 95]. There has been some confusion as to the correct names for the terverticillate taxa of *Penicillium* and of late it has been shown that a substantial number of secondary metabolite-producing isolates have been misidentified[41]. For example, the species *P. roqueforti* commonly used as a starter culture for the production of blue cheese has recently been re-classified into three species. The three new species are now known as *P. roqueforti*, *P. carneum* and *P. paneum*. Morphologically, *P. roqueforti* is one of the easiest Penicillia to recognize because of its conidiophore stipes, large globose smooth conidia, good growth on creatine sucrose agar and the blackish-green colony reverse on most laboratory media[74]. Random Amplified Polymorphic DNA (RAPD) analysis together with a comparison of the secondary metabolite profiles produced by the three groups showed that the *P. roqueforti* isolates all produce PR toxin, roquefortine C, mycophenolic acid and fumigaclavine A, while the *P. carneum* isolates produce patulin, roquefortine C, isofumiclavines, penitrem A and mycophenolic acid. *P. paneum* produces

secondary metabolites in five chromophore families including the known mycotoxins patulin, roquefortine C, marcfortines and botryodiploidin[21,43]. Due to the large number of toxic metabolites produced by the different strains of *P. roqueforti* this information becomes rather important. The need arises for better methods to classify and identify at the species level to ensure that the most appropriate strains (ie. poor toxin producers) are used for the production of cheeses and that starter cultures are not contaminated.

Two other common and consistently toxigenic species that have often been misidentified in the literature are *P. crustosum* and *P. simplicissimum*. Reports have used the names *P. cyclopium*, *P. verrucosum*, *P. palitans* and *P. crustosum* for isolates producing the tremorgenic mycotoxin penitrem A. Producers of verruculogen, another tremorgenic mycotoxin have been reported as *P. verruculosum*, *P. paraherquei*, *P. piscarium* and *P. estinogenum*. However, from comparative taxonomic studies, it has been concluded that all of these isolates belong to only two species. *P. crustosum* isolates produce penitrem A and *P. simplicissimum* isolates produce verruculogen[73].

1.5 Metabolomics

A practical definition of metabolomics has been proposed by Smedsgaard *et al.* (2005) as the complete pool of low molecular weight metabolites in a cell at any given time[91]. Recently the area of metabolomics, a newer complementary technique to functional genomics, has been used to provide integral information due to the fact that a large number of genes may be involved in the production of one single metabolite. Not always is there a one-to-one relationship between a gene and a metabolite, and the metabolite profiles are therefore usually the result of the expression of many genes and the function of many enzymes. It is therefore inherently difficult to interpret the patterns of the metabolites and particularly to infer something about the gene functions based on

metabolite profiling alone. The study of metabolomics relies heavily on the advanced analytical techniques developed to determine the metabolite profiles of a sample and to quantify the individual metabolites[91].

Profiles of secondary metabolites and the use of molecular methods have been exploited both as tools for clarifying the phylogenetic relationships among species as well as for classifying species. Several methods have been used such as DNA sequence determination of various regions including the ribosomal genes and fingerprinting methods such as RAPD analysis[21]. The ribosomal DNA region has previously been used for taxonomic analysis. In particular, the internal transcribed spacer (ITS) regions have been shown to be useful for identification at the genus and species level[54,71]. Advancements in the development of analytical methodology have allowed the use of secondary metabolite profiling for fungal identification and have been used to revise the taxonomy within the genera *Penicillium*[44].

1.6 Chemotaxonomy

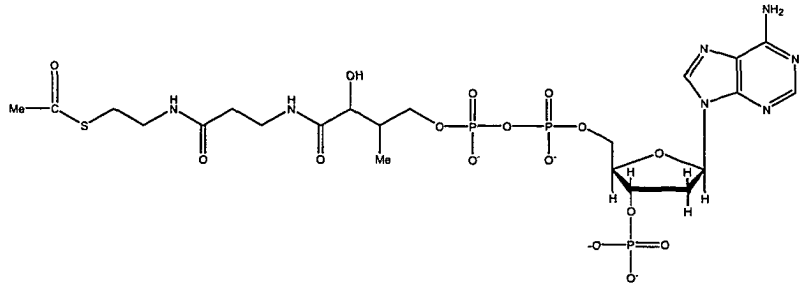
Single spore isolates were used during the chemotaxonomic study of *Penicillium* species in this study. Variability in properties and the spontaneous occurrence of variant lines within single strains has been reported in many genera of filamentous fungi, including *Penicillium*[46,23]. This is significant during the selection and maintenance of strains of industrial importance and in the construction of taxonomic schemes. Morphological and biochemical features may give rise to distinct lines and studies have shown that this might have significant effects on secondary metabolite production. In order to construct reliable taxonomic schemes, it is important that the occurrence and degree of variability is considered not only between strains of individual taxa but also within single strains[23].

In 1989, Frisvad and Filtenborg analyzed over 4000 isolates of terverticillate Penicillia for production of mycotoxins and other secondary metabolites[41]. Nearly all taxa and chemotypes produced one or more secondary metabolite consistently. Entire profiles of secondary metabolites were specific for every taxon studied but several mycotoxins were produced by more than one species. A high number of misidentified *Penicillium* isolates were reclassified and production of several mycotoxins in different taxa was reported for the first time. These techniques have improved the classification schemes of *Penicillium* species but they have also shown that taxonomy based on one character alone might be insufficient to provide a clear classification. Varieties within a species have several characters in common but may differ in at least one morphological, physiological or chemotaxonomic aspect from other varieties in the species. Frisvad and Samson (2004) have now revised and proposed a polyphasic approach to the taxonomy of *Penicillium* subgenus *Penicillium*[43]. In their latest review they describe the 58 current accepted species of *Penicillium*. They propose new groups in natural sections and series that they suspect are both ecologically and phylogenetically consistent. *P. brevicompactum* belongs to the new section *Coronata* and *P. chrysogenum* belongs to the section *Chrysogena*[43].

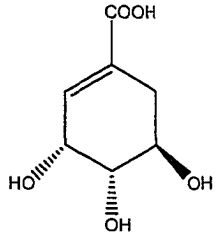
Ciegler *et al.* were the first group to use single secondary metabolites in *Penicillium* taxonomy[30]. Frisvad and Filtenborg later suggested that profiles of secondary metabolites rather than single metabolites should be used in the taxonomy of these species[40]. Chemical profiles of secondary metabolites are of great importance since they can be objectively recorded and standardized, whereas morphological characters are more difficult to assess.

1.7 Primary and secondary metabolism

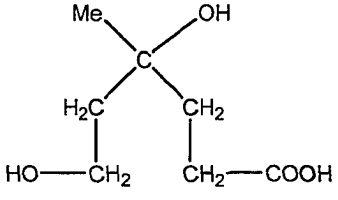
Primary metabolism refers to the pathways involved in energy metabolism and biosynthesis of precursors for the formation of major structural components in living organisms. It is essential for the growth and life of the organism and the major pathways are photosynthesis, glycolysis, the Krebs cycle and the catabolic and anabolic processes of fatty acids, amino acids and nucleic acids. These pathways result in the production of several primary metabolites and intermediates including acetyl-CoA, shikimic acid and mevalonic acid (Figure 1.5). These compounds are used by fungi during secondary metabolism.



acetyl CoA



shikimic acid



mevalonic acid

Figure 1.5 Structures of acetyl-CoA, shikimic acid and mevalonic acid.

Acetyl CoA is formed from the oxidative decarboxylation of pyruvic acid, formed via the glycolytic pathway and by the β-hydroxylation of fatty acids. Acetyl CoA is then used in the acetate pathway of secondary metabolism to produce phenols and fatty acid derivatives. Shikimic acid is produced by the combination of phosphoenolpyruvate (a

glycolic pathway intermediate) and erythrose-4-phosphate (from the pentose phosphate cycle). It is then used in the shikimate pathway resulting in a variety of aromatic compounds including phenols, cinnamic acid derivatives, lignans and alkaloids. Mevalonic acid is formed from three molecules of acetyl-CoA. The mevalonate pathway produces a large variety of terpenoids and steroids. Despite their diversity, secondary metabolites are all produced from a few key intermediates of primary metabolism.

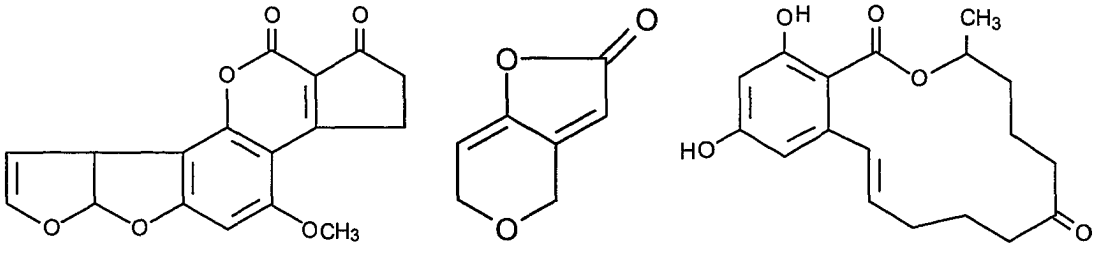
1.8 Secondary metabolite classification

Most species of fungi produce unique secondary metabolites in addition to compounds that they share with other taxa. Fungi will only produce secondary metabolites when primary growth of the cell is limited or stressed by the lack of an essential nutrient such as its carbon source, nitrogen, oxygen or phosphorous. In addition, secondary metabolites may be produced from stress elicited by competitors[60]. The reasons why most filamentous fungi produce such a diverse profile of secondary metabolites is still unclear. It has been suggested that they are probably produced as a result of stimuli, and are directed against or support actions on receptor systems or as outward directed differentiation products[29]. The current view suggests that secondary metabolites are produced by the fungi as a defence mechanism[109].

Secondary metabolites are subdivided according to their biosynthetic origin, e.g. polyketide, terpene, diketopiperazine and cyclopeptide. This becomes important since there are only a limited number of members of a biosynthetic pathway that are expressed under a given set of conditions[65]. In addition, this allows for the detection of a specific gene cluster through the identification of different compounds from a particular biosynthetic pathway. Based on Pitt's taxonomic scheme (2000), isolates in the subgenus

Penicillium are regarded as co-specific if they possess at least three different biosynthetic families of secondary metabolites in common[74].

Polyketides exhibit remarkable diversity both in terms of their structure and function. Polymerization of acetate in the presence of malonate results in the formation of a polyketide chain. The chain may be further processed by cyclization, lactonization, or by the formation of thioesters or amides. Further transformations starting from these simple primer units result in more intricate compounds that include toxins, immunosuppressants and antibiotics. This is a class of secondary metabolites known to possess a wealth of pharmacologically important activities, including antimicrobial, antifungal, antiparasitic and antitumor. They are ubiquitous in distribution and have been reported not only in fungi but from other organisms as diverse as bacteria, plants, insects, dinoflagellates, mollusks and sponges. Aflatoxins, patulin and zearalenone are three examples of important fungal polyketides formed during secondary metabolism (Figure 1.6)[17].

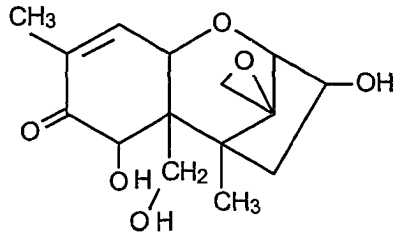


aflatoxin B_1 patulin zearalenone

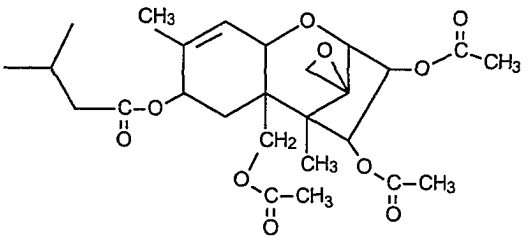
Figure 1.6 Structures of aflatoxin, patulin and zearalenone.

Terpenes are the second major class of secondary metabolites produced by fungi. After mevalonic acid undergoes pyrophosphorylation, decarboxylation, and dehydration, it forms isopentenyl pyrophosphate. Isopentenyl pyrophosphate is the precursor for the

formation of sterols, steroids, carotenoids and terpenes. The most important fungal metabolites in this class are the trichothecenes. This class of secondary metabolites includes some of the most economically damaging compounds due to their common occurrence in crops intended for human or animal use (Figure 1.7)[92].



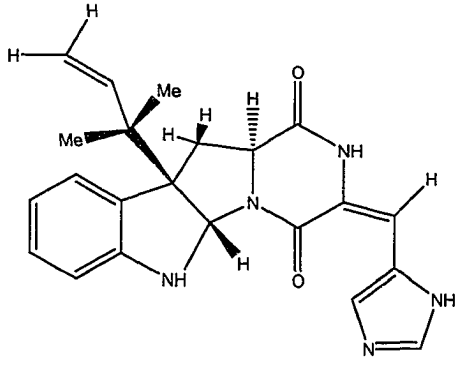
deoxynivalenol



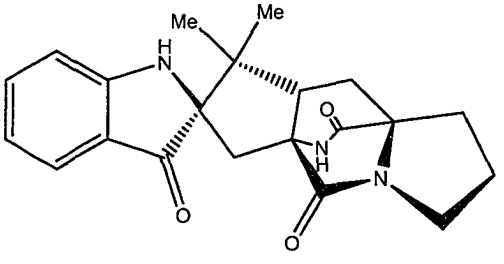
T-2 toxin

Figure 1.7 Structures of the trichothecenes: deoxynivalenol and T-2 toxin.

The shikimic acid pathway converts simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway to the aromatic amino acids phenylalanine, tyrosine, and tryptophan. This pathway leads to the third major class of fungal metabolites, the diketopiperazines. These compounds are then used to form secondary metabolites such as roquefortine C and the brevianamides (Figure 1.8)[110].



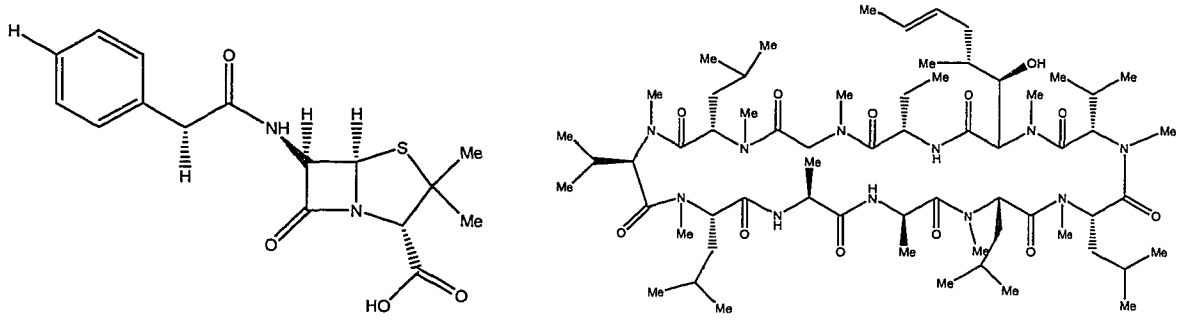
roquefortine C



brevianamide A

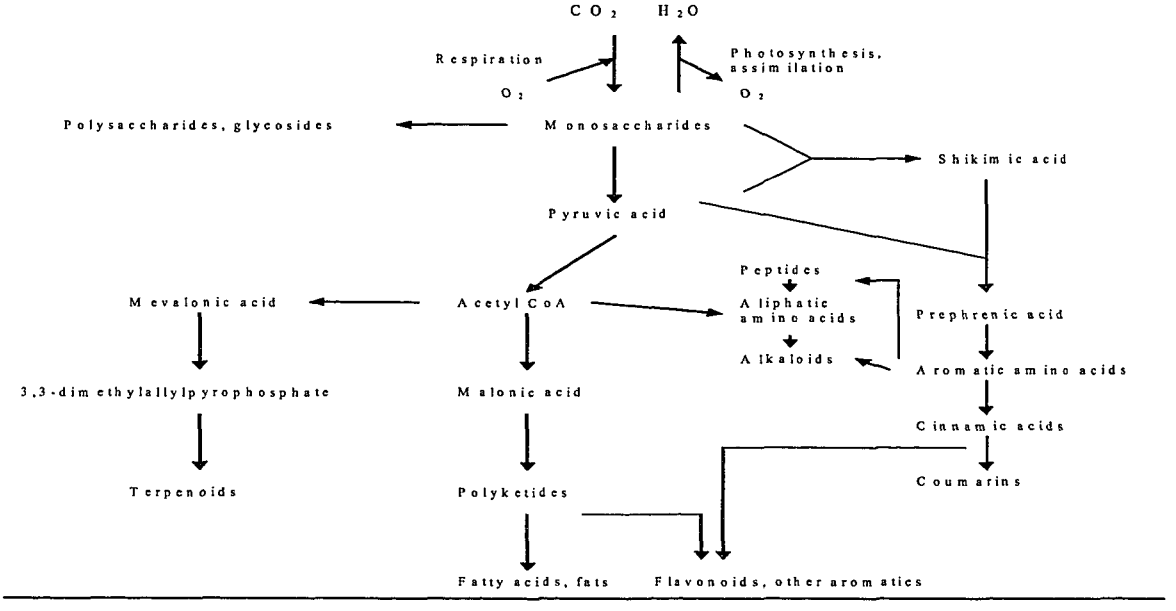
Figure 1.8 Structures of roquefortine C and brevianamide A.

The cyclopeptide class comprises compounds derived from linear peptides or from cyclic peptides. Penicillin and cyclosporin are good examples of each type of metabolite from this group (Figure 1.9).



penicillin G cyclosporin

Figure 1.9 Structure of penicillin G and cyclosporin.



Scheme 1. Main pathways of secondary metabolism[99].

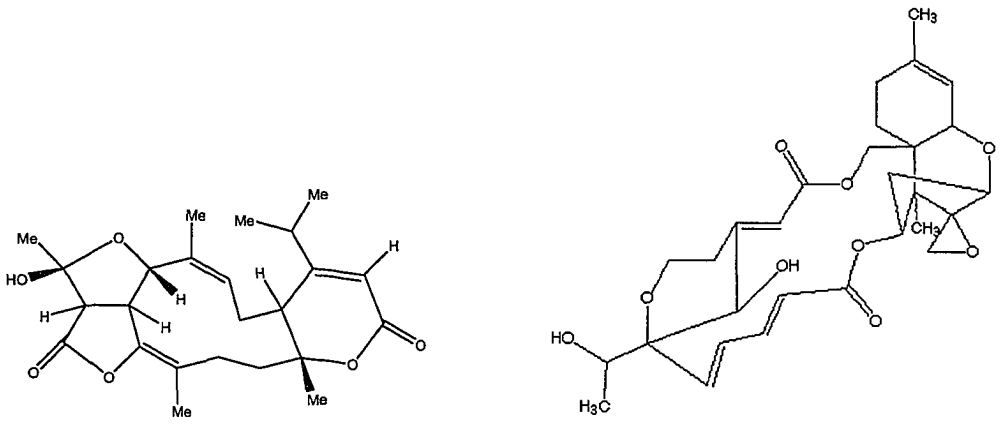
1.9 Analytical Techniques

The most common methods to determine secondary metabolite profiles include thin layer chromatography (TLC), high pressure liquid chromatography with diode array detection (HPLC-DAD), gas chromatography (GC) and liquid chromatography-electrospray ionization mass spectrometry (LC-ESMS)[40,65]. Developments in chromatography and mass spectrometry have greatly increased resolution and sensitivity. For example, LC-NMR and LC-ESMS allow for the detection of secondary metabolites directly from crude extracts. LC-ESMS takes advantage of the very limited fragmentation pattern produced by this ionization technique resulting in mass spectra that almost exclusively show protonated ions from a broad range of extractable metabolites, this in turn can be used as a mass profile for that sample. In addition, there are now methods available including LC-HRMS that allow for rapid metabolite screening from a small amount of sample. As a result, a broad range of metabolites can now be determined in one analysis without losing sensitivity[65].

1.10 Expected fungal metabolites

Not all isolates of a particular taxon will produce all expected secondary metabolites, due to the environmental conditions present or the genetics of the particulate isolate. This might be partly due to the fact that secondary metabolite production is influenced by moisture, temperature, substrate, CO_2-O_2 relationship, mechanical damage, conidial load, competing microbes, microbial interactions and the maturity of the fungus[68,105]. For instance, when *Stachybotrys* isolates mainly from water-damaged buildings were studied by Andersen *et al* (2003), two different chemotypes and one new species were identified[4]. The new species was named *S. chlorohalonata* and differed morphologically from *S. chartarum*. Of the *S. chartarum* species, one chemotype

produced macrocyclic trichothecenes, satratoxins H and G, roridin E and verrucarins J and B, and the second chemotype does not produce satratoxins but produce atranones (Figure 1.10)[47].

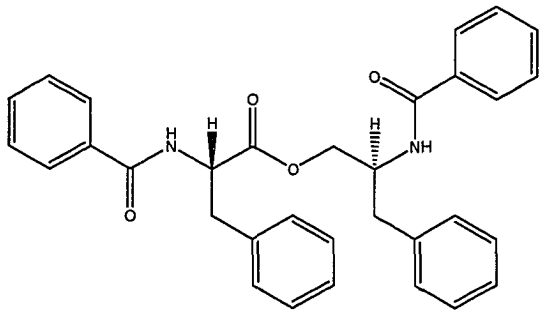


atranone C satratoxin

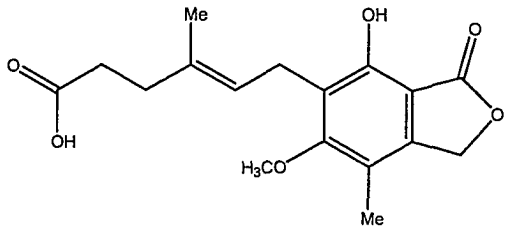
Figure 1.10 Structures of satratoxin and atranone.

P. brevicompactum produce the Raistrick phenols, a characteristic pattern of secondary metabolites which fluoresce blue in short wave UV light. The Raistrick phenols is a collective name given for three closely related compounds 2,4-dihydroxy-6-(2-oxopropyl) benzoic acid, 2,4-dihydroxy-6-(1-hydroxy-2-oxopropyl) benzoic acid and (2,4-dihydroxy-6-(1,2-dioxopropyl) benzoic acid (Figure 1.11). These secondary metabolites were first isolated together with 3,5-dihydroxyphthalic acid from *P. brevicompactum* in 1932[33]. When found together with mycophenolic acid and the brevianamides (A, B, C, D, E or F), the profile is sufficient to unequivocally identify an isolate (Figures 1.11)[82]. However, some strains of this species are also known to produce asperphenamate, pebrolide and botryodiploidin (Figure 1.11)[55]. *P. brevicompactum* is one of the most common *Penicillium* species found in indoor environments because of its ability to grow at very low water activities[45]. When *P. brevicompactum* was inoculated

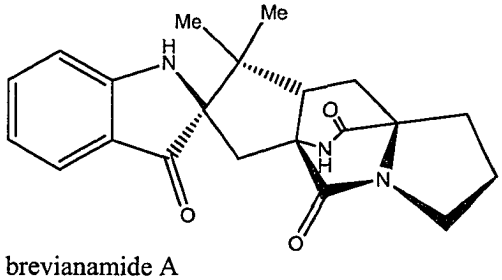
on building materials (gypsum board and chipboard with or without wall paper), it produced mycophenolic acid, asperphenamate and tanzawaid acid analogue[63].



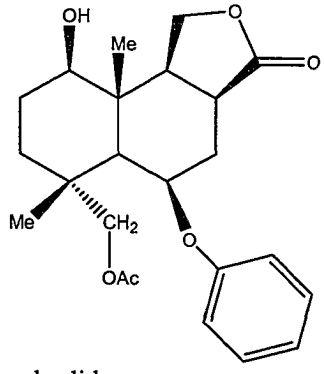
asperphenamate



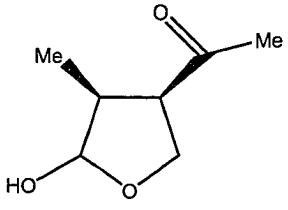
mycophenolic acid



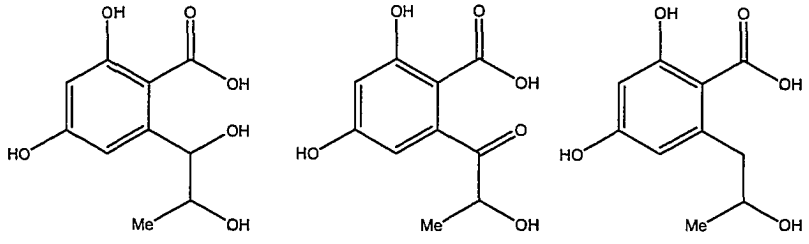
brevianamide A



pebrolide



botryodiploidin

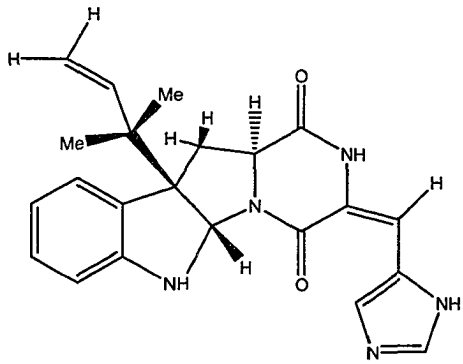


Raistrick phenols

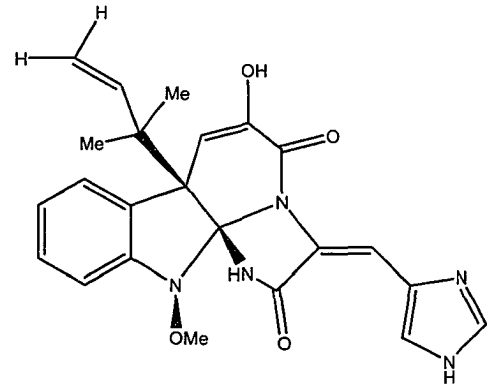
Figure 1.11 Secondary metabolites from *P. brevicompactum.*

Strains of *P. chrysogenum* produce penicillins F and G, roquefortine C and meleagrin (Figure 1.12). The production of these compounds unequivocally identifies this species[82]. The yellow colour exudate in *P. chrysogenum* may be caused by chrysogine,

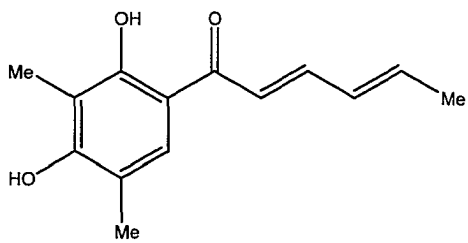
secalonic acid, sorbicillin or xanthocillins (Figure 1.12)[43]. Like *P. brevicompactum,* this species is ubiquitous. It is particularly common on spices, dry cereals, cheeses, meat, tropical seeds, fruits, vegetables and in the rhizosphere soil of vegetables[41]. *P. chrysogenum* is very common in indoor environments requiring fairly high water activities for growth[45]. Small quantities of chrysogine, 2-pyrovoylaminobenzamide and meleagrin have been detected on building materials (Figure 1.12)[63].



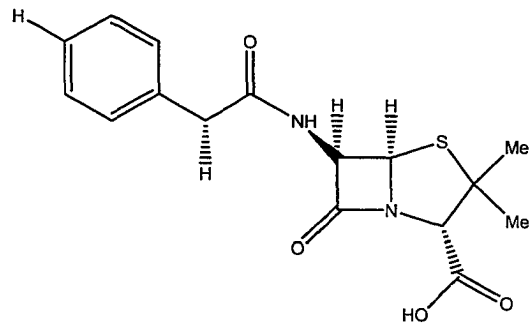
roquefortine C



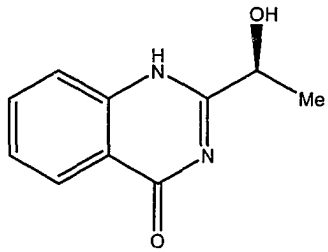
meleagrin

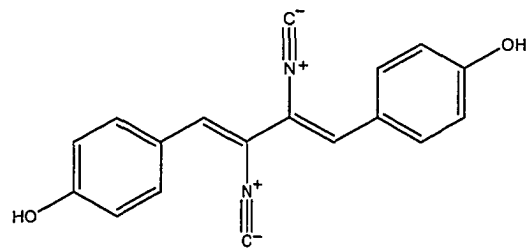


sorbicillin

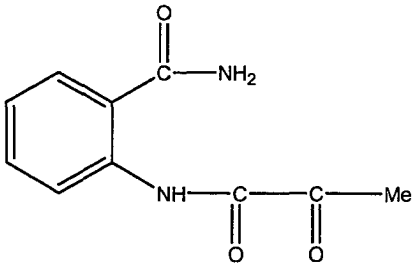


penicillin G



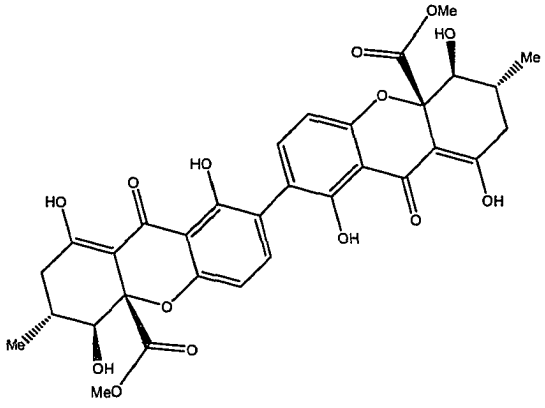


chrysogine



2-pyrovoylaminobenzamide

xanthocillin x



secalonic acid

Figure 1.12 Secondary metabolites reported from *P. chrysogenum.*

1.11 Mycophenolic acid

Mycophenolic acid was first isolated from *P. stoloniferum* Thom (= *P. brevicompactum*) by Gosio in 1896 and it is one of the oldest known antibiotics[35]. Its total synthesis and a biosynthetic pathway have been proposed although its structure was not determined until 1948 by Birkinshaw (Figure 1.13)[20,35,16]. It has been shown that the polyketide origin of the ring system comes from methionine and the side chain is the result of the addition of a farnesyl unit[101]. Mycophenolic acid is a major secondary metabolite produced by *P. brevicompactum*, *P. bialowiezense*, *P. roqueforti* and *P. carneum*[43]. This compound has been extensively studied because of its significant antiviral and antitumor activity[5]. In addition, it has an important use as an immunosuppressant in organ transplant patients[36].

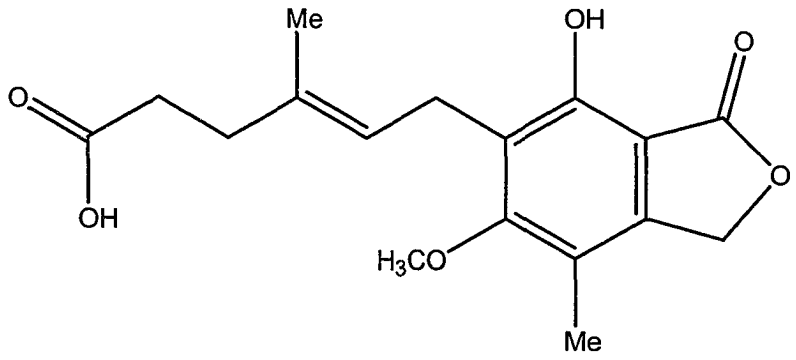


Figure 1.13 Structure of mycophenolic acid.

1.11.1 Toxicity

The toxicity of mycophenolic acid appears to be very low in comparison to other metabolites isolated from strains of *Penicillium* species. The LD_{50} for rats was determined to be 2500 mg/kg when administered orally, and 500 mg/kg when administered intravenously (IV)[112]. Mice showed similar results with LD_{50} values of 700 mg/kg orally, and 450 mg/kg when administered via IV[112]. Chronic toxicity was also tested in rabbits for one year with daily oral doses ranging from 80 to 320 mg/kg with no apparent signs of toxicity[2]. Carter *et al.* (1969) showed that rats lost weight and were dead within nine weeks when given daily oral doses of 30 mg/kg, they also showed that rhesus monkeys who received 150 mg/kg daily developed abdominal colic, lost weight, and had bloody diarrhea after two weeks of feeding[27]. The impact of mycophenolic acid on humans was also tested, 35 patients received daily oral doses of 2.4 to 7.2 g for periods of 1 or 2 years. Common symptoms reported were cramps, nausea and diarrhea[56]. Mycophenolic acid has been shown to have antibiotic activity against bacteria, dermatophytic fungi and it can also interfere with viral multiplication[76]. Additionally, the AMES test using Salmonella was negative, indicating no mutagenic or carcinogenic risk[108].

1.12 Brevianamide A

The brevianamide family comprise a small but structurally interesting family of indole alkaloids formed from tryptophan, proline and one isoprene unit. Brevianamide A was originally isolated as the major fluorescent metabolite from *P. brevicompactum* in 1969 and it has otherwise only been found in *P. viridicatum*[13]. Its structure and absolute stereochemistry were elucidated by X-ray crystallography of a semi-synthetic derivative (5-bromobrevianamide A)[34]. Figure 1.14 shows the chemical structure of brevianamide A with its correct stereochemistry.

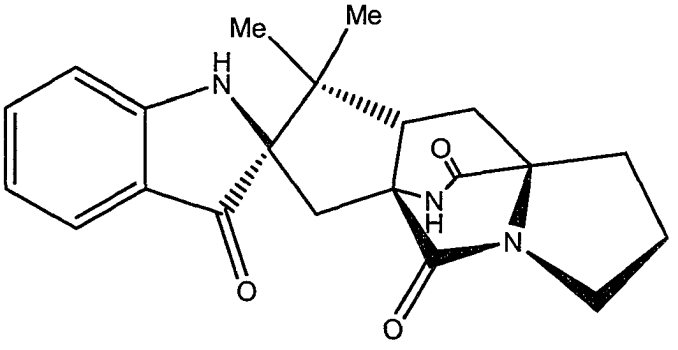


Figure 1.14 Structure of brevianamide A.

1.12.1 Toxicity

The embryotoxic effects of brevianamide A have been investigated in two, three and four day chick embryos. The results were evaluated on the eighth day of development and the observed embryotoxicity ranged from 1 to 100 μg/embryo. The embryotoxic effects demonstrated in chick embryos correlated with the well-know literary data on mammals[106]. Brevianamide A was not toxic when fed or injected into female mice at doses up to 40 mg/animal and to male mice at doses up to 100 mg/animal. In addition, it showed no antibiotic properties against several common bacterial and fungal microorganisms[113].

1.13 Asperphenamate

Asperphenamate is a major secondary metabolite produced by *P. brevicompactum*, *P. olsonii* and *P. bialowiezense*. Figure 1.15 shows the chemical structure of asperphenamate with its correct stereochemistry.

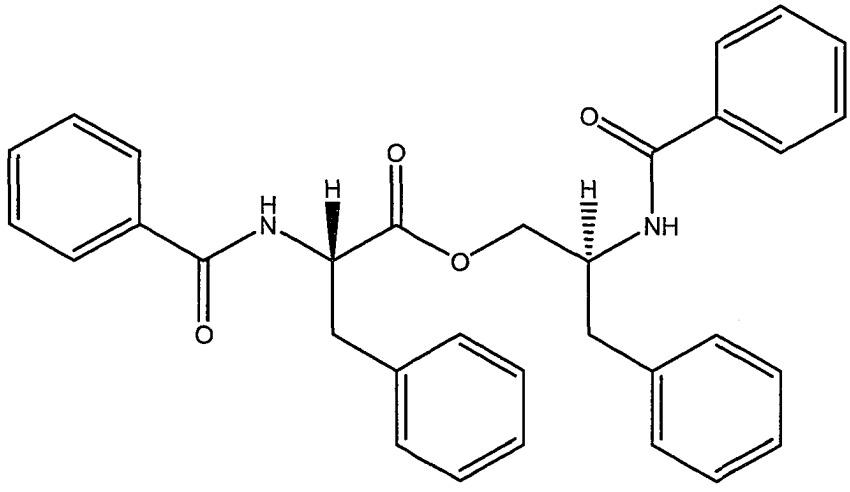


Figure 1.15 Structure of asperphenamate.

1.13.1 Toxicity

To my knowledge there are no toxicity studies that have been published to date on asperphenamate.

1.14 Roquefortine C

Roquefortine C is an indole alkaloid mycotoxin derived from tryptophan that was independently discovered from the mycelium of a strain of *P. roqueforti* and from blue cheese[88,67]. Roquefortine C is the major secondary metabolite produced by *P. roqueforti* and *P. crustosum*. It has also been found in *P. paneum, P. carneum, P. chrysogenum, P. flavigenum, P. atramentosum, P. persicinum, P. expansum, P. marinum, P. sclerotigenum, P. griseofulvum, P. concentricum, P. glandicola, P. coprobium, P. vulpinum*. Figure 1.16 shows the chemical structure of roquefortine C.

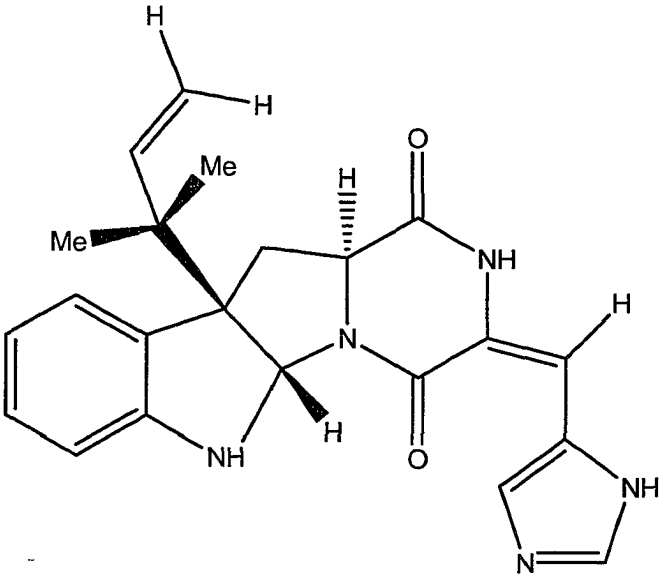


Figure 1.16 Structure of roquefortine C.

1.14.1 Toxicity

A number of toxicity studies have been conducted on roquefortine. Schoch *et al.* (1983 and 1984) conducted mutagenic studies using the Ames test on strains of *P. roqueforti* used for cheese production[84,85]. The results showed no signs of mutagenic activity by either the fungus or roquefortine. Rats that received intraperitoneal injections of roquefortine showed a range of LD_{50}'s. Ueno and Ueno (1978), reported values of 15-20 mg/kg[103]. Similar experiments performed on mice by Arnold *et al.* (1978) reported LD_{50} values for male and female CR57 mice as 169 and 184 mg/kg respectively and 189 and 184 mg/kg for Swiss-Webster mice[7]. Scott *et al.* (1976) referred to a French study on the neurotoxic activity of roquefortine in mice, similar symptoms were also observed in day-old cockerels dosed with roquefortine[88,107].

The in vitro effect of roquefortine C on mitogen induced lymphocyte proliferation was determined by using purified lymphocytes from 6 piglets. Dose response curves were generated and concentrations producing 50% inhibition (IC_{50}) of cell proliferation were estimated. The inhibition potencies were presented relative to ochratoxin A. The IC_{50} for

roquefortine C was 85μmol/L and its relative inhibition was 98.5 times lower than that of ochratoxin A[10].

The carcinogenic effect of *P. roqueforti* was studied using 800 rats that were fed both a strain of mold used for commercial cheese production and also the finished cheese products. The results of the experiment indicated that there were no significant adverse effects between control and treated animals for either the final cheese products or for the starter strains used for their production[39].

An important study on the impact of roquefortine on 18 female sheep was conducted by Tuller *et al.* (1998). They dosed the animals by means of a gel capsule via a stomach tube with the equivalent to 0, 5, and 25 mg/kg of silage over a 16-18 day period[100]. The results showed that no clinical signs of intoxication were recognized over the period of the experiment. Neither chemical nor pathological factors were altered by the presence of roquefortine.

1.15 Meleagrin

Meleagrin was first reported in 1979 by Nozawa *et al.* as a yellow pigment with the molecular formula $C_{23}H_{23}N_5O_4$[66]. According to Frisvad and Samson (2004) meleagrin is produced by *P. chrysogenum, P. flavigenum, P. confertum, P. atramentosum, P. concentricum, P. glandicola, P. coprobium, P. vulpinum, P. albocoremium, P. allii, P. radicicola* and *P. tulipae*[43]. No chemical structure was proposed until 1984, but it was suggested that meleagrin belongs to the tremorgenic mycotoxin group that includes compounds like fumitremorgin A, fumitremorgenic B, verruculogen and roquefortine C and D[49]. In 1995 Reshetilova *et al.* explored the biogenetic relation between meleagrin and roquefortine C. As part of their experiments they were able to incorporate ^{14}C from exogenous roquefortine C into molecules of

meleagrin, glandicolines A and B and oxaline in cell suspensions and cultures of fungi. Roquefortine C was shown to be a precursor of these alkaloids. In addition, the biosynthetic pathway for the transformation of roquefortine C into oxaline was presented (Scheme 2)[79]. Figure 1.17 shows the chemical structure of meleagrin with the proposed stereochemistry by Nozawa *et al.*, (1984)[49].

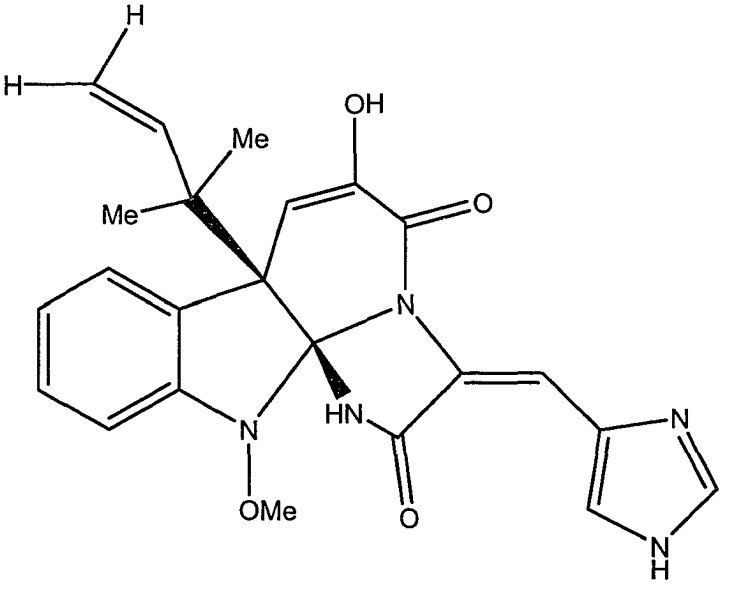
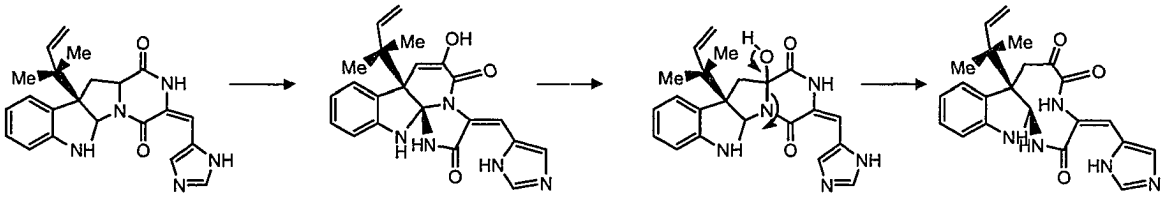
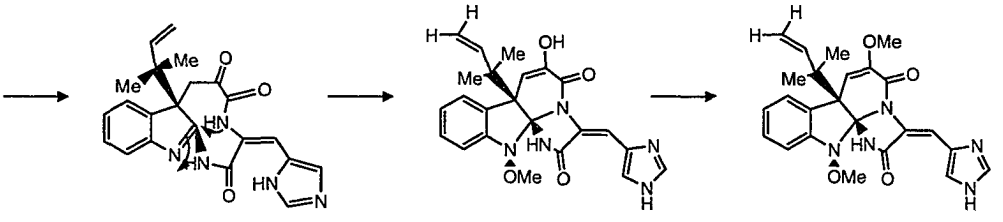


Figure 1.17 Proposed structure of meleagrin.



roquefortine C



meleagrin oxaline

Scheme 2. Proposed biosynthetic pathway for oxaline[93,110].

1.15.1 Toxicity

Meleagrin has been regarded as relatively non-toxic[63]. Special attention should be paid to this secondary metabolite since it has been isolated in large quantities from *P. chrysogenum* strains growing in building environments and because roquefortine C has been shown to be a precursor for meleagrin[63,21].

1.16 Penicillin G

There are several types of penicillins (F, G, K, N and V) and structural studies established that all the penicillins contain a common nucleus comprising a β-lactam ring (Figure 1.18). Penicillin V and penicillin G are active against most aerobic Gram-positive organisms. Penicillin G is one of the most widely used antibiotic agents today, used for infections due to streptococci, susceptible staphylococci and meningococci. Their bactericidal effect is caused by the inhibition of bacterial cell wall synthesis. Penicillin G must penetrate the cell wall to attach to specific proteins on the inner surface of the bacterial cell membrane; from there they interfere with the production of peptidoglycans followed by lysis of the cell in a hypo or iso osmotic environment. The β-lactam nucleus present in penicillin G is sensitive to both acidic and basic conditions. At pH 10 it loses all biological activity if left for 15 minutes at room temperature[69]. Penicillin G is produced industrially by the fermentation of *P. chrysogenum* and it has been found in *P. chrysogenum* strains growing in damp buildings[63,51].

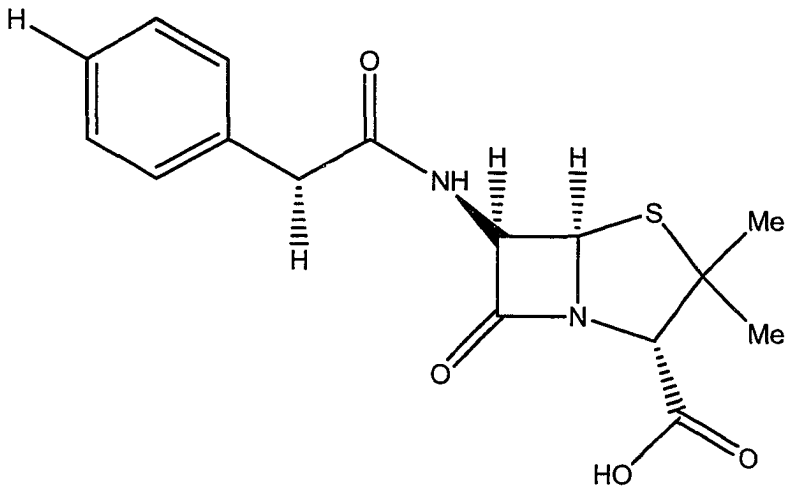


Figure 1.18 Structure of penicillin G[104].

1.16.1 Toxicity

Previous studies by Perelmutter *et al.* (1975) showed that penicillin G causes allergy symptoms[70]. Penicillin is a chemical hapten with a low molecular weight that needs to bind to a tissue macromolecule, usually a protein, to become immunogenic. The breakdown products of penicillin include the penicilloyl group. This group is known as the major determinant because it is the major penicillin metabolic product (approximately 85% to 90% of the penicillin breakdown products). The minor determinant mixture is composed of the parent penicillin molecules, penicilloate, penicilloylamine, penilloate, and other simple chemical products of penicillin. Immediate reactions following penicillin administration are mediated through IgE antibodies against either the major or minor determinants, or both[18].

They found that when rats were given penicillin G in their drinking water at concentrations of 0.1 to 1 U/mL over a period of 1 to 3 months they produced serum IgE and IgG antibodies. Penicillins are one of the most common cause of drug allergy and hypersensitivity reactions and allergies are the most common adverse effect associated with this drug. It appears that all of the penicillins are equal in their potential for causing

allergic reactions, and all penicillins are cross-sensitizing and cross-reacting. The most important antigenic intermediate of penicillin appears to be the penicilloyl moiety that is formed when the β-lactam ring is opened. Allergic reactions may occur in many forms including anaphylactic shock, serum sickness type reactions, skin rashes, fever, interstitial nephritis, eosinophilia, hemolytic anemia, and vasculitis[6].

1.17 Cluster Analysis

Cluster analysis is used in many fields of science such as archeology, psychology, marketing, and biology. In biology, it has been used to analyze microbial populations, microarrays, genetic studies and more. Although, the procedure for doing cluster analysis is not well established, due to the many algorithms possible, it is a very useful method.

Cluster analysis was first used by Tryon in 1939 and it encompasses a number of different algorithms and methods for grouping objects of similar kind into respective categories. It is an exploratory data analysis tool which aims at sorting different objects into groups in a way that the degree of association between two objects is maximal if they belong to the same group and minimal otherwise. It is important to mention that clustering algorithms do not focus on statistical significance testing[94]. In fact, cluster analysis is not a typical statistical test as it is a collection of different algorithms that put objects into clusters according to well defined similarity rules. It is usually performed when there are no previous hypotheses regarding the grouping of the data and is it used as the means to find the most significant solution possible. In other words, it classifies unknown groups. There are no readily available guidelines to select a specific algorithm for the analysis of a set of data and one must rely on the interpretation of the results observed.

The results of cluster analysis are commonly visualized using a dendogram or binary tree. The objects are represented as nodes in the dendogram and the branches illustrate when the cluster method joins subgroups containing that object. The length of the branch indicates the distance between the subgroups when they are joined. Adequately interpreting a dendogram leads to choosing the adequate algorithm. To be significant, the clustering method must give a dendogram that clearly differentiates groups of objects by significant distances[94].

The first step in doing cluster analysis is to calculate the distance between the objects to be grouped. This is usually done by the computing package used, but several options are available[94]. The most common algorithm to determine distances is the Euclidean distance method followed by the square Euclidean distance, the Chebychev distance and the power distance methods. Once the distance between the objects has been found, cluster analysis involves the division of the objects into groups based on the distances. This is done by means of the K-means cluster analysis, hierarchical clustering , single linkage, complete linkage, unweighted pair-group average, weighted pair-group average, unweighted pair-group centroid, weighted pair-group centroid, Ward's Method or by minimum variance clustering.

The Ward's method was used in this study and it initially treats all strains as separate clusters, and then combines clusters by maximising the within-cluster homogeneity. To measure homogeneity a within-cluster sum of squares is used. At each step, all possible solutions are considered and clusters are formed using the solution that gives the smallest within-cluster sums of squares. The within-cluster sum of squares that is minimised is also known as the Error Sum of Squares. For each case the squared

Euclidean distance to the cluster means is calculated. As each case initially starts off as a cluster, the error sum of squares is zero. The next step would then be to form the first cluster of size two and the others all of size one. The error sum of squares is calculated for all possible solutions and the cluster solution that produces the least error sum of squares is chosen and the process is repeated. This continues until there is just one cluster containing all local authorities.

Once the clusters have been formed, a check must be carried out to ensure that each local authority is assigned to its correct cluster. Due to the agglomerative nature of the technique the cluster centroid will have changed at each step, as new strains are added. This might mean that by the end of the process some strains are more similar to strains in other clusters than they are to strains in their own cluster. To ensure that all authorities are in the right cluster, a k-means analysis was carried out. This technique reassigns strains to the cluster with the smallest distance between the strains and the cluster centroid. The interpretation of the dendogram is somewhat subjective and can be complemented with other tests such as discriminant function analysis.

2. PROJECT AIM

At the beginning of this project it was important to know whether *P. chrysogenum* isolates associated with indoor environments or present in building materials were potential penicillin producers. The presence of penicillin or penicillin-like compounds is not desirable in indoor environments as they can trigger allergic reactions.

As the project advanced the goal became to know whether the chemotypes of the isolates under study were consistent in their production of known secondary metabolites irrespective of geographic or environmental origin. It was also important to determine the relationship between these groups of isolates since studies on *S. chartarum* have shown

that isolates from water-damaged buildings in Northern Europe and the United States segregated into two distinct groups based on morphology, physiology and chemistry[4]. Variability in properties and the occurrence of variant lines within single strains has been reported in many genera of filamentous fungi, including *Penicillium*[22]. This variability may be of considerable importance in the built environment.

In addition, it was necessary to isolate the major secondary metabolites produced by these strains and to provide clear evidence of their purity in order to submit them as standards for toxicity studies.

3. MATERIALS AND METHODS

3.1 Fungal strains

Eleven fungal strains of *P. brevicompactum* were used for this project. Seven strains were isolated from indoor air or contact plates and were provided by Paracel Laboratories Ltd. (Ottawa, Ontario). These strains were identified by J. D. Miller, verified by K.A. Seifert and deposited at the Agriculture and Agri-Food Canada Collection of Fungal Cultures (DAOM). The other four strains, isolated from lumber, were obtained directly from DAOM and were stored at 5°C on 2% malt extract agar slants. These strains were identified by B.T. Grylls, K.A. Seifert and J.C. Frisvad. The origin of all the strains is listed below (Table 3.1).

Table 3.1 *Penicillium brevicompactum* strains

Strain Id #	Year	Substratum and Locality
DAOM 215331	1990	Spruce lumber, St. Pamphile, Que
DAOM 215333	1990	Douglas fir, Vancouver, BC
DAOM 215334	1990	HemLock, West coast, Canada
DAOM 215335	1990	Spruce lumber, St. Pamphile, Que
DAOM 234044	2003	Indoor air, RCS sampler, using rose bengal agar as the isolation medium. Ottawa, ON
DAOM 234045	2003	Indoor air, RCS sampler, using rose bengal agar as the isolation medium. Ottawa, ON
DAOM 234046	2004	Contact plates, using malt agar extract as the isolation medium. Calgary, AB
DAOM 234047	2004	Indoor air, RCS sampler, using rose bengal agar as the isolation medium. Saskatoon, SK
DAOM 234048	2004	Indoor air, RCS sampler, using rose bengal agar as the isolation medium. BC
DAOM 234049	2004	Contact plates, using malt agar extract as the isolation medium. Calgary, AB
DAOM 234050	2004	Contact plates, using malt agar extract as the isolation medium. Ottawa, ON

Thirty fungal strains of *P. chrysogenum* were used for this project. Thirteen of these were obtained from DAOM and stored at 5°C on 2% malt extract agar slants. These particular strains had previously been included in a study of the genotypic variation of *P. chrysogenum* strains from building environments[86]. Strains DAOM 167036, DAOM 178623, DAOM 190864, DAOM 171025, DAOM 175758, DAOM 155628, DAOM 175157, and DAOM 155627 were identified by Dr. J. Bissett. DAOM 215701 was

identified by J.C. Frisvad. DAOM 212336 and DAOM 215337 were identified by B.T. Grylls, K.A. Seifert and J.C. Frisvad. The other seventeen strains were isolated from outdoor air, indoor air or from building materials and were provided by Paracel Laboratories Ltd. (Ottawa, Ontario). These strains were identified by J. D. Miller, verified by K.A. Seifert and deposited at DAOM. The origin of the strains is listed below (Table 3.2).

Table 3.2 *Penicillium chrysogenum* strains

Strain Id #	Year	Substratum and Locality
DAOM 155627	1976	Paper, Canadian Conservation Institute, Ottawa, ON
DAOM 155628	1976	Paper, Canadian Conservation Institute, Ottawa, ON
DAOM 167036	1977	Soil, spruce forest Lacolle, Que
DAOM 171025	1979	Surface washings of salami, Food Research Institute Agriculture Canada, Central Experimental Farm, Ottawa, ON
DAOM 175157	1980	Foam particles, from urea-formaldehyde foam in a house in Niagara Falls, ON
DAOM 175758	1980	Air plates, Employment Canada Office, Montreal, Que
DAOM 178623	1980	Paper product
DAOM 190864	1984	Stored grains (wheat & barley)
DAOM 193710		Cheese
DAOM 212336	1990	Wooden studs, Edmonton, AB
DAOM 215337	1990	HemLock lumber, Vancouver, BC
DAOM 215701	1984	*Sesanum indicum*, seed in Korea
DAOM 59494C		Dried culture of DAOM 59494A from Honduras

DAOM 234051	2003	Indoor air, RCS sampler, using rose bengal agar as the isolation medium. Saskatoon, SK
DAOM 234052	2003	Pipewrap, using rose bengal agar as the isolation medium. Ottawa, ON
DAOM 234053	2003	Indoor air, RCS sampler, using rose bengal agar as the isolation medium. Ottawa, ON
DAOM 234054	2003	Isolated from dust. Calgary, AB
DAOM 234055	2003	Outdoor air, RCS sampler, using rose bengal agar as the isolation medium. Ottawa, ON
DAOM 234056	2003	Wallboard. Calgary, AB
DAOM 234057	2003	Isolated from dust. Desoronto, ON
DAOM 234058	2003	Indoor air, RCS sampler, using rose bengal agar as the isolation medium. Saskatoon, SK
DAOM 234059	2003	Isolated from insulation material. Surrey, BC
DAOM 234060	2004	Outdoor air, RCS sampler, using rose bengal agar as the isolation medium. Ottawa, ON
DAOM 234061	2004	Indoor air, RCS sampler, using rose bengal agar as the isolation medium. Carp, ON
DAOM 234062	2004	Wallboard. Ottawa, ON
DAOM 234063	2003	Isolated from wood. Ottawa, ON
DAOM 234064	2003	Isolated from drywall. Ottawa, ON
DAOM 234065	2003	Isolated from culture swab. Ottawa, ON
DAOM 234066	2003	Unknown location, RCS sampler, using rose bengal agar as the isolation medium. Sudbury, ON
DAOM 234067	2003	Indoor air, RCS sampler, using rose bengal agar as the isolation medium. Ottawa, ON

3.2 Slants, transfer and single spore isolate preparation

The fungal strains obtained from DAOM were transferred aseptically onto sterilized 2% malt extract agar slants. The slants were incubated at 25 °C for 7 days. Ten slants per strain were prepared and stored at 5°C.

All the strains from Paracel Laboratories were transferred onto sterilized 2% malt extract agar plates. After 7 days of growth at 25°C in the dark, single spore isolates were prepared as follows. A pipetteful of sterile distilled water and Tween 20 (1 drop of Tween 20 per liter of water) was added into each culture plate. The plates were stirred gently, the liquid was drawn with a pipette and diluted into 20mL of sterile water. One drop of this dilution was placed and spread evenly onto 2% malt extract agar plates using a sterile bent glass rod. After a day in the incubator, spores could be seen under the microscope. Single spores were transferred into 2% malt extract agar plates and allowed to grow for 10 days at 25°C in the dark. The single spore cultures were then transferred aseptically onto sterilized 2% malt extract agar slants prepared as described above. The slants were incubated at room temperature for 7 days. Ten slants per strain were prepared and stored at 5°C.

3.3 Culture growth

Slants of *P. brevicompactum* and *P. chrysogenum* were macerated in 50 mL of sterile distilled water, from which 2.5 mL or a 5% (v/v) aliquot was used to inoculate each one of three 1L Roux bottles. The Roux bottles contained 200 mL of sterile Czapek-Dox supplemented with yeast extract (30g/L sucrose, 1g/L K_2HPO_4, 5g/L yeast extract, 0.5g/L $MgSO_4$, 3g/L $NaNO_3$, 0.01g/L $FeSO_4$, 0.5g/L KCl) or Czapek-Dox/1% corn-steep medium (same as above except 1g/L corn steep liquor powder was added) and were incubated in the dark at 25°C (Figure 3.1).

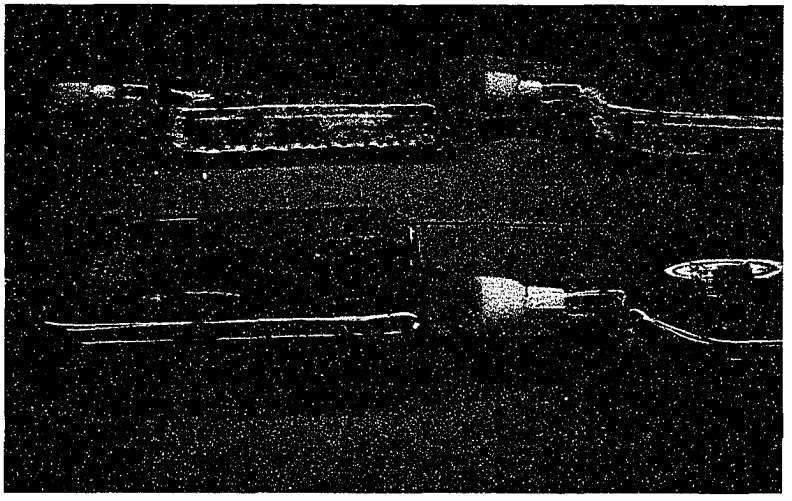


Figure 3.1 *P. chysogenum* cultures growing in Roux flasks.

All cultures were harvested after 14 days of growth and filtered through a Buchner funnel using Whatman #1 filter paper and vacuum pressure. The volume of filtrate from each flask was measured and the pH was recorded before the filtrates were stored at 5°C for later processing. The mycelium was rinsed with two times equal volumes of distilled water to remove any unwanted sugars and was dried using vacuum pressure and wrapped in aluminum foil. It was kept frozen for later processing. These steps were repeated for all the remaining strains.

3.4 Modification to growth conditions for penicillin production enhancement

Czapek-Dox/1% corn-steep media was modified by the replacement of sucrose with glucose in an attempt to enhance the production of penicillins in *P. chrysogenum* strains. Spore suspensions were prepared by macerating slants of *P. chrysogenum* in 50 mL of sterile distilled water, from which 2.5 mL was used to inoculate 50 mL of sterile seed medium (30g/L glucose, 10g/L lactose, 10g/L corn-steep liquor, 2g/L $(NH_4)_2SO_4$, 5g/L $CaCO_3$, 0.5g/L KH_2PO_4) in 250 mL Erlenmeyer flasks. The flasks were then incubated in the dark at 25°C on a New Brunswick Scientific triple tier rotary shaker (220

rpm, 3.81cm throw) for 48 hours to prepare the inoculum for the fermentation. After 48 hours the cultures were used to inoculate 200mL of sterile fermentation media (62g/L lactose, 13g/L Pharmamedia, 5.5g/L $(NH_4)_2SO_4$, 5.2 g/L $CaCO_3$, 2.6g/L $CaSO_4$, 2g/L Na_2SO_4, 5.2g/L glycerol, 0.3g/L KH_2PO_4, 5.8g/L phenoxyacetic acid) in 3 X 1L Roux bottles. All cultures were harvested after 14 days of growth at 25°C in the dark and were filtered through a Buchner funnel using Whatman #1 filter paper and vacuum pressure. The mycelium was rinsed with two times equal volumes of distilled water to remove any unwanted sugars. The mycelium was dried using vacuum pressure and wrapped in aluminum foil and stored in the freezer for later processing.

3.5 Large scale fermentation for metabolite isolation

It was observed that metabolite production was much higher in the Czapek-Dox/1% corn-steep media. Czapek-Dox/1% corn-steep media was therefore used as the media of choice for the rest of the project. *P. brevicompactum* strains DAOM 215331 and DAOM 215335 were chosen for large scale fermentation because of their metabolite production. Cultures were grown in Glaxo bottles (20 x 1 L) for 14 days at 25°C before harvest.

3.6 Culture extraction

The filtrates, pH 6, were extracted twice with equal volumes of ethyl acetate (Fisher Scientific, Canada) in separatory funnels. The combined ethyl acetate extracts were dried using Na_2SO_4 (BDH, Canada) and were concentrated *in vacuo* (Caframo VV 2000 rotary evaporator) to afford dark yellow crude extracts. Each sample was re-suspended in ethyl acetate, transferred into pre-weighed vials and dried under a gentle stream of nitrogen gas. The samples were kept at 5°C for later processing. In addition, the remaining filtrates were extracted with an equal volume of chloroform to ensure that all

extractable metabolites were obtained. The chloroform extracts were processed in the same manner as the ethyl acetate extracts.

The mycelium was wrapped in aluminium foil, freeze-dried (Cryocool Neslab CC-100) for 48 hours, weighed and ground to a powder. The mycelium was stirred in ethyl acetate (30 g/L) overnight. Filtration, drying and evaporation of the solvent *in vacuo* gave crude extracts. Each sample was re-suspended in ethyl acetate, transferred into pre-weighed vials and dried under nitrogen gas. The samples were kept at 5°C for later processing.

3.7 Extraction of penicillin

The pH of the culture filtrates was recorded and then adjusted to pH 2.5 prior to extraction by using 1N HCl. The filtrates were then extracted with 200 mL of butyl acetate (J.T. Baker, USA) in separatory funnels. Re-extraction into an aqueous medium was carried out with 5 mL of phosphate buffer (pH 6.0) and 15 mL of 5% (w/v) $NaHCO_3$ in order to re-adjust to pH 6.0[6]. To remove any water from the samples the butyl acetate extracts were dried by using Na_2SO4 powder. The extracts were then concentrated *in vacuo,* at 30°C and medium speed. Each sample was then re-suspended in butyl acetate, dried under nitrogen gas in pre-weighed vials and dissolved again in 5 mL of solvent prior to analysis by TLC and HPLC-DAD.

The frozen mycelium was freeze-dried for 48 hours. The weights were recorded immediately after the mycelium was removed from the freeze-drier. The mycelium was extracted with 30 mL of butyl acetate in 1L Erlenmeyer flasks and stirred for 8 hours. The extracts were filtered through a Buchner funnel using Whatman # 1 filter paper and vacuum pressure. They were dried though a funnel containing filter paper and Na_2SO4 powder. The extracts were then dried to completion using a rotary evaporator set to 30°C

and medium speed. Each sample was re-suspended in butyl acetate, dried under nitrogen gas in pre-weighed vials and dissolved again in 5 mL of solvent prior to analysis by HPLC-DAD.

3.8 Screening by thin layer chromatography (TLC)

TLC analysis of the filtrate and cell extracts was performed to screen and evaluate the secondary metabolite production of the cultures in the two types of media used. The extracts were spotted using TLC plates (250μm silica, 60Å, Whatman, New Jersey) and a variety of solvent systems. A comparison of the TLC solvent systems was tested using both standards and sample extracts. The solvent mixtures tested included the following: chloroform/acetone (9:1), chloroform/methanol (9:1), hexane/ethyl acetate (3:1) and toluene/ethyl acetate/90% formic acid (5:4:1). It was determined that the chloroform/methanol (9:1) system provided the best separation. Variations of this ratio in order to alter the polarity of the solvent system were tested and used throughout the rest of the study.

Standards of roquefortine C and mycophenolic acid (minimum 90%, Sigma, Canada) were used to specifically screen for the production of these metabolites and test the validity of the method. The TLC plates were developed until the solvent had travelled to 1 cm from the top of the plate at which point the plate was removed from the tank and allowed to air dry. The plates were sprayed with a development reagent (sulphuric acid/ethanol, 1:1 v/v) and once again dried. As the plates dried, the metabolite spots began to appear, these were compared to standards for identification. The number of unknown spots and their intensity on the plates was initially used to identify the most productive strains.

3.9 Analysis by High Pressure Liquid Chromatography (HPLC-DAD)

3.9.1 Analysis of secondary metabolites

The HPLC-DAD method for the determination and quantification of secondary metabolites from both filtrate and mycelium was adapted from Nielsen *et al.*, (2003)[64]. This method targets the separation and detection of fungal metabolites from liquid media. A gradient mobile phase consisting of an acetonitrile/water/0.05% TFA mixture at a flow rate of 1mL/min was used (Table 3.3). The HPLC-DAD analysis was performed using a 1100 series Hewlett Packard HPLC-diode array detector (DAD) with built in degasser, and four pumps. A Synergi Max-RP, 250 x 4.6 mm, 4µm, 80Å HPLC column (Phenomenex, California) was used for compound separation and the detector was set at 215nm, 254nm and 354nm for optimal absorption of all compounds. For prep-HPLC, the same conditions were used at a flow rate of 15mL/min. All extracts were filtered through 13mm PTFE (0.45µm) syringe filters (Waters) prior to injection.

Table 3.3 HPLC gradient program for secondary metabolite analysis

Time (min)	Acetonitrile/0.05% TFA	Water	Flow rate (mL/min)
0	15	85	1
40	0	100	1
45	0	100	1
53	85	15	1
58	85	15	1

3.9.2 Analysis of penicillin G

The HPLC-DAD method for the determination and quantification of penicillin from both filtrate and mycelium was adapted from Anné *et al.*, (1982). This gradient method targets the separation and detection of penicillins and β-lactam compounds from liquid media[6]. A gradient mobile phase used was 30mM K_2HPO_4/KH_2PO_4 (50:50) and acetonitrile at pH 5.5 (Table 3.4). A Synergi Max-RP, 250 x 4.6 mm, 4µm, 80Å HPLC

column (Phenomenex, California) was used for compound separation and the detector was set at 220nm, for optimal absorption of penicillin and at 254nm and 354nm for optimal absorption of all other compounds that might be present. All extracts were filtered through 13mm PTFE (0.45μm) syringe filters prior to injection.

Table 3.4 HPLC gradient program for penicillin analysis

Time (min)	K_2HPO_4/KH_2PO_4, (pH 5.5)	Acetonitrile	Flow rate (mL/min)
0	100	0	1
16	100	0	1
31	80	20	1
36	80	20	1
41	100	0	1
45	100	0	1

3.10 Recovery

A penicillin G standard was obtained from Sigma (St. Louis, USA). A 5 mg/mL stock solution of penicillin G in water was mixed and serially diluted to create a range of concentrations for use in the construction of a standard calibration curve. The standard curve was based on the peak intensity of penicillin G at 220 nm. The area units of the resulting peaks were plotted against μg of penicillin G injected onto the HPLC column. A range of concentrations from 1 mg/mL up 4mg/mL, representing 10 μg to 50 μg of penicillin G on the column was used. The same procedure was repeated to prepare roquefortine C and meleagrin standards.

For percent recovery studies, 50 mL of Czapek-Dox/1% corn-steep media was spiked with 5μg of penicillin and a separate 50mL of media was spiked with 1μg each of roquefortine C and meleagrin standards. This was repeated for two other concentrations at 10 and 30 μg of penicillin and 10 and 20 μg roquefortine C and meleagrin. For

penicillin recovery, the pH of the media was adjusted to pH 2.5 prior to extraction by using 1N HCl. The samples were then processed as described previously for the extraction of penicillin. Each sample was then re-suspended in butyl acetate, dried under nitrogen in clean vials and dissolved again in 5 mL of solvent. A 10 µl aliquot of extract was injected on to the HPLC column.

To calculate the recoveries of roquefortine C and meleagrin, the media was extracted with 100 mL of ethyl acetate and the samples were processed as described above in the culture extraction section. Peak identity was confirmed by comparing the resulting spectra to an authentic standard UV spectrum. This allowed the calculation of percent recovery of penicillin G, roquefortine C and meleagrin by testing the experimental extraction process. All of the recovery analyses were performed in triplicate.

Roquefortine C has been shown to be sensitive to light and exposure greatly reduces its recovery. For this reason, all experiments were performed under reduced light conditions with the lights off, away from natural light sources[96]. All cultures were grown in darkness (covered in aluminum foil), filtered and extracted in darkness, and all samples and standards were stored in amber vials.

3.11 LC-MS, LC-MS/MS and NMR analysis

LC-MS was used to help in the determination and confirmation of unknown compounds from both the filtrate and mycelium. A Hewlett Packard 1050 HPLC system (pump, inline degasser, UV detector, and computer) was attached to a Quattro LC-triple quadropole electrospray ionization (EI) mass spectrometer (Micromass, UK). The instrument was set for analysis in positive ion mode with full scan spectra between *m/z* 200-1000. The EI-MS was controlled using MassLynx software version 3.5 that was also

used for analysis of mass spectrum data. This allowed for the analysis of extracts under the identical HPLC conditions as described above. The effluent was divided using a micro-splitter valve (Upchurch Scientific, WA) such that the flow to the electrospray was approximately 100μL/min. This was diverted through capillary tubing set at -3.8 kV into the EI-MS. The source block temperature was 80 °C and the cone voltage was -47 V. Nitrogen served as the drying and nebulizing gas at a flow rate of 70 L/h, 5.5 x 10^5 Pa. The majority of the flow was diverted into the UV detector as usual to provide a chromatogram of the sample mixture. The sample entering the electrospray was compared to the complementary UV trace. Comparisons were made between both to determine the molecular weights of individual peaks from the samples for aid in identification of the secondary metabolites. The same samples were then analyzed using LC-MS/MS to provide fragmentation patterns of the parent ions determined above to further aid the identification of metabolites.

NMR spectra were acquired on a Bruker ARX500 spectrometer at 500.13 MHz (^{1}H) or 125.18 MHz (^{13}C) operating at 303°K, using a 5 mm inverse triple (H/C/N) probe. NMR spectra were determined in acetonitrile-d_3 (CD_3CN) and chemical shifts were referenced to the solvent at 1.94 ppm for ^{1}H and 1.39 ppm for ^{13}C and are reported relative to tetramethysilane (TMS). Chemical shift assignments were made with ^{1}H/^{1}H (COSY), and ^{1}H/^{13}C (HMQC, HMBC) inverse correlation spectra using standard Bruker pulse sequences.

3.12 Secondary metabolite isolation and purification

3.12.1 *P. brevicompactum* strain DAOM 215331

A chromatography column was prepared by mixing 50g of silica gel/g of extract (230-400 mesh, 40-63 μm, 60Å) with enough chloroform to make a workable slurry. The

column was packed using forced air to compress the silica gel taking care not to let the column dry. Fine sand was added (0.5 cm) to serve as a protective layer and to act as a filter for the sample.

Initially, the columns were eluted with a gradient system starting with 5% ethyl acetate/hexane increasing to 30% ethyl acetate/hexane. The first eleven fractions collected from the columns were mostly triglycerides and fatty acids as shown by ^{1}H NMR. Fractions twelve to thirty-one were fatty acids. Fractions thirty-two to eighty-nine each consisted of several spots that proved difficult to separate. A second system made up of 10% methanol/chloroform gave excellent separation after TLC and it was determined that this system should be used during column chromatography. The column was eluted with a gradient system starting with 0% methanol/chloroform increasing to 10%-15% methanol/chloroform as needed.

The crude ethyl acetate extract (350 mg) from the filtrate of strain DAOM 215331 was dissolved in a minimum amount of solvent and was loaded onto the column. The extract was eluted using a stepwise solvent gradient. The solvent gradient used was MeOH/$CHCl_3$ (0-15%) and it was added in 200 mL aliquots. Three mL fractions were collected in glass test tubes. The fractions were analysed by TLC to check their purity as well as to screen for major spots. Similar fractions were combined and analyzed by ^{1}H NMR. From the chromatography column of strain DAOM 215331, three major fractions (F1-3) were obtained.

Fraction 1 (179.6 mg) was subjected to preparative TLC (silica gel 60, 20 x 20, 2000μm, Whatman, USA). The plates were developed multiple times in 2% MeOH/$CHCl_3$ to obtain maximum separation. Four distinct bands were evident and these

were scrapped off the plate and extracted separately in 50mL of a 10% MeOH/$CHCl_3$ solution. The extracts were filtered, evaporated *in vacuo* and analyzed by ^1H NMR. Impure brevianamide A (148 mg) was collected from the first extract. Further purification of brevianamide A was achieved by preparative HPLC (Synergi Max-RP, 250 x 30 mm, 4μm, 80 Å, Phenomenex, California) using the solvent gradient system described previously (Table 3.4). Recrystallization from $CHCl_3$ gave 42 mg of bright yellow crystals. The purity of brevianamide A was confirmed by ^1H NMR (one spot by TLC analysis, R_f=0.39 in 10% MeOH/$CHCl_3$), optical rotation and melting point.

A pale yellow solid (1.60 g) identified as mycophenolic acid was obtained from Fraction 2. The solid was washed with acetone twice before it recrystallized from 5% MeOH/$CHCl_3$ to give 500 mg of white crystals.

Fraction 3 (2.0 g) was a thick, dark brown solid that had to be separated again over silica gel eluting with a $CHCl_3$/MeOH solvent gradient (0-15% MeOH/$CHCl_3$). The fractions were analyzed by TLC and all fractions containing similar spots were combined and dried to give a light brown solid (1.44 g). This compound was recrystallized from 5% MeOH/$CHCl_3$ resulting in a pale pink solid (600 mg). Further purification of ~100 mg of this material was done by preparative plate TLC in 5% MeOH/$CHCl_3$ yielded of a white solid (50 mg). This compound was identified as a Raistrick phenol derivative.

The crude ethyl acetate extract (3.74 g) from the mycelium of DAOM 215331 was also separated on a silica gel column similar to that described for its filtrate resulting in two major fractions (F4-5). Fraction 4, the non-polar fraction was a dark orange liquid consisting mainly of triglycerides (1.11 g). Fraction 5 was impure brevianamide A (50

mg). Brevianamide A was purified by preparative plate TLC as before and recrystallized from $CHCl_3$ to yield pure yellow crystals (18 mg).

3.12.2 *P. brevicompactum* strain DAOM 215335

The crude ethyl acetate extract (3.96 g) from the filtrate of DAOM 215335 was purified using the same procedure as described for DAOM 215331 to give 3 major fractions (F6-8). A pale brown solid (1.52 g) identified as mycophenolic acid was obtained from fraction 6. It was purified as described for DAOM 215331 yielding white crystals (300 mg). Fraction 7 consisted of triterpenoids (200 mg). Fraction 8 was a light brown liquid (100mg) that was again identified as the Raistrick phenol derivative. Further purification of the Raistrick phenol derivative by preparative plate TLC yielded of the pure material (10 mg).

The crude ethyl acetate extract (10.9 g) from the mycelium of DAOM 215335 was divided into 2 equal parts. Part 1 (5.0g) was separated using silica gel chromatography as described above to give 2 main fractions (F9-10). Fraction 9 (2.8g) was mainly triglycerides. Fraction 10 yielded mycophenolic acid (100 mg).

Part 2 (5.9g) was first extracted with 100mL of hexane for 15 minutes yielding 4.92 g of extract. This fraction contained mainly triglycerides and fatty acids. The residue (740 mg) was separated on a silica gel column as described above to afford of a pale yellow solid (240 mg). The solid was further washed with hexane and acetone to provide white crystals identified later as asperphenamate (20 mg).

3.12.3 *P. chrysogenum* strain DAOM 234067

The crude ethyl acetate extract (6.56 g) from the mycelium of strain DAOM 234067 was defatted with hexane. The defatted extract (3.81 g) was purified using

column chromatography as described above eluting with a stepwise solvent gradient of 0-15% MeOH/$CHCl_3$ in 200mL aliquots to provide 3 major fractions.

Fraction 1 yielded impure ergosterol (300 mg). Fraction 2 was identified to be of impure meleagrin (1 g). Fraction 3 was found to be roquefortine C (20 mg). The major fraction was recrystallized twice from acetone to provide meleagrin as a pale yellow solid (350 mg) which was pure by ^1H NMR, TLC analysis (one spot) and optical rotation.

The crude ethyl acetate extract (1.0 g) from the filtrate of strain DAOM 234067 was also purified using column chromatography eluting with a step-wise solvent gradient of 0-15% MeOH/$CHCl_3$ in 200 mL aliquots. The main fraction was impure meleagrin (80 mg). This fraction was further purified by preparative plate TLC in 5% MeOH/$CHCl_3$ to yield relatively pure meleagrin (50 mg).

3.12.4 *P. chrysogenum* strain DAOM 234065

The crude ethyl acetate extract (7.0 g) from the mycelium of DAOM 234065 was purified using column chromatography over silica gel. This time the sample was eluted with hexane followed by a 0-5% MeOH/$CHCl_3$ gradient in 400mL aliquots to provide two main fractions. Fraction 1 (5.07g) contained mainly triglycerides and fraction 2 consisted of ergosterol (1.40 g).

The crude ethyl acetate extract (6.83 g) from the filtrate of DAOM 234065 was separated using the same procedure described for the filtrate of DAOM 234067 to give 3 fractions. Fraction 2 (10mg) was determined to be impure meleagrin. The impure meleagrin was recrystallized twice from acetone to yield pure meleagrin (7 mg). Fraction 3 gave roquefortine C as a pale yellow solid (3.01 g).

3.13 Derivatization of meleagrin

The synthetic derivatives meleagrin acetate, N-methyl meleagrin and N-methyl oxaline, were prepared in order to verify the structure of meleagrin.

Acetylation of meleagrin with acetic anhydride in CH_2Cl_2 (Et_3N, DMAP) at room temperature afforded meleagrin acetate. Upon methylation with diazomethane (CH_2N_2), N-methyl oxaline was produced as the major product. A minor product that appeared to be N-methyl meleagrin by ^1H NMR was also formed.

3.13.1 Meleagrin acetate

To initiate the reaction, 0.25 mL of acetic anhydride (2.3×10^{-3} mol) was added to a solution of meleagrin (50mg, 1.15×10^{-4} mol) in 10 mL of CH_2Cl_2, 0.25mL of triethyl amine (2.3×10^{-3} mol) and 10 mg of DMAP at room temperature (25°C). The reaction mixture was left to stir overnight before it was washed successively with 5 mL of distilled deionized water, 5 mL of 5% HCl and an extra 5 mL of water. The organic layer was dried over sodium sulphate, filtered and concentrated *in vacuo*. The crude product was purified by preparative plate TLC in 2% MeOH/$CHCl_3$ to yield meleagrin acetate as a pale yellow solid (20 mg) (m.p. 225-230°C (lit. 247°C))[49,66].

3.13.2 N-methyl oxaline

Diazomethane solution was prepared by adding 15 mL of a 0.7M solution of KOH in ethanol to a solution of N-methyl-N-nitrosotoluene-p-sulfonamide (3.5 g) in 40 mL of diethyl ether for 5 min. The solution was distilled at 55-60°C until a yellow etheral CH_2N_2 fraction was produced. The yellow fraction was collected over an ice bath at 0°C. The etheral CH_2N_2 solution was added in excess to a stirred solution containing meleagrin (50 mg) in 25 mL of CH_2Cl_2 with a few drops of MeOH at 0°C until it remained permanently yellow. The excess CH_2N_2 was evaporated in the fumehood

overnight. The solvent was removed *in vacuo* and the residue was re-dissolved in 30 mL of ethyl acetate. It was then washed successively with 10 mL of water, 10 mL of brine and 20 mL of water. The organic layer was dried over sodium sulphate, filtered and concentrated *in vacuo*. The crude product was further purified using multiple elution preparative plate TLC in 2% MeOH/$CHCl_3$ to yield two main products, oxaline and N-methyl oxaline (15 mg) as a pale yellow solid (m.p. 225°C). N-methyl meleagrin was formed as a minor product.

The melting points of all the isolated the compounds were measured and along with the optical rotation, MS and NMR spectra, were used to characterize and confirm their purity.

3.14 Statistical analysis

Cluster analysis was performed in order to determine the presence of a potential link between the different strains of *P. chysogenum* and their production of secondary metabolites. A subset of eight *P. chrysogenum* isolates obtained from DAOM and a subset of twenty *P. chrysogenum* isolates from water-damaged buildings and building materials (Paracel Labs, Ottawa) were examined. The strains were determined to be producers of penicillin G, roquefortine C, meleagrin and xanthocillin x. A data matrix of twenty-seven isolates and 8 variables (secondary metabolite and secondary metabolite yield) was constructed for cluster analysis. The matrix was normalized by calculating the secondary metabolite yield in μg/g/L of liquid culture. The total amount of secondary metabolites detected per strain was added up and individual values for each respective secondary metabolite were entered as a fraction of this total in the final matrix.

The data was analyzed by the algorithms offered by the statistical program Systat 10.2. The squared Euclidean distance measure was used and the Ward's method was

chosen as the one giving the most significant and meaningful dendogram. To be able to account for the difference in orders of magnitude and calculate these distances, the data were log transformed.

4. RESULTS

4.1 Culture growth

The results of testing the growth of *P. brevicompactum* and *P. chrysogenum* on different media are displayed in Table 4.1. The dry weight of the mycelium is included as an indicator of how well the cultures grew on that particular media. The average mycelium weight increased when the cultures were grown on Czapek-Dox/1% corn-steep media as compared with growth in the Czapek-Dox. Average growth in Czapek-Dox/1% corn-steep media was 1.3 times better for *P. brevicompactum* strains and 1.6 times better for *P. chrysogenum* strains. The results of testing the growth of *P. chrysogenum* on the Czapek-Dox/1% corn-steep media versus the fermentation media for the enhancement of penicillin production are displayed in Table 4.2. The average mycelium weight increased when the cultures were grown on Czapek-Dox/1% corn-steep media as compared with growth in the fermentation media. Average growth in Czapek-Dox/1% corn-steep media was 2.6 times better.

Table 4.1 Comparison of dry mycelium weights of *P. brevicompactum* strains when grown on two types of media.

Species and strain	Av. mycelium weight (g) Czapek Dox/1% corn steep	Av. mycelium weight (g) Czapek Dox
P. brevicompactum		
DAOM 215331	1.56	1.30
DAOM 215333	1.91	1.60
DAOM 215334	2.27	1.71
DAOM 215335	2.52	2.46
DAOM 234044	2.45	1.95
DAOM 234045	3.01	2.48
DAOM 234046	2.83	2.21
DAOM 234047	3.52	2.13
DAOM 234048	2.58	1.99
DAOM 234049	2.96	2.55
DAOM 234050	2.24	1.52
Average	**2.53**	**1.99**
St. dev.	**0.54**	**0.42**

Table 4.2 Comparison of dry mycelium weights of *P. chrysogenum* strains when grown on three types of media.

Species and strain	Av. mycelium weight (g) Czapek Dox/1% corn steep	Av. mycelium weight (g) Czapek Dox	Av. mycelium weight (g) Fermentation media
P. chrysogenum			
DAOM 155627	2.54	1.32	0.96
DAOM 155628	2.99	1.40	1.03
DAOM 167036	2.84	1.41	1.10
DAOM 171025	1.86	1.92	1.17
DAOM 175157	2.57	1.95	1.07
DAOM 175758	0.93	0.73	0.76
DAOM 178623	3.48	2.21	1.09
DAOM 190864	2.10	1.13	0.82
DAOM 193710	1.16	0.99	0.74
DAOM 215336	3.26	1.35	1.08
DAOM 215337	2.47	1.52	1.25
DAOM 215701	3.60	1.30	0.58
DAOM 59494C	2.71	1.61	0.89
DAOM 234051	2.51	1.85	0.90
DAOM 234052	3.49	2.46	1.11
DAOM 234053	2.68	1.95	1.02
DAOM 234054	1.96	1.48	1.07
DAOM 234055	2.75	2.12	1.13
DAOM 234056	2.93	1.13	0.56

DAOM 234057	3.48	1.99	1.21
DAOM 234058	2.11	1.35	1.23
DAOM 234059	1.58	1.12	0.70
DAOM 234060	3.06	1.39	1.10
DAOM 234061	2.42	1.50	1.29
DAOM 234062	3.58	1.71	1.45
DAOM 234063	2.63	2.06	0.82
DAOM 234064	3.28	1.53	1.23
DAOM 234065	2.55	2.02	1.19
DAOM 234066	2.97	2.61	0.93
DAOM 234067	3.01	2.13	1.09
Average	**2.65**	**1.64**	**1.02**
St. dev.	**0.68**	**0.45**	**0.22**

4.2 Recovery of penicillin G, roquefortine C and meleagrin from the spiked media

The standard calibration curve of penicillin G was prepared by plotting the peak area counts versus the amount of penicillin G on the HPLC column (μg). The standard curve developed from the final gradient program showed a linear relationship with an R^2 value of 0.99 over the full concentration range used (Figure 4.1). These steps were repeated for roquefortine C (R^2=0.98) (Figure 4.2) and meleagrin (R^2=0.99) (Figure 4.3).

Using the equations from the respective standard calibration curves, the percent recovery of penicillin G, roquefortine C and meleagrin from the spiked media extracts was determined to be 84.11 ± 4.17 %, 84.67 ± 3.72 % and 76.33 ± 2.59 %. The percent recovery calculations indicated that the extraction method was suitable for the analysis of penicillin G, roquefortine C and meleagrin. The effectiveness of the extraction method and of the different extraction solvents was confirmed and compared to studies by others. Industrial recovery yields for penicillin G from *P. chrysogenum* cultures is approximately 85%[37]. The mean recovery for roquefortine C from food and feed samples using similar methodology is between 70-85%[81].

4.4 Analysis of *Penicillium* strains by HPLC-DAD for metabolite profiles

All the strains used in this study were analyzed using HPLC-DAD to determine their respective chemotaxonomic profiles. In addition, the amount of secondary metabolites produced was used in order to screen for the most active strains. The most active strains were then grown in large scale fermentations in hope of producing large amounts of metabolites to be able to isolate and purify them for future use in toxicity studies. The resulting retention times, chromatograms and UV spectra for the filtrate and mycelium extracts were compared to chromatograms of standards and to chromatograms of previously published data for identification purposes. This was done for some of the expected secondary metabolites of *P. brevicompactum* such as mycophenolic acid and botryodiploidin. Although brevianamide A, asperphenamate and meleagrin were also expected in this species, no standards were available at the time of analysis. Characterization of these compounds became possible only after the compounds were isolated and purified later on during this study. Figures 4.4 and 4.5 show the filtrate and mycelium chromatograms of strain DAOM 215331 from *P. brevicompactum* and are presented here as a representative strain for this species.

After isolation, purification and characterization of brevianamide A, the standard was used to search for its production by other *P. brevicompactum* strains. It eluted at a retention time of 14.1 minutes and was identified as one of the major metabolites in the HPLC-DAD chromatograms of the filtrate and mycelium extracts of *P. brevicompactum* DAOM 215331. Mycophenolic acid was seen in the filtrate extract of DAOM 215331 and in the filtrate and mycelium extracts of DAOM 215335 eluting at 20.0 minutes. Asperphenamate was only seen in the mycelium extract of DAOM 215335 eluting at 28.9

minutes. The Raistrick phenol derivative was seen in the mycelium extracts of both strains eluting at 2.4 minutes (Figure 4.5).

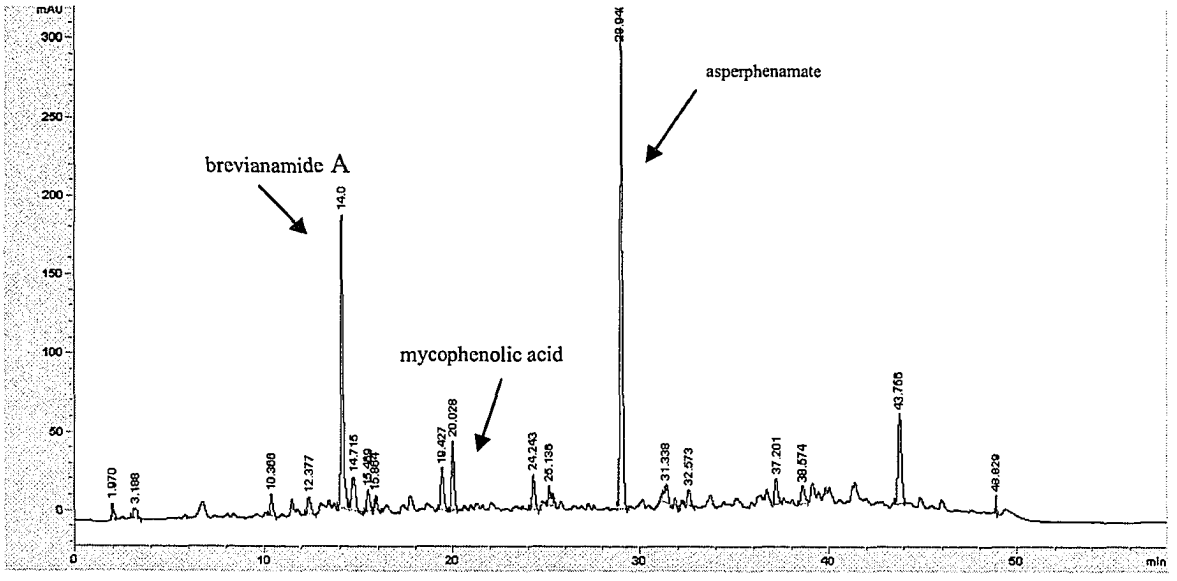


Figure 4.4 HPLC-DAD chromatogram of the filtrate extract of *P. brevicompactum* DAOM 215331 indicating the retention times of brevianamide A (14.1 min), mycophenolic acid (20.0 min) and asperphenamate (28.9 min). (@ 354 nm)

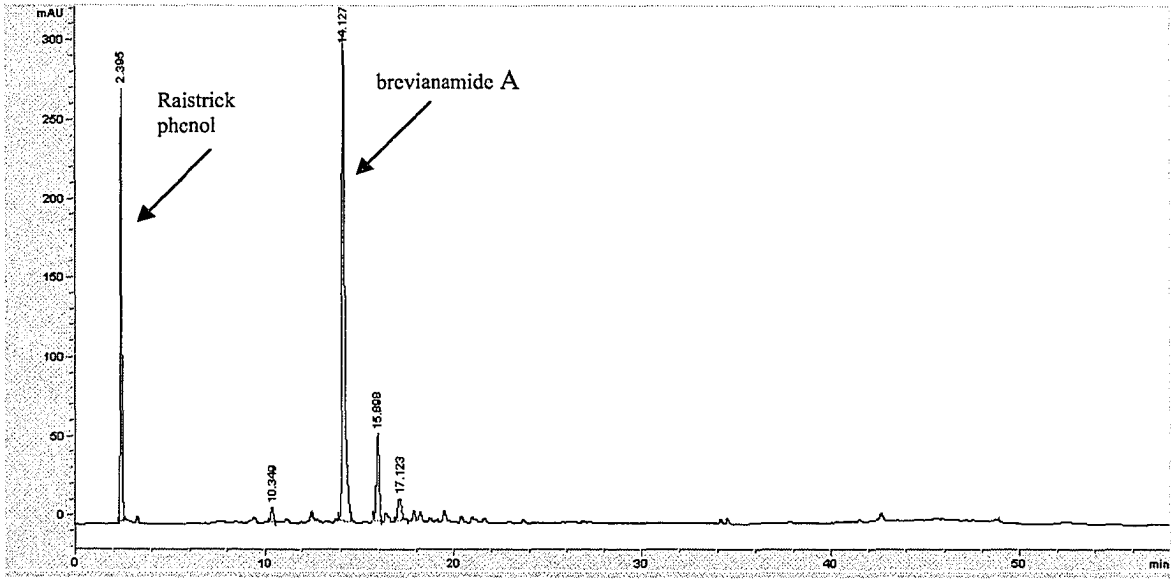


Figure 4.5 HPLC-DAD chromatogram of the mycelium extract of *P. brevicompactum* DAOM 215331 indicating the retention times of the Raistrick phenol derivative (2.4 min) and brevianamide A (14.1 min). (@ 354 nm)

Chromatograms of roquefortine C, penicillin G, xanthocillin x and sorbicillin standards were compared to chromatograms of *P. chrysogenum* extracts. After isolation, purification and characterization of meleagrin, the standard was used to search for its production by *P. chrysogenum* strains. It eluted at a retention time of 24.2 minutes and was identified as one of the most consistent metabolites produced by this species but was not seen in the mycelium extract of DAOM 234065. Roquefortine C was seen at 15.8 minutes in the mycelium extract of DAOM 234067.

4.5 Identification and isolation of secondary metabolites from *P. brevicompactum*

The ethyl acetate extracts from the filtrate and mycelium of *P. brevicompactum* strains DAOM 215331 and DAOM 215335 were processed in order to identify and isolate the major secondary metabolites present. The secondary metabolites found in the extracts were brevianamide A, mycophenolic acid, asperphenamate and a Raistrick phenol derivative (Table 4.3).

All of these secondary metabolites except for the Raistrick phenol derivative have been included in the metabolite profile of *P. brevicompactum*[3]. Raistrick phenols are characteristic of this fungal species and have been shown to form lactols during isolation and under acidic conditions[3,102]. Hence, it is plausible to derive the Raistrick phenol derivative from the Raistrick phenol (2,4-dihydroxy-6-(1-hydroxy-2-oxopropyl)benzoic acid) (Appendix A)[3].

Mycophenolic acid was isolated from the filtrate and mycelium fractions of both fungal strains, DAOM 215331 and DAOM 215335. Brevianamide A was isolated from strain DAOM 215331 while asperphenamate was isolated from strain DAOM 215335. The Raistrick phenol derivative was isolated from the mycelium fractions of both strains in smaller amounts (Table 4.3).

Table 4.3 Secondary metabolites isolated from the ethyl acetate extracts of *P. brevicompactum* strains DAOM 215331 and DAOM 215335.

Metabolites	DAOM 215331		DAOM 215335	
	Filtrate (7.16 g)	Mycelia (3.74 g)	Filtrate (3.96 g)	Mycelia (10.13 g)
Brevianamide A (mg)	42	18		
Mycophenolic Acid (mg)	500		300	100
Asperphenamate (mg)				20
Raistrick Phenol Derivative (mg)		50		10

4.5.1 Brevianamide A

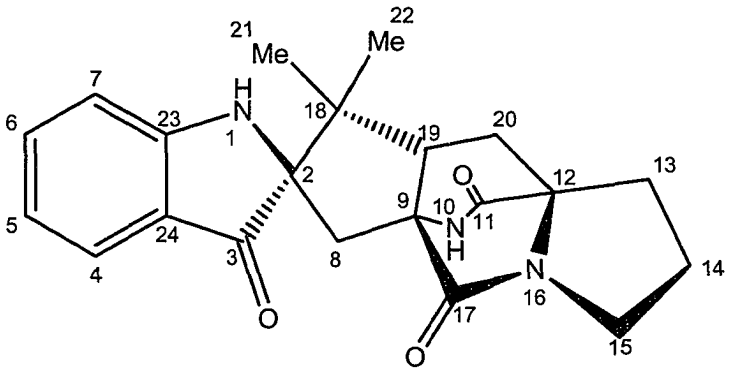


Figure 4.6 Structure of brevianamide A showing numbering.

Brevianamide A was isolated from the ethyl acetate extracts of the filtrate and mycelia of *P. brevicompactum* strain DAOM 215331 as bright yellow crystals with a melting point of 200-212°C (lit. 90-220°C)[14] and 2-3 mg/L yield (lit. 0.1-10 mg/L yield)[14]. In comparison, the melting point of brevianamide B, the diastereomer has been reported as 324-328°C [12].

The purification of brevianamide A required silica gel chromatography using a solvent gradient of MeOH/$CHCl_3$ (0-15%), followed by preparative plate TLC, preparative HPLC and recrystallization from chloroform. The TLC plate of brevianamide A revealed one yellow fluorescent spot under UV light (366 nm). The R_f value was 0.39 when developed in a 10% mixture of MeOH/$CHCl_3$.

The EI mass spectrum displayed the $[M]^+$ at m/z 365 corresponding to the expected molecular formula of $C_{21}H_{23}N_3O_3$. This was confirmed by high resolution electron impact mass spectrometry (HRMS) (Figure A.4). The primary fragmentation pattern involved the loss of mass 69 (C_5H_9) from the molecular ion to give a stable ion at m/z 296 which was in conformity with literature values [14,12].

The presence of an N-H and C=O functional groups were apparent by the strong IR absorptions at 3317-3226 and 1620-1679 cm^{-1} respectively. The ^1H NMR data were consistent with previously published values (Table 4.4)[14]. Diagnostic resonances were observed for the aromatic protons at δ 6.82-7.60 typical of a symmetrically ortho-substituted benzene AA'XX' system, methylene protons at δ 2.35 (t, J=6.7 Hz, H-15) as well as for the methyl singlets at δ 1.05 (s, 3H, H-21) and at δ 0.80 (s, 3H, H-22) (Figure A.5). The ^1H NMR data also resembled that reported for brevianamide B with respect to the bicylo moiety (the ring system made up of tryptophan, proline and substituted proline derivatives where the olefinic unit of the isoprene moiety has been oxidatively cyclized across the α-carbon atoms of the cyclic dipeptide)[111].

Table 4.4 ^{13}C and ^{1}H NMR chemical shift assignments for brevianamide A (500 MHz, CD_3CN, coupling constants in parentheses).

Position #	^{13}C (ppm from TMS)	^{1}H (ppm from TMS)
1 (NH)		3.35 (br s, NH)
2	112.2	
3	202.5	
4	125.2	7.45 (d, J=7.8 Hz, 1H)
5 and 7	119.8	6.75 (t, J=7.8 Hz, 2H)
6	139.5	6.90 (t, J=7.8 Hz, 1H)
8	37.8	2.60 (m, 2H)
9	55.0	
10 (NH)		7.62 (br s, 1H)
11	171.2	
12	80.0	
13	30.0	2.45 (m, 2H)
14	26.5	2.02 (m, 2H)
15	48.2	2.35 (t, J=6.7 Hz, 2H)
17	174.8	
18	45.0	
19	54.6	2.60 (m, 1H)
20	44.8	1.80 (m, 3H)
21	24.2	1.05 (s, 3H)
22	20.0	0.80 (s, 3H)
23	162.2	
24	122.5	

The carbon spectra clearly indicated the resonance for each of the carbons in the molecule. (Figure A.6).

4.5.2 Mycophenolic Acid

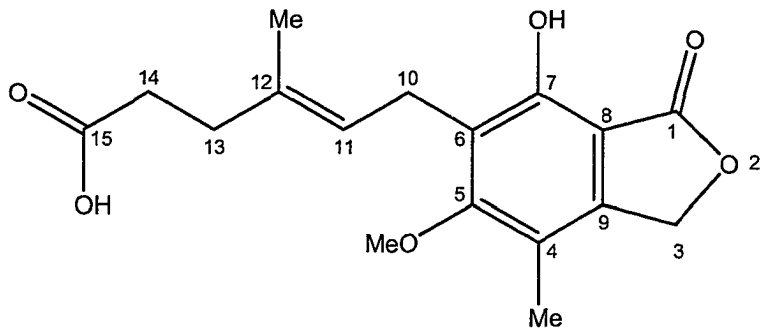


Figure 4.7 Structure of mycophenolic acid showing numbering

Mycophenolic acid was isolated from the filtrates of *P. brevicompactum* strains DAOM 215331 and DAOM 215335 and from the mycelia of DAOM 215335. After purification by silica gel column chromatography utilizing the same conditions as described for brevianamide A, followed by washing with acetone and recrystallization from MeOH/$CHCl_3$, the pure compound was obtained as white crystals with a melting point of 141-142°C (lit.139-141°C)[35] and in ~30 mg/L yield. The analytical TLC plate of mycophenolic acid revealed one blue fluorescent spot under UV light (366 nm). The R_f value was 0.38 when developed in a 10% mixture of MeOH/$CHCl_3$.

The molecular composition of mycophenolic acid, $C_{17}H_{20}O_6$, was confirmed by the molecular ion observed at m/z 320 in its EIMS spectrum and by HRMS (Figure A.7). The primary fragmentation pattern involved the loss of mass 73 ($C_3H_5O_2$) from the molecular ion to give a stable ion at m/z 247 and the loss of mass 113 ($C_6H_9O_2$) to give a stable ion at m/z 207. The fragmentation pattern matched well with those reported in the literature[35]. The free OH, CO and CO_2 groups exhibited strong IR bands at 3417 and 1706, 1625 cm^{-1} respectively.

The ^1H NMR and ^{13}C NMR spectra recorded for mycophenolic acid were in agreement with previously published values (Table 4.5 and Figure A.8 and A.9)[35].

Table 4.5 ^{1}H NMR and ^{13}C chemical shift assignments for mycophenolic acid (200 MHz, CD_3CN, coupling constants in parentheses).

Position #	^{13}C (ppm from TMS)	^{1}H (ppm from TMS)
1	165.0	
3	70.0	5.20, (s, 2H)
4	118.3	
5	173.7	
5 (OMe)	61.7	3.76, (s)
6	107.5	
7	153.9	
8	92.5	
9	146.2	
10	16.3	3.34, (d, J=7.0 Hz, 2H)
11	122.3	5.21, (m, 1H)
12	135.2	
13	35.1	2.32, (m, 2H)
14	32.9	2.45, (m, 2H)
15	174.7	
16 (Me)	11.6	2.15, (s, 3H)
17 (Me)	23.2	1.77 (s, 3H)
OH		7.75 (br s)

4.5.3 Asperphenamate

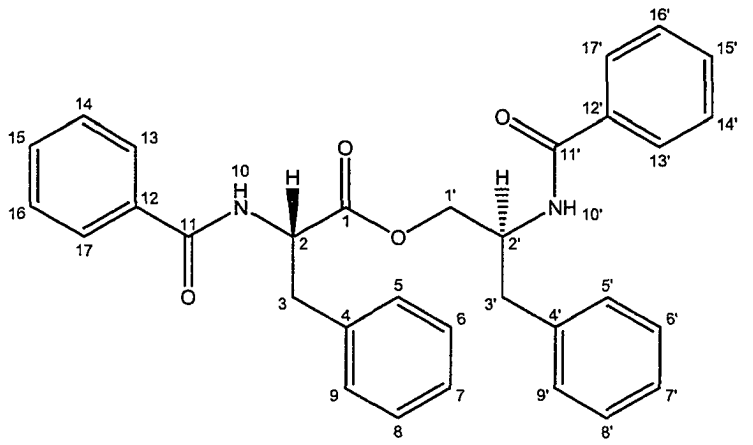


Figure 4.8 Structure of asperphenamate showing numbering

Asperphenamate was isolated from the filtrate of *P. brevicompactum* strains DAOM 215331 and DAOM 215335 as a white solid. The melting point was determined to be 215-217°C (lit. 210°C, lit. 206-207°C, lit. 201-204°C)[57,15,31] in ~1 mg/L yield, after silica gel column chromatography using a $MeOH/CHCl_3$ solvent gradient (0-15%) followed by washing with acetone.

The molecular ion at m/z 506 matched the proposed molecular formula of $C_{32}H_{30}N_2O_4$. This was confirmed by high resolution electron impact mass spectrometry (HRMS). The fragment ion peaks in the EIMS spectra were observed at m/z 415, 251, 146 and 105 and were identical with reported values [57]. The IR spectrum showed a broad N-H stretch at 3306 cm^{-1} with strong CO bands at 1751, 1734 and 1638 cm^{-1}. The ^1H and ^{13}C NMR data was revised from that published by McCorkindale et. al, (1978) (Figure 4.6, Figure A.10 and A.11)[57].

Table 4.6 ^{13}C and ^{1}H NMR chemical shift assignments for asperphenamate (200 MHz, $CDCl_3$, coupling constants in parentheses).

Position #	^{13}C (ppm from TMS)	^{1}H (ppm from TMS)
1	172.4	
2	55.7	4.75 (dd, J = 12.5 Hz, 1H)
3	37.5	3.12, 3.25 (J_{AB} = 12.5 Hz, $J_{3,2}$ = 5.0 Hz, 2H)
1'	66.5	4.00, 4.45 (J_{AB} = 12.5 Hz, $J_{3',2'}$ = 5.0 Hz, 2H)
2'	51.2	4.55 (m)
3'	37.6	2.85 (J = 6.5 Hz, 2H)
4, 4'	139.2, 138.3	
5, 9, 5', 9'	130.2, 129.4	7.12 (m, 4H)
6, 8, 6', 8'	129.4, 128.6	7.20 (m, 4H)
7, 7'	127.5,	7.15 (m, 2H)
11	168.1	
11'	167.6	
12, 12'	132.7	
13, 13', 17, 17'	128.0	7.75 (m, 4H)
14, 14', 16, 16'	128.6	7.44 (m, 4H)
15, 15'	132.2, 132.5	7.51 (m, 2H)

4.5.4 Raistrick Phenol Derivative 5, 7-Dihydroxy-3(1-hydroxyethyl)phthalide

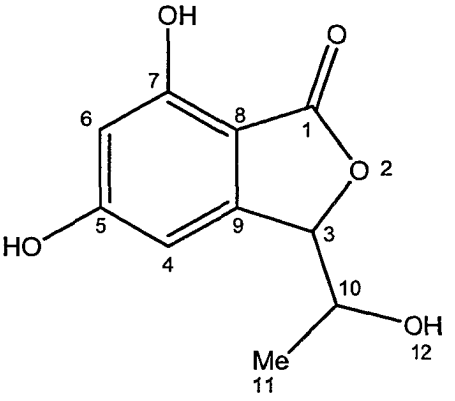


Figure 4.9 Structure of Raistrick phenol derivative showing numbering

The Raistrick phenol derivative was isolated from the mycelium extract of strain DAOM 215335 as a white solid with a melting point of 204-208°C (lit. 206-208°C)[102]. The isolation was achieved after repeated column chromatography eluting with MeOH/$CHCl_3$ (0-15%) followed by preparative HPLC and recrystallization from MeOH.

The EIMS displayed a molecular ion at m/z 210 corresponding to the molecular formula of $C_{10}H_{10}O_5$. This was further established by HRMS. The IR spectrum showed prominent bands at 3329 and 1710 cm^{-1} due to the free OH and benzoate ester functional groups respectively. The ^1H NMR data was very similar to data published by Uchida *et. al.*, (1998) for 5, 7-dihydroxy-3(1-hydroxyethyl)phthalide, a phenol isolated from *Ceratocystis ulmi*, *C. ips* and *Alternaria kikuchiana* (Table 4.7 and Figures A.13 and A.14)[8,32,48,102].

Table 4.7 ^{13}C and ^{1}H NMR chemical shift assignments for 5, 7-Dihydroxy-3(1-hydroxyethyl)phthalide (200 MHz, CD_3CN, coupling constants in parentheses).

Position #	^{13}C (ppm from TMS)	^{1}H (ppm from TMS)
1	172.5	
3	85.7	5.25 (d, J= 4.7 Hz)
4	103.7	6.50 (d, J= 1.0 Hz)
5	165.0	
6	103.7	6.37 (d, J= 3.0 Hz)
7	158.7	
8	120.0	
9	152.5	
10	70.0	4.00 (m)
11	18.7	1.13 (d, J= 6.3 Hz, 3H)
OH (12)		3.40
OH (5, 7)		7.87

4.6 Isolation and identification of secondary metabolites from *P. chrysogenum*

Penicillin G was one of the major metabolites produced by strains of *P. chrysogenum*. It was initially identified by comparison of the retention time on the HPLC-DAD chromatograms to the retention time of the standard and by using UV absorption spectra. Its identity was later confirmed by LC-MS. The molecular formula of penicillin G was established as $C_{16}H_{18}O_5N_2S$ by HRMS. The characteristic EIMS ions at m/z 335 [M^+], 160, 367 were comparable with literature values[64].

The secondary metabolites isolated from the culture medium of *P. chrysogenum* strains DAOM 234067 and DAOM 234065 were identified to be meleagrin and roquefortine C. The synthetic derivatives, meleagrin acetate and N-methyl oxaline, were prepared in order to verify the structure of meleagrin. Oxaline is included here for comparison only (Figure A.3)[80,110].

4.6.1 Meleagrin and related compounds

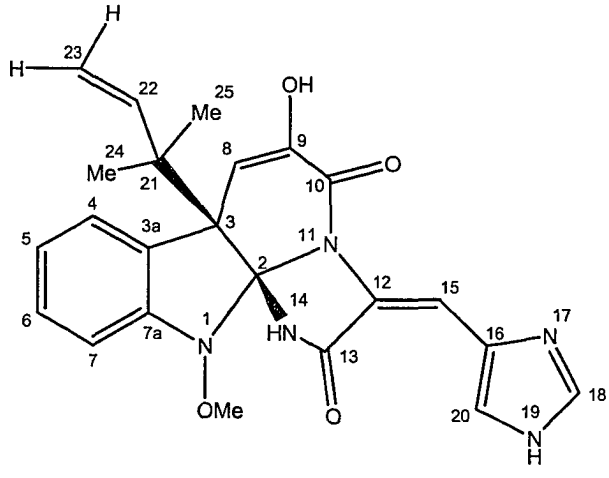


Figure 4.10 Structure of meleagrin showing numbering.

Meleagrin was the major metabolite found in the mycelium and filtrate of strain DAOM 234067. A small amount of meleagrin was also found in the filtrate of strain DAOM 234065. Roquefortine C was only present in the mycelia of strain DAOM 234067 (Table 4.8).

Table 4.8 Secondary metabolites isolated from the ethyl acetate extracts of *P. chrysogenum* strains DAOM 234067 and DAOM 234065.

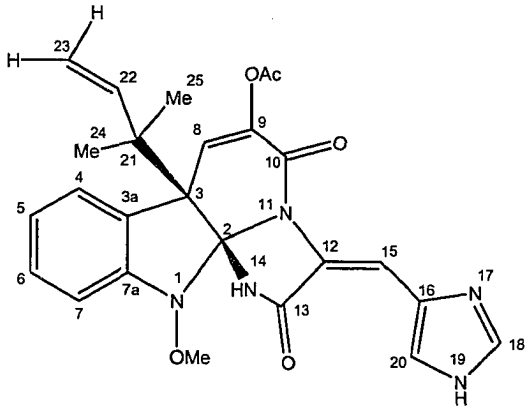
Metabolites	DAOM 234067		DAOM 234065	
	Filtrate (1.0 g)	Mycelium (6.56 g)	Filtrate (0.5 g)	Mycelium (7.0 g)
Meleagrin (mg)	40	350	10	
Roquefortine C (mg)		20		

Meleagrin was isolated as a pale yellow solid with a melting point of 197-201°C (lit. 250°C)[49,66]. It was isolated from the ethyl acetate extracts and purified after column chromatography eluting with a MeOH/$CHCl_3$ (0-15%) solvent gradient, followed by recrystallization from acetone.

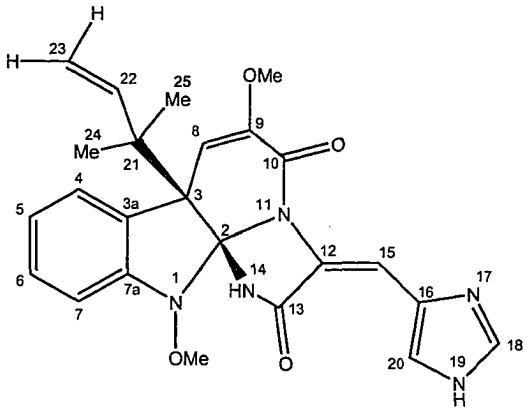
The ^1H NMR spectrum of meleagrin at 25 °C disclosed a feature that was incompatible with its structure. That is, the ABX system comprising of protons at positions 22 and 23 appeared as a broad unresolved one-proton singlet at δ 6.12 and as a two-proton unsymmetrical triplet at δ 5.02 (E) 5.07(Z) respectively, instead of the expected doublets (Figure A.16). The ^1H NMR spectrum of N-methyl oxaline was compared with literature values for oxaline and its structure was for comparison purposes[80,110]. N-methyl oxaline has not been reported in the literature.

Confirmation of the structure and stereochemistry of meleagrin was achieved by properly characterizing the molecule and its derivatization products (Figure 4.11). This was accomplished by performing complete NMR assignments on a 40 mg sample of

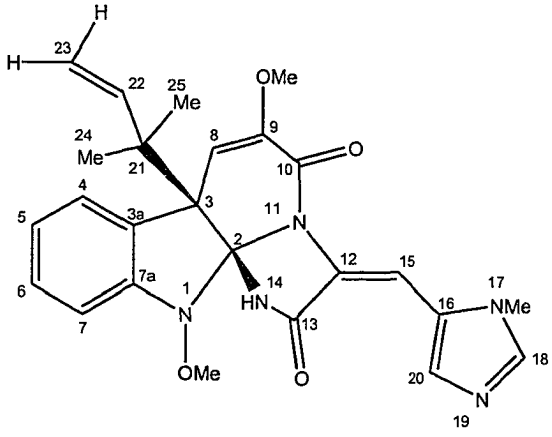
meleagrin, 10 mg samples of meleagrin acetate and N-methyl oxaline and a 5mg sample of N-methyl meleagrin in CD_3CN. The ^1H NMR spectrum of meleagrin was also run at 75 °C with 15 μL of D_2O as an extra step in order to provide the identity of all exchangeable protons (Figure A.17).



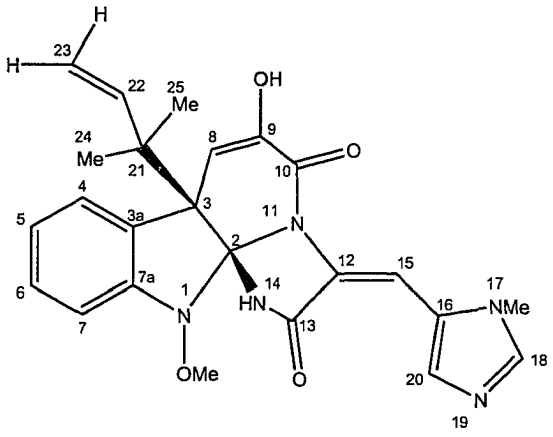
meleagrin acetate



oxaline



N-methyl oxaline



N-methyl meleagrin

Figure 4.11 Structures of the secondary metabolites meleagrin acetate, oxaline, N-methyl meleagrin and N-methyl oxaline.

When the ^1H NMR spectrum of meleagrin was run at 75 °C the ABX system at H-22 and H-23 sharpened up and now appeared as a well resolved doublet at δ 6.28 and as two, two-proton doublets at δ 5.26 (E) and 5.20(Z) respectively (Figure A.17). The same result was seen in the ^1H NMR spectrum of N-methyl meleagrin and N-methyl oxaline as

expected (Figure A.21 and A.23). This data was consistent with reported values, the chemical shifts and coupling constants are shown in (Table 4.9)[49,66]. The carbon spectra of meleagrin clearly indicated one resonance for each of the 23 carbons in the molecule. (Figure A.18).

To ensure assignments, ^{13}C spectra and the standard set of homonuclear (gradient DQF-COSY) and heteronuclear correlations (gradient HMQC and HMBC) were performed on N-methyl oxaline at 500 MHz and the rest were assigned by comparison. (Figure A.26 and A.27). The DQF-COSY for N-methyl oxaline is displayed in Figure A.25. The three isolated spin systems correspond to the aromatic regions (C 4-7), to the allylic side chain (C22-23) and to C 24 and C 25. The O-Me (C 9) singlet can be seen at δ 3.56.

The HMQC spectrum for N-methyl oxaline was used to assign the protonated carbons (Figure A.26). The spectrum clearly shows the correlation for all the protonated carbons for example, it can be seen that the singlet at δ 7.37 is attached to C 20. The gradient HMBC experiment was used to make the non-protonated carbon assignments (Figure A.27). The gradient HMBC confirms ^{1}H/long range interactions for example, the two methyl resonances with protons at δ 1.20 and δ 1.32 show a correlation to C 21 (δ 42.9), C 3 (δ 54.6) and C 22 (δ 144.2) as well as to the expected single bond correlations to δ 23.6 and δ 25.4. Quaternary carbons show correlations to protons that are two and three bonds away. This information was used to provide the correct assignment to C 21.

Table 4.9 ^1H NMR chemical shift assignments for meleagrin (no D_2O added) and comparison of its chemical shifts with meleagrin acetate and N-methyl oxaline.[80,110,49,66]. (500 MHz, CD_3CN, coupling constants in parentheses).

Position #	^1H (meleagrin at 25° C) (ppm from TMS)	^1H (meleagrin at 75° C) (ppm from TMS)	^1H (N-methyl meleagrin) (ppm from TMS	^1H (meleagrin acetate) (ppm from TMS)	^1H (N-methyl oxaline) (ppm from TMS)
4	7.62 (d, J=7.7 Hz,1H)	7.79 (d, J=7.1 Hz,1H)	7.66 (d, J=7.1 Hz,1H)	7.60 (d, J=8.0 Hz,1H)	7.58 (d, J=7.7 Hz,1H)
5	7.08 (t, J=7.6 Hz, 1H)	7.26 (t, J=7.1 Hz, 1H)	7.10 (t, J=7.1 Hz, 1H)	7.10 (t, J=8.0 Hz, 1H)	7.10 (t, J=7.6 Hz, 1H)
6	7.23 (t, J=7.8 Hz, 1H)	7.48 (t, J=7.2 Hz, 1H)	7.32 (t, J=7.2 Hz, 1H)	7.25 (t, J=8.0 Hz, 1H)	7.30 (t, J=7.8 Hz, 1H)
7	7.01 (d, J=7.8 Hz, 1H)	7.19 (d, J=7.8 Hz, 1H)	6.97 (d, J=7.8 Hz, 1H)	7.00 (d, J=8.0 Hz, 1H)	6.97 (d, J=7.8 Hz, 1H)
8	5.47 (s, 1H)	5.66 (s, 1H)	5.37 (s, 1H)	5.45 (s,1H)	5.28 (s, 1H)
14 (NH)	12.59 (br s, 1H)	12.75 (br s, 1H)	12.57 (br s, 1H)	12.72 (s, 1H)	13.12 (br s, 1H)
15	8.23 (s, 1H)	8.42 (s, 1H)	8.37 (s, 1H)	8.22 (s,1H)	8.35 (s, 1H)
18	7.65 (s, 1H)	7.67 (s, 1H)	7.71 (s, 1H)	7.65 (s, 1H)	7.67 (s, 1H)
19 (NH)	6.50 (br s,1H)	6.70 (br s,1H)		9.54 (br s, 1H)	
20	7.31 (s, 1H)	7.52 (s, 1H)		7.30 (s, 1H)	7.37 (s, 1H)
22	6.12 (br s, J=17.3 Hz, J=10.4 Hz, 1H)	6.28 (dt, J=17.3 Hz, J=10.4 Hz, 1H)	6.12 (dt, J=17.3 Hz, J=10.4 Hz, 1H)	6.11 (dd, J=17.3 Hz, J=10.4 Hz, 1H)	6.12 (dd, J=17.3 Hz, J=10.8 Hz, 1H)
23 (E, Z)	5.00 (br d, J=17.4 Hz, J=10.8 Hz, 2H, E) 5.06 (br d, J=17.2 Hz, J=10.2 Hz, 2H, Z)	5.26 (dt, J=17.4 Hz, J=10.8 Hz, 2H, E) 5.20 (dt, J=17.4 Hz, J=10.8 Hz, 2H, Z)	5.03 (br d, J=17.4 Hz, J=10.8 Hz, 2H, E) 5.00 (br d, J=17.2 Hz, J=10.2 Hz, 2H, Z)	5.00 (dd, J=17.3 Hz, J=10.4 Hz, 2H) 5.05 (dd, J=17.3 Hz, J=10.4 Hz, 2H)	5.00 (dd, J=13.4 Hz, J=6.7 Hz, 2H) 5.03 (dd, J=17.3 Hz, J=10.4 Hz, 2H)
24	1.24 (s, 3H)	1.43 (s, 3H)	1.20 (s, 3H)	1.28 (s, 3H)	1.32 (s, 3H)

25	1.29 (s, 3H)	1.48 (s, 3H)	1.31 (s, 3H)	1.23 (s, 3H)	1.20 (s, 3H)
1 (N-OMe)	3.71 (s, 3H)	3.90 (s, 3H)	3.72 (s, 3H)	3.70 (s, 3H)	3.70 (s, 3H)
9 (OH)	7.62 (br s)	7.78 (br s)	7.59 (br s)		
9 (OAc)				2.20 (s, 3H)	
9 (OMe)					3.56 (s, 3H)
17 (N Me)			2.39 (s, 3H)		2.38 (s, 3H)

Table 4.10 ^{13}C NMR chemical shift assignments for meleagrin and comparison of its chemical shifts with meleagrin acetate and N-methyl oxaline[80,110,49,66].

Carbon #	^{13}C (meleagrin) (ppm from TMS)	^{13}C (meleagrin acetate) (ppm from TMS)	^{13}C (N-methyl oxaline) (ppm from TMS)
2	66.0	65.4	65.7
3	53.0	53.4	54.6
4	125.5	125.1	125.3
5	124.4	123.5	124.1
6	129.3	129.1	129.7
7	110.8	110.1	110.9
8	146.9	146.9	147.1
10	165.9	166.5	166.3
12	122.7	122.6	123.7
13	159.7	156.1	158.7
15	112.7	112.0	112.8

16	125.8	125.8	126.3
18	137.7	136.9	138.5
20	135.5	134.5	135.4
21	43.3	42.5	42.9
22	142.3	142.2	144.2
23	114.9	114.5	113.8
24	24.0	23.7	23.6
25	24.1	23.7	25.3
O Ac		168.65	
O Ac Me		20.32	

4.6.2 Roquefortine C

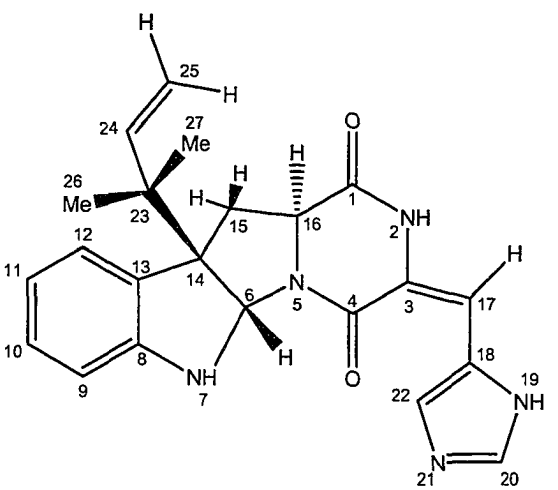


Figure 4.12 Structure of roquefortine C showing numbering

Roquefortine C was purified by recrystallization, twice from MeOH/H_2O and once from MeOH/hexane resulting in approximately 460 mg of fine white crystals. The melting point was determined to be 195-200°C (lit. 195-200°C)[87]. Only one spot was

seen after TLC analysis with an R_f= 0.30 when developed in 10% MeOH/$CHCl_3$. Its purity was confirmed by ^1H NMR.

The molecular formula of roquefortine C was $C_{22}H_{23}N_5O_2$ by HRMS. The characteristic EIMS ions at m/z 389 [M^+], 320 (base peak), 198, 162, 130, 108, and 69 were comparable with literature values (Figure A.28)[96,87].

The IR spectrum showed the presence of the tertiary amide at 1681 cm^{-1} and the N-H functional group at 3197 cm^{-1}. The ^{13}C and ^1H NMR data was in accordance with those recorded in the literature (Table 4.11, Figure A.29 and A.30)[96].

Table 4.11 ^{13}C and ^1H NMR chemical shift assignments for roquefortine C (500 MHz, $CDCl_3$, coupling constants in parentheses).

Position #	^{13}C (ppm from TMS)	^1H (ppm from TMS)
1	166.7	
2		8.97 (br s, NH)
3	121.5	
4	159.4	
5		
6	78.4	5.65 (s, 1H)
7		4.95 (br s, NH)
8	149.8	
9	109.1	6.60 (d, J=7.8 Hz, 1H)
10	129.1	7.10 (dt, J=7.8 Hz, J=1.3 Hz, 1H)

11	119.1	6.78 (dt, J=7.8 Hz, J=1.0 Hz, 1H)
12	125.1	7.20 (dd, J=7.8 Hz, J=1.12 Hz, 1H)
13	128.5	
14	61.5	
15	36.8	2.60 (α) (dd, J=12.4 Hz, J=5.9 Hz, 1H)
		2.50 (*β*) (dd, J=11.5 Hz, J=12.4 Hz, 1H)
16	58.8	4.07 (dd, J=6.0 Hz, J=11.5 Hz, 1H)
17	111.3	6.28 (br s, 1H)
18	125.3	
19		12.97 (br s, 1H)
20	136.8	7.72 (br s, 1H)
21		
22	135.2	7.29 (br s, 1H)
23	40.9	
24	143.3	5.99 (dd, J=11.0 Hz, J=17.2 Hz, 1H)
25	114.7	5.16 (*E*) (dd, J=1.0 Hz, J=10.8 Hz, 1H)
		5.11 (*Z*) (dd, J=0.9 Hz, J=17.4 Hz,1H)
26	22.5	1.14 (s, 3H)
27	22.9	1.01 (s, 3H)

4.7 Metabolites identified from *Penicillium* strains

Seven out of eleven *P. brevicompactum* strains were producers of roquefortine C, four strains produced meleagrin, mycophenolic acid and asperphenamate. Interestingly,

only two strains produced brevianamide A (Table 4.12). Strain DAOM 215331 was the most active strain producing meleagrin, mycophenolic acid, asperphenamate and brevianamide A.

Seventeen out of thirty *P. chrysogenum* strains produced meleagrin, twenty strains produced roquefortine C, fourteen strains were penicillin G producers and ten strains produced xanthocillin x. Ten out of the seventeen strains isolated from building materials produced xanthocillin x (Table 4.13).

Table 4.12 Production of secondary metabolites identified from *P. brevicompactum* strains.

P. brevicompactum	Meleagrin (ug/g/L)	Roquefortine C (ug/g/L)	Mycophenolic acid (ug/g/L)	Asperphenamate (ug/g/L)	Brevianamide A (ug/g/L)
DAOM 215 331	59.82		238.26	5546.35	6732.22
DAOM 215 333		609.75		4546.14	
DAOM 215 334				6025.50	
DAOM 215 335			1499.40	60.96	5875.59
DAOM 234 044		10499.24			
DAOM 234 045	16.87	491.41			
DAOM 234 046	86.70	370.01			
DAOM 234 047	20.85	913.56			
DAOM 234 048		8281.38			
DAOM 234 049			2339.26		
DAOM 234 050		18011.76	176.28		

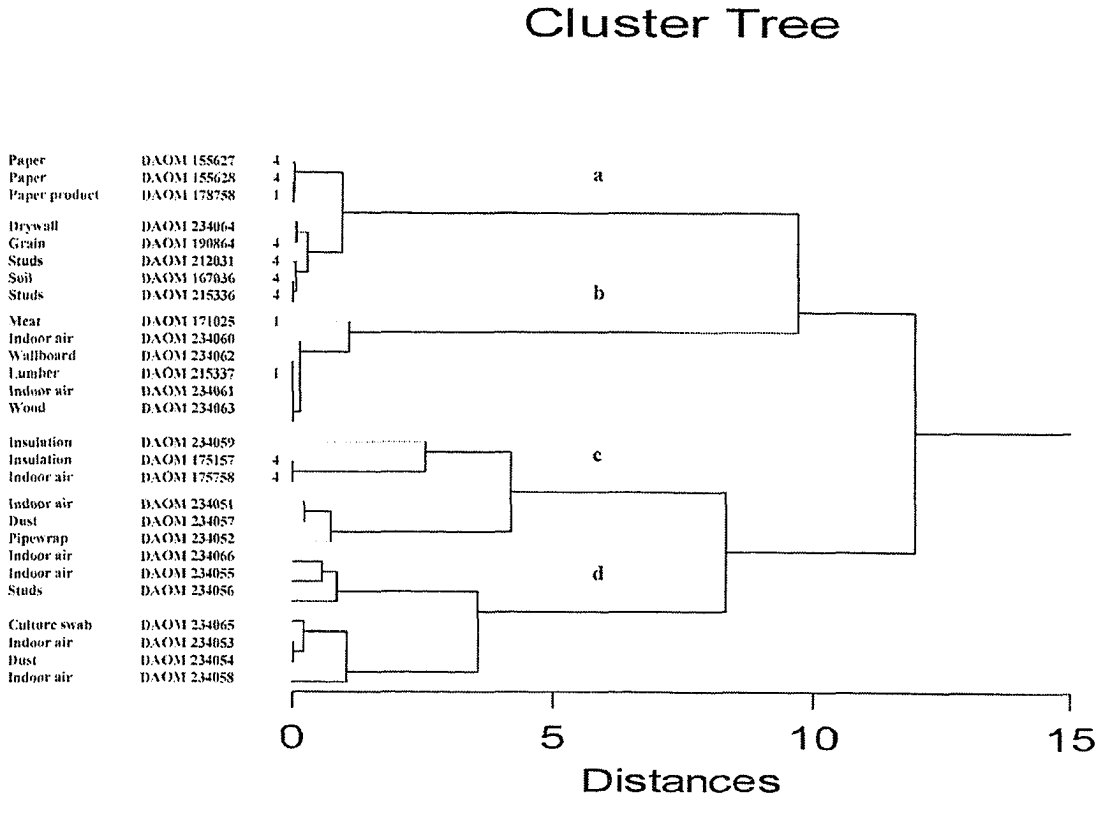
Table 4.13 Production of secondary metabolites identified from *P. chrysogenum* strains.

P. chrysogenum	Meleagrin (ug/g/L)	Roquefortine C (ug/g/L)	Penicillin G (ug/g/L)	Xanthocillin x (ug/g/L)
DAOM 171 025	7.10	938.32	7020.00	
DAOM 178 758	3.90	1.41	1980.10	
DAOM 193 710				
DAOM 215 337		593.82	4644.31	
DAOM 212 031	2.90	6.20		
DAOM 155 627	4.21	1.33		
DAOM 155 628	4.75	1.60	2020.01	
DAOM 167 036	5.52	14.62		
DAOM 175 157	54.00			
DAOM 175 758	11.43		3360.12	
DAOM 190 864	12.70	17.00		
DAOM 215 336	53.74	145.58	4786.26	
DAOM 594 94C				
DAOM 234 051	23.03		4656.11	7.62
DAOM 234 052	298.59		6178.14	426.86
DAOM 234 053		254.64		180.09
DAOM 234 054		297.99		232.73
DAOM 234 055	136.69	550.67	5137.53	106.50
DAOM 234 056	15.65	767.06	5789.32	120.48
DAOM 234 057	107.41		7028.06	123.42
DAOM 234 058			9672.23	
DAOM 234 059		5.01	12732.72	115.20
DAOM 234 060			25361.34	
DAOM 234 061	0.11	152.34		
DAOM 234 062		87.58	17652.24	
DAOM 234 063		65.41		
DAOM 234 064		14.49		
DAOM 234 065	57.55	51.51		
DAOM 234 066		251.16		77.09
DAOM 234 067	139.03	41.98		147.18

4.8 Cluster analysis

Cluster analysis produced only one tree and had a cophenetic correlation of 0.87227. (Figure 4.13). The resulting dendrogram shows two distinct, main clusters or clades and four minor clusters (a-d). A clade is a group of biological taxa that includes all descendants of one or more ancestor. Little or no variation was detected after HPLC-DAD analysis, as almost all the isolates seemed to produce a similar profile of secondary metabolites. The two main clusters divided according to the presence of xanthocillin x. Strains in clade (a) produced roquefortine C and meleagrin in equal amounts but no

xanthocillin x was detected. Strains in clade (b) produced roquefortine C, a trace of meleagrin but again no xanthocillin x was detected. Strains in clade (c) produced roquefortine C or meleagrin together with xanthocillin x. Strains in clade (d) produced roquefortine C, meleagrin and xanthocillin x in equal amounts (Figure 4.13a). Sixty-seven percent of isolates in clades a and b produced penicillin G and 37% of clades c and d were producers of penicillin G. Strains DAOM 171025, DAOM 234060, DAOM 234062, DAOM 215337, DAOM 234061 and DAOM 234063 accumulated roquefortine C exclusively.



1 and 4 refer to the clade classification by Scott et al[86]. The numbers show how these strains were distributed in our cluster analysis.

Figure 4.13 *P. chrysogenum* strain dendogram after cluster analysis.

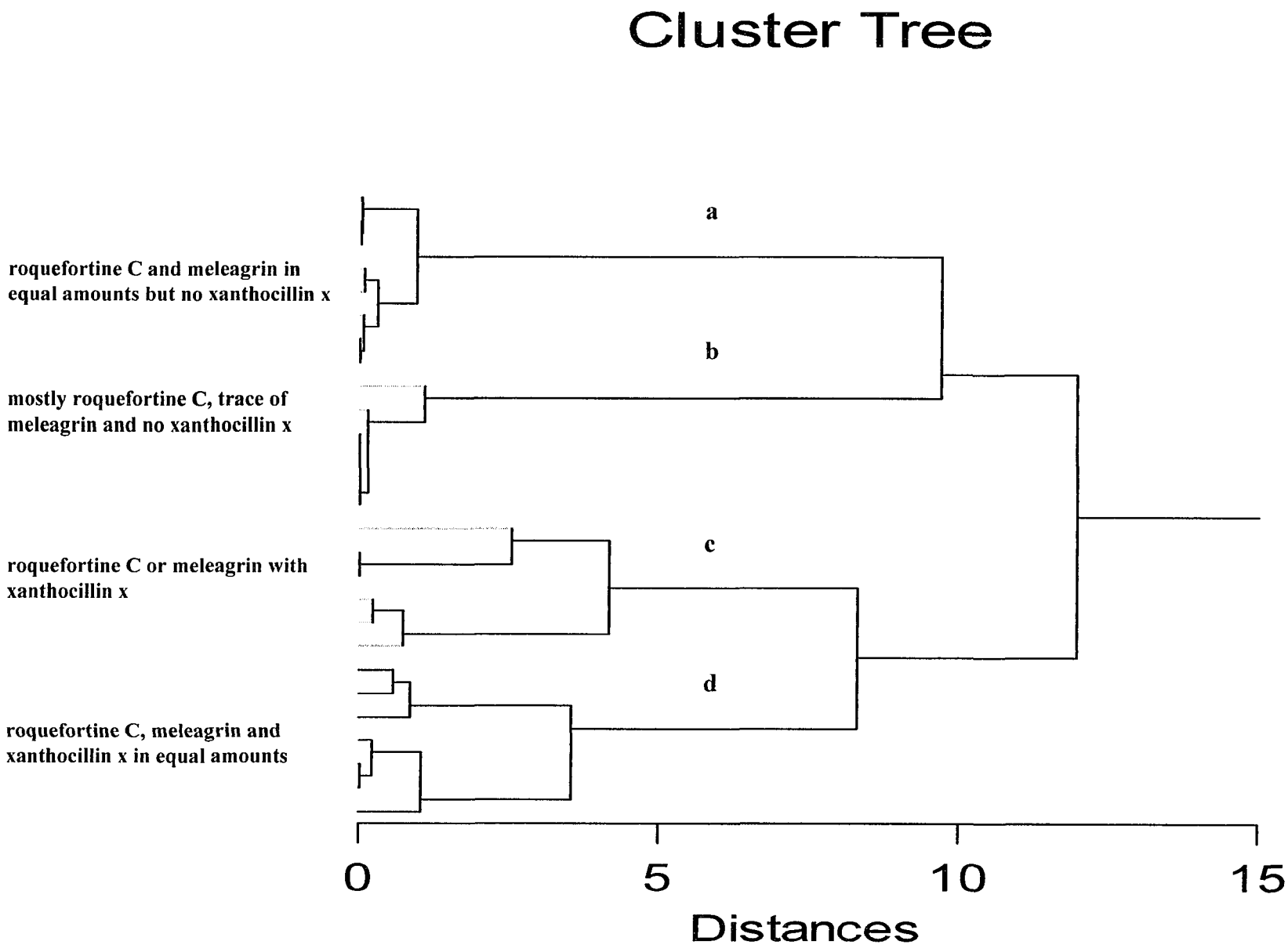


Figure 4.13a *P. chrysogenum* strain dendogram after cluster analysis.

5. DISCUSSION

5.1 Fungal strains

P. brevicompactum and *P. chrysogenum* are among the most widely encountered *Penicillium* species growing indoors but can also be found outdoors. With the new understanding that building associated strains of fungi might represent different populations from those of the same species encountered outdoors, it was important to obtain strains that had been isolated from indoor environments and building materials. The building associated Canadian strains were isolated and used together with thirteen strains isolated from wood, lumber, paper and food. These thirteen strains had been previously studied by Scott *et al.* (2004) and were separated into 4 clades according to genetic variation (Figure 4.13). These were included in the experimental set as a comparison to biotypes of the indoor isolates and also to investigate the relationship between their chemotaxonomic profiles.

5.2 Culture growth

It has been demonstrated that the pattern of secondary metabolites synthesized by particular strains of fungi may depend on geographical location[50]. It was also important to choose a medium that would provide the necessary nutrients and result in cell growth without inhibiting the production of metabolites.

Different types of media have been used for growth, taxonomic comparison and identification of *Penicillium* species for many years. In 1910, Charles Thom studied the cultural variability of *Penicillium*, and pointed to the morphological individuality of the genera as compared to isolates of other species. In addition, he explained how this individuality is expressed uniquely on each different medium employed. He stressed the importance of having media with ingredients that were consistent from batch to batch and

explained how even small differences in these ingredients could lead to significantly different results[98]. The Czapek/Dox formula was first used by Thom in 1930. This is a completely synthetic medium that utilizes sucrose and sodium nitrate as the sources of carbon and nitrogen. Although it lacks trace elements considered necessary for fungal growth, most *Penicillium* species are able to grow and reproduce on it. This media has remained popular over the years since Raper and Thom used it for their extensive works on this species. In the early 1940's, scientists at the USDA introduced the addition of corn-steep liquor to the Czapek/Dox formula, a by-product of cornstarch manufacture, to provide the cultures with an extra source of nitrogen[78]. When a number of nitrogen sources were investigated for the growth of *Fusarium oxysporum*, Cheilas et al. (2000) reported that enzymatic activity was generally higher when organic nitrogen sources were used in the media. Of all the organic nitrogen sources they tested, the highest activity was observed with corn-steep liquor[28].

Czapek/Dox/1% corn steep liquor was the best medium for the growth of *P. brevicompactum* and *P. chrysogenum* isolates, as determined by the dry weight of the mycelium. During a preliminary trial, it was observed that the fungal biomass decreased if the cultures were incubated for more than two weeks at 25 °C. It was first hypothesized that since secondary metabolism begins after nutrients utilized during primary metabolism become limited, incubating the cultures for a longer period of time might increase secondary metabolite production. However, this was not the case as the biomass deteriorated rapidly after 15 days without any significant increase in the amount of secondary metabolites present.

Roux bottles were chosen for the experiment to help increase the metabolite yields by providing low but changing oxygen tension[96]. Maintenance of adequate levels of O_2, usually measured as dissolved oxygen, can often be critical to the success of the fermentation. There have been numerous studies investigating the effects of dissolved oxygen on fungal bioprocesses. Generally, in these studies moderately enhanced levels of O_2 supply resulted in improvement in growth, product formation and acceptable morphological changes, while the negative impact of higher levels of dissolved oxygen on morphology and product synthesis were generally assumed to be a consequence of oxidative stress[9]. It was also important to keep the cultures in the dark since the sensitivity to light of some secondary metabolites such as roquefortine C and the brevianamides has been previously reported[96,113].

5.3 Identification of secondary metabolites

Culture filtrates were extracted in ethyl acetate, chloroform and in the case of penicillin, in butyl acetate. The crude extracts were screened by HPLC-DAD and LC-MS in order to determine the best extraction solvent. It was seen that, as expected, chloroform extracted mostly non-polar compounds including ergosterol and triterpenoids. Ethyl acetate resulted in extracts that consisted of low molecular weight polar secondary metabolites. Butyl acetate was a good solvent for the extraction of penicillin, as confirmed later during recovery studies.

The results of several surveys of Canadian lumber in the 1990's revealed that *P. brevicompactum* is one of the most common inhabitants of the sapwood of conifer lumber in North America and it is often reported in indoor air[89]. *P. brevicompactum* strains have been reported to produce mycophenolic acid, brevianamide A, B and F, asperphenamate, pebrolide and botryodiploidin in indoor environments. Most of the

building associated strains of *P. brevicompactum* isolated from Canadian homes in this study produced roquefortine C, mycophenolic acid or meleagrin but only the wood strains produced significant amounts of asperphenamate and brevianamide A.

P. chrysogenum strains have been reported to produce roquefortine C, meleagrin, chrysogine and penicillin G in water-damaged buildings. Most of the building associated strains of *P. chrysogenum* isolated from Canadian homes in this study produced penicillin G, roquefortine C, meleagrin and xanthocillin x. This finding has not previously been reported in the literature.

5.4 Meleagrin

The biosynthetic pathway for the transformation of roquefortine C into oxaline with meleagrin as an intermediate has been proposed by several groups[92,79]. According to Samson *et al.*, *P. confertum* is the only *Penicillium* species reported to produce meleagrin with no apparent accumulation of either roquefortine C or oxaline[43]. Roquefortine C is produced by *P. crustosum, P. griseofulvum, P. expansum, P. roqueforti, P. carneum, P. paneum*[43]. In addition, several species are known to produce both meleagrin and roquefortine C and these include *P. chrysogenum, P. flavigenum, P. glandicola, P. vulpinum* and *P. concentricum*[43]. The only species reported to accumulate all three metabolites is *P. atramentosum* (Figure 4.14)[43].

Among the strains tested in our study, some were mostly meleagrin producers (DAOM 175758, DAOM 175157, DAOM 234051, DAOM 178758, DAOM 155628, DAOM 155627), some were mostly roquefortine C producers (DAOM 234061, DAOM 171025, DAOM 234056, DAOM 234066, DAOM 234056) and some strains produced both in relatively equal amounts (DAOM 234065 and DAOM 190864) (Figure 4.13). The effect of dry mycelium weight on the accumulation of secondary metabolites was taken

into consideration. It was found that there was no significant relationship between the biomass and the amount of secondary metabolites produced.

A search of the literature revealed a discrepancy with regard to the published ^{1}H NMR data and the stereochemistry of meleagrin. Derivatization of meleagrin into meleagrin acetate, N-methyl meleagrin and N-methyl oxaline was therefore necessary in order to validate its structure. The synthetic derivative meleagrin acetate was prepared upon acetylation with acetic anhydride and N-methyl oxaline was prepared upon methylation with diazomethane. N-methyl meleagrin was expected as the major product of the methylation reaction as it was reported by Kawai et al. (1984)[49]. However, N-methyl meleagrin was only formed as a minor product during this reaction. Interestingly, methylation of meleagrin with CH_2N_2 by Kawai *et. al.*, resulted in the formation of N-methyl meleagrin and not N-methyl oxaline[49]. This is thought to be due to the higher reactivity of the imidazole ring (pKa ~7) compared to the enol (pKa~10) and amide moieties (pKa ~15). Figure 5.1 shows the correct structure and stereochemistry of meleagrin.

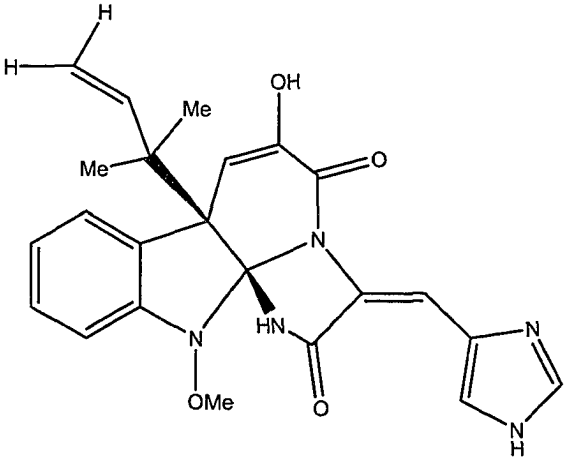


Figure 5.1 Structure and stereochemistry of meleagrin.

Table 4.14 Production of roquefortine C, meleagrin and oxaline by different *Penicillium* species with respect to their classification by series and section[43].

Secondary metabolite	Species	Series	Section
meleagrin only	*P. confertum*	*Mononematosa*	*Chrysogena*
roquefortine C only	*P. crustosum*	*Camemberti*	*Viridicata*
	P. griseofulvum	*Urticicolae*	*Penicillium*
	P. expansum	*Expansa*	*Penicillium*
	P. roqueforti	*Roqueforti*	*Roqueforti*
	P. carneum	*Roqueforti*	*Roqueforti*
	P. paneum	*Roqueforti*	*Roqueforti*
meleagrin and roquefortine C	*P. chrysogenum*	*Chrysogena*	*Chrysogena*
	P. flavigenum	*Chrysogena*	*Chrysogena*
	P. glandicola	*Claviformia*	*Penicillium*
	P. vulpinum	*Claviformia*	*Penicillium*
	P. concentricum	*Claviformia*	*Penicillium*
meleagrin, roquefortine C and oxaline	*P. atramentosum*	*Camemberti*	*Viridicata*

4.5 Toxicity studies

P. brevicompactum strains DAOM 215331 and DAOM 215335 were identified as strong producers of brevianamide A, mycophenolic acid, asperphenamate and a Raistrick phenol derivative. These strains were chosen for large scale fermentation for the isolation of these metabolites to be used for studies of the toxigenic potential of building-associated fungi and to investigate their biological activity. Building-associated *P.*

chrysogenum strains DAOM 234067 and DAOM 234065 were identified as good producers of roquefortine C and meleagrin. This species had not previously been considered to produce major secondary metabolites but it was shown that this view might need to be altered as most of the indoor strains were producers of roquefortine C and penicillin G.

Brevianamide A, mycophenolic acid and roquefortine C were identified, isolated and purified by means of several chromatographic methods. For the toxicology studies the documentation of the purity of these compounds is of outmost importance. Establishing the purity of the samples during the isolation process is quite difficult, especially when the retention times are similar for two desired peaks during HPLC analysis. HPLC coupled with MS and/or NMR offer effective ways of obtaining purity information about the samples.

Brevianamide A was isolated as a yellow solid with a melting point of 200-212°C (lit. 190-220°C)[14]. Mycophenolic acid was isolated as white crystals; melting point 141-142°C (lit. 139-141°C)[35]. Roquefortine C was obtained as white crystals, melting point 195-200°C (lit. 195-200°C)[87]. For all three compounds, ^{13}C and ^{1}H NMR spectra, optical rotations and the IR maxima were similar to the literature values (see Results section). The purity based on ^{1}H NMR, optical rotation and melting point was assessed at >98%.

Rand *et al.* (2005) conducted the toxicity experiments using brevianamide A, mycophenolic acid and roquefortine C isolated from the indoor strains of *P. brevicompactum* and *P. chrysogenum* in this study[77]. They were able to show that these three compounds induced a transient dose and time dependent inflammatory response expressed as differentially elevated and persistent macrophage, neutrophil, macrophage

inflammatory protein-2, tumor necrosis factor-α and interleukin-6 concentrations in the bronchioalveolar lavage fluid (BALF) of intratracheally exposed mice[77]. In addition, brevianamide A and mycophenolic acid induced cytotoxicity evident as significantly increased lactose dehydrogenase concentration in mouse BALF at 250nM/animal and at 6 and 24 hours post instillation[77]. The toxins also stimulated vascular leakage, observed as increased BALF albumin concentrations in lungs of moderately and high dosed animals[77]. The results demonstrated that these toxins result in differing inflammatory and/or cytotoxic response patterns. This is important because it indicates that differences in the lung inflammatory response patterns stimulated by toxin exposures might prove to be significant in the development of biomarkers of exposure.

4.6 Cluster analysis

It has been estimated that approximately one hundred genes may be involved in the production of a single secondary metabolite[24]. Characters that result in variations are in some cases the final expression of relatively large biosynthetic pathways. These pathways contain a number of control and regulation genes and so a relatively small alteration of the genome can significantly alter secondary metabolite production[23]. In this study, the secondary metabolites which showed variation are derived via different metabolic routes. Penicillin G is a β-lactam derived from aminoadipic acid, cysteine and valine. Roquefortine C and meleagrin are derived from dimethylallylpyrophosphate, tryptophan and histidine. Xanthocillin x is formed from tyrosine from the Shikimic acid pathway[26].

Scott *et al.*, studied the phylogenetic relationships of species in the *P. chrysogenum* group using isolates collected from houses in Wallaceburg, Ontario[86]. The Wallaceburg study investigated the relationship between house construction and

operating characteristics, condensation on interior surfaces, biological contamination and health factors[52]. The phylogenetic relationships were inferred from an analysis of partial sequences of the β-tubulin gene.

Statistical analysis by Scott *et al.*, revealed two lineages and four well-supported clades among the strains tested. Clade 1 included strains DAOM 215337, DAOM 171025 and DAOM 178623 and eleven isolates (5%) from Wallaceburg house dust. Clade 2, a sister of clade 1, contained two Wallaceburg isolates. Clade 3 included six Wallaceburg isolates. Clade 4 included the majority of the Wallaceburg isolates (96%) along with the British strain isolated by Alexander Fleming (NRRL 824) and thus related to this known penicillin producer. The study showed that *P. chrysogenum* strains from clade 1 are uncommon in the indoor dust microbiota relative to isolates from clade 4. In addition, Scott *et al.* suggested the creation of new taxa to accommodate the different lineages observed based on the phylogenetic positions of the major penicillin-producing strains[86].

However, in contrast to the study by Scott *et al.*, our indoor isolates and penicillin-producing strains were evenly distributed throughout the experimental set (Figure 4.13). The production of penicillin G from strains growing on water-damaged building materials had not been previously reported. The penicillin producing strains DAOM 234052, DAOM 234056, DAOM 234059 and DAOM 234062 were isolated from pipewrap, wallboard and insulating material (Figure 4.13).

The results of our study do not support the view that *P. chrysogenum* should be split into new species. It can be observed that genetics have the potential to give a clear classification of the organism, however since large parts of the genome can be quite similar within some genera it is necessary that the right genes are chosen carefully or that

a sufficiently large part of the genome is sequenced to give a usable delimitation of the different fungal species[82]. Taxonomy of this species based on a polyphasic approach of a large number of isolates should be pursued.

6. Conclusions

The chemotypes of *P. brevicompactum* and *P. chrysogenum* fungal strains isolated from of water-damaged buildings were determined. It was apparent that the strains were consistent in their production of known secondary metabolites irrespective of geographic or environmental origin. This predictability may be of considerable importance in the built environment. In addition, the structure and stereochemistry of meleagrin were confirmed, after a search of the literature revealed a discrepancy with regard to the published NMR data of this compound.

It was established that *P. chrysogenum* isolates associated with indoor environments or present in building materials are potential penicillin producers. Allergic reactions can be triggered by these compounds and thus presence of penicillin or penicillin-like compounds is not desirable in indoor environments. However, taxonomy of this species based on a polyphasic approach of a larger number of isolates should be pursued to confirm this view.

In addition, the major secondary metabolites produced by these species were isolated and clear evidence of their purity was presented. These compounds were submitted to test their toxigenic potential in intratracheally instilled mice.

7. Going Forward

There is a need to isolate, identify and screen more *Penicillium* isolates growing in water-damaged buildings. This will not only provide more information on the identity and frequency of species occurring indoors, but also on the secondary metabolites that they produce. Additionally, water-damaged building materials should be tested for the presence of secondary metabolites. This will help in the determination of the differences between the metabolites present in raw materials versus those grown in the laboratory on synthetic media. Furthermore, there is a need for more toxicological testing so that associations can be made between these metabolites and the health of the building occupants.

Reference List

1. Clearing the air: asthma and indoor air exposures. Institute of Medicine. 2000. Washington, D.C., National Academy Press.

2. Adams, E., Todd, G., and Gibson, W. Long term toxicity study of mycophenolic acid in rabbits. Toxicology and Applied Pharmacology 34[3], 509-512. 1975.

3. Andersen, B. Consistent production of phenolic compounds by *Penicillium brevicompactum* for chemotaxonomic characterization. Antonie van Leeuwenhoek 60[2], 115-123. 1991.

4. Andersen, B., Nielsen, K. F., Thrane, U., Szaro, T., Taylor, J. W., and Jarvis, B. B. Molecular and phenotypic descriptions of *Stachybotrys chlorohalonata* sp. nov. and two chemotypes of *Stachybotrys chartarum* found in water-damaged buildings. Mycologia 95[6], 1227-1238. 2003.

5. Ando, K., Susuki, S., Tamura, G., and Arima, K. Antiviral and antitumor antibiotics. IV. Antiviral activity of mycophenolic acid. Journal of Antibiotics 21, 649-652. 1968.

6. Anne, J. Comparison of penicillins produced by inter-species hybrids from Penicillium chrysogenum. European Journal of Applied Microbiology and Biotechnology 15, 41-46. 1982.

7. Arnold, D. L., Scott, P. M., McGuire, P. F., Hawig, J., and Nera, E. A. Acute toxicity studies on roquefortine and PR toxin, metabolites of *Penicillium roqueforti* in the mouse. Food and Cosmetics Toxicology 16, 369-371. 1978.

8. Ayer, W. A., Browen, L. M., Feng, M. C., Orszanska, H., and Saeedi Ghomi, H. The chemistry of the blue stain fungi. Part 1. Some metabolites of *Ceratocystis* species associated with mountain pine beetle infected lodgepole pine. Canadian Jounal of Chemistry 64, 904-909. 1986.

9. Bai, Z, Harvey, LM, and McNeil, B. Oxidative stress in submerged cultures of fungi. Critical Reviews Biotechnology 23[4], 267-302. 2003.

10. Bernhoft, A., Keblys, M., Höfer, C. C., Morrison, E., and Larsen, H. J. S. Combined effects of selected *Penicillium* mycotoxins on in vitro proliferation of porcine lymphocytes. Mycopathologia 158[3], 317-324. 2004.

11. Besl, H. and Bresinsky, A. 1997. Chemosystematics of *Suillaceae* and *Gomphidiaceae* (suborder Suillineae). Plant Systematics and Evolution 206, 223-242. 2005.

12. Birch, A. J. and Russell, R. A. Biosynthesis. XLIV. Structural elucidations of brevianamides B, C, D, and F . Tetrahedron 28, 2999-3008. 1972.

13. Birch, A. J. and Wright, J. J. The brevianamides: a new class of fungal alkaloid. Journal of the Chemical Society.Chemical communications , 644-645. 1969.

14. Birch, A. J. and Wright, J. J. Studies in relation to biosynthesis. XLII. The structural elucidation and some aspects of the biosynthesis of the brevianamides A and E. Tetrahedron 26, 2329-2344. 1970.

15. Bird, B. A. and Campbell, I. M. Phytochemistry 21[9], 2405-2406. 1982.

16. Birkinshaw, J. H., Bracken, A., Morgan, E. N., and Raistrick, H. Biochemistry of micro.ovrddot.organisms. LXXVIII. Molecular constitution of mycophenolic acid, a metabolic product of Penicillium brevi-compactum Dierckx. 2. Possible structural formulas for mycophenolic acid. Biochemical Journal 43, 216-223. 1948.

17. Blackwell, B. A., Miller, J. D., and Greenhalgh, R. ^{13}C NMR study of the biosynthesis of toxins by *Fusarium graminearum*. The Journal of Biological Chemistry 260[7], 4243-4247. 1985.

18. Blaiss MS. and Erffmeyer JE. Proving penicillin allergy. Postgraduate Medicine 87[2], 33-35. 1990.

19. Bondy, G. S. and Peska, J. J. Immunomodulation by fungal toxins. Journal of Toxicology and Environmental Health B Crit Reviews 3, 109-143. 2000.

20. Bowen, L., Clifford, K. H., and Phillips, G. T. Biosynthesis of mycophenolic acid. The synthesis of 6-farnesyl-5,7-dihydroxy-4-methylphthalide in cell-free

preparation from *Penicillium brevicompactum*. Journal of the Chemical Society.Chemical communications , 949-951. 1977.

21. Boysen, M, Skouboe, P., Frisvad, J. C., and Rossen, L. Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles. Microbiology 142, 541-549. 1996.

22. Bridge, P. D., Hawksworth, D. L., Kozakiewicz, Z., Onions, A. H. S., and Paterson, R. R. M. Morphological and biochemical variation in single isolates of *Penicillium*. Transactions of the British Mycological Society 87, 389-396. 1986.

23. Bridge, P. D., Hawksworth, D. L., Kozakiewicz, Z., Onions, A. H. S., and Paterson, R. R. M. Investigation of variation in phenotype and DNA content between single-conidium isolates of single *Penicillium* strains. Journal of General Microbiology 133, 995-1004. 1987.

24. Bu'lock, J. D. Mycotoxins as secondary metabolites. Steyn, P. S. The biosynthesis of mycotoxins: a study in secondary metabolism. 1-16. 1980. New York, Academic Press.

25. Burnett, J. Fungal populations and species. 2003. Oxford, UK, Oxford University Press.

26. Cable, K. M., Herbert, R. B., Knaggs, A. R., and Mann, J. The biosynthesis of xanthocillin x monomethyl ether in Dichotomomyces cejpii. Experiments on the origin of the isocyanide carbon atoms. Journal of the Chemical Society.Perkin Transactions 1 , 595-599. 1991.

27. Carter, S. B., Franklin, T. J., Jones, D. F., Leonard, B. J., Mills, S. D., Turner, R. W., and Turner, W. B. Mycophenolic acid: an anti-cancer compound with unusual properties. Nature 223, 848-850. 1969.

28. Cheilas, T., Stoupis T., Christakopoulos P., Katapodis P., Mamma D., Hatzinikolaou D.G., Kekos D., and Macris B.J. Hemicellulolytic activity of *Fusarium oxysporum* grown on sugar beet pulp. Production of extracellular arabinase. Process Biochemistry 35, 557-561. 2000.

29. Christophersen, C. Theory of the origin, function and evolution of secondary metabolites. Atta-ur-Rahman. Studies in natural products chemistry 18

Stereoselective synthesis (part K). 677-737. 1996. Amsterdam, Elsevier.

30. Ciegler, A., Fennell, D. I., Sansing, G. A., Detroy, R. W., and Bennett, R. H. Mycotoxin producing strains of *Penicillium viridicatum*: classification into subgroups. Applied Microbiology [26], 271-278. 1973.

31. Clark, A. M. and Hufford, C. D. Phytochemistry 17, 552-553. 1978.

32. Claydon, N. and Grove, J. F. and Hosken M. Phenolic metabolites of *Ceratocystis ulmi*. Phytochemistry 13, 2567-2571. 1974.

33. Clutterbuck, P. W, Oxford, A. E., Raistrick, H., and Smith, G. The metabolic products of the *Penicillium brevicompactum* series. Biochemical Journal 26, 1441-1458. 1932.

34. Coetzer, J. The structure and absolute configuration of 5-bromobrevianamide A. Acta Crystallographica B30, 2254-2256. 1974.

35. Danheiser, R. L., Gee, S. K., and Perez, J. J. Total synthesis of mycophenolic acid. Journal of the American Chemical Society 108, 806-810. 1986.

36. David-Neto, E., Pereira Araujo, L., Sumita Nairo, M., Mendes, M. E., Ribeiro Castro, M. C., Alves, C., Kakehashi, E., Romano, P., Yagyu, E. M., Queiroga, M., Nahas, W. C., and Ianhez, L. E. Mycophenolic acid pharmacokinetics in stable pediatric renal transplantation . Pediatric nephrology 18[3], 266-272. 2003.

37. Elander, R. P. Industrial production of beta-lactam antibiotics. Applied Microbiology and Biotechnology 61[5-6], 385-392. 2003.

38. Esch, R. E., Hartsell, C. J., and Crenshaw, R. Common allergenic pollens, fungi, animals and arthropods. Clinical Reviews in Allergy and Immunology 21, 261-292. 2001.

39. Frank, H. K., Orth, R., Ivankovic, S., Kuhlmann, M., and Schmahl, D. Investigations on carcinogenic effects of *Penicillium caseicolum* and *P. roqueforti* in rats. Experentia 33, 515-516. 1977.

40. Frisvad, J. C. and Filtenborg, O. Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites. Applied and Environmental Microbiology 46, 1301-1310. 1983.

41. Frisvad, J. C. and Filtenborg, O. Terverticillate Penicillia: chemotaxonomy and mycotoxin production. Mycologia 81[6], 837-861. 1989.

42. Frisvad, J. C. and Filtenborg, O. Secondary metabolites as consistent criteria in *Penicillium* taxonomy and a synoptic key to *Penicillium* subgenus *Penicillium*. R.A.Samson and J.I.Pitt. Modern concepts in *Penicillium* and *Aspergillus* classification. 373-384. 1990. New York, Plenum Press.

43. Frisvad, J. C. and Samson, R. A. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium* a guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. Studies in Mycology 49, 1-173. 2004.

44. Frisvad, J. C., Smedsgaard, J., Larsen, T. O., and Samson, R. A. Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. Studies in Mycology 49, 201-242. 2004.

45. Hocking, A. D. and Pitt, J. I. Water relations of some Penicillium species at 25°C. Transactions of the British Mycological Society 73, 141-145. 1979.

46. Hooker, A. L. Cultural variability in Septoria avenae through successive single-macrospore transfers. Phytopathology 47, 460-468. 1957.

47. Jarvis, B. B. Chemistry and toxicology of molds isolated from water-damaged buildings. Advances in Experimental and Medical Biology 504, 43-52. 2002.

48. Kameda, K., Aoki, H., Tanaka, H., and Namikin, M. Studies on metabolites of Alternaria kikuchiana Tanaka, a phytopathogenic fungus of Japanese pear. Agricultural and Biological Chemistry 37, 2137-2146. 1973.

49. Kawai, K., Nozawa, K., Nakajima, S., and Iitaka, Y. Studies on fungal products. VII. The structures of meleagrin and 9-O-p-bromobenzoylmeleagrin. Chemical Pharmaceutical Bulletin 32[1], 94-98. 1984.

50. Kozlovskii AG, Zhelifonova VP, Adanin VM, Antipova TV, Shnyreva AV, and Viktorov AN. The biosynthesis of low-molecular-weight nitrogen-containing secondary metabolite-alkaloids by the resident strains of *Penicillium chrysogenum* and *Penicillium expansum* isolated on the board of the Mir space station. Mikrobiologiia 71[6], 773-777. 2002.
Ref Type: Generic

51. Laich, F., Fierro, F., and Martin, J. F. Production of penicillin by fungi growing on food products: identification of a complete penicillin gene cluster in *Penicillium griseofulvum* and a truncated cluster in *Penicillium verrucosum*. Applied and Environmental Microbiology 68[3], 1211-1219. 2002.

52. Lawton, M. D., Dales, R. E., and White, J. The influence of house characteristics in a Canadian community on microbiological contamination. Indoor air 8, 2-11. 1998.

53. Lewis, F. A. Regulating indoor microbes: the OSHA proposed rule on IAQ. International conference on fungi and bacteria in indoor air environments. Eastern New York Occupational Health Program. 5-9. 1995. Saratoga Springs, New York.

54. Logrieco, A, Peterson, S. W., and Wicklow, D. T. Ribosomal RNA comparisons among taxa of the terverticillate penicillia. Samson, R. A. and Pitt, J. I. Modern concepts in *Pencillium* and *Aspergillus* systematics. 343-355. 1990. New York, Plenum Press.

55. Mantle, P G. Secondary metabolites of *Penicillium* and *Acremonium*. Peberdy, J. F. *Penicillium* and *Acremonium*. 161-243. 1987. New York, London, Plenum Press.

56. Marinari, R., Fleischmajer, R., Schragger, A. H., and Rosenthal, A. L. Mycophenolic acid in the treatment of psoriasis: long-term administration. Archives in Dermatology 113[7], 930-932. 1977.

57. McCorkindale, N. J., Baxter, R. L., Roy, T. P., Shields, H. S., Stewart, R. M., and Hutchinson, S. A. Synthesis and chemistry of N-benzoyl-O-[N'-benzoyl-L-phenylalanyl]-L-phenylalaninol, the major mycelial metabolite of Penicillium canadense. Tetrahedron 34, 2791-2795. 1978.

58. Miller, J. D. and Day, J. D. Indoor mold exposure: epidemiology, consequences and immunothapy. Canadian Journal of Allergy & Clinical Immunology 2, 25-32.

1997.

59. Miller, J. D., Gilbert, N. L., and Dales, R. E. Fungal contamination in public buildings: health effects and investigation methods. Health Canada . 2004.

60. Miller, J. D. and Greenhalgh, R. Trichothecene chemotypes of three *Fusarium* species. Mycologia 83[2], 121-130. 1991.

61. Miller, J. D., Laflamme, A. M., Sobol, Y., Lafontaine, P., and Greenhalgh, R. Fungi and fungal products in some Canadian houses. International Biodeterioration 24[2], 103-120. 1988.

62. Nielsen, K. F. Mycotoxin production by indoor molds. Fungal Genetics and Biology 39, 103-117. 2003.

63. Nielsen, K. F., Gravesen, S., Nielsen, P. A., Andersen, B., Thrane, U., and Frisvad, J. C. Production of mycotoxins on artificially and naturally infested building materials. Mycopathologia 145[1], 43-56. 1999.

64. Nielsen, K. F. and Smedsgaard, J. Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardized liquid chromatography-UV-mass spectrometry methodology. Journal of Chromatography A 1002, 111-136. 2003.

65. Nielsen, K. F., Smedsgaard, J., Larsen, T. O., Lund, F., Thrane, U, and Frisvad, J. C. Chemical identification of fungi: metabolite profiling and metabolomics. Fungal biotechnology in agricultural, food and environmental applications. 19-35. 2004. New York, Marcel Dekker.

66. Nozawa, K. and Nakajima, S. Isolation of a radicicol from *Penicillium luteo-aurantium*, and meleagrin, a new metabolite, from *Penicillium meleagrinum*. Journal of Natural Products , 374-377. 1979.

67. Ohmomo, S., Sato, T., Utagawa, T., and Matazo, A. Isolation of festuclavine and three new indole alkaloids, roquefortine A, B and C from the cultures of Penicillium roqueforti. Journal of the Agricultural Chemistry Society of Japan 49, 615-623. 1975.

68. Ominski, K. H., Marquardt, R. R., and Sinha, R. N. Ecological aspects of growth and mycotoxin production by storage fungi. Miller, J. D. and Treholm, H. L. Mycotoxins in Grain. Compounds other than aflatoxin. 287-312. 1997. St. Paul, Minnesota, Eagan Press.

69. Peberdy, J. F. *Penicillium* and *Acremonium*. Biotechnology Handbooks. 1, 114-116. 1987. New York and London, Plenum Press.

70. Perelmutter, L. and Liakopoulou, A. Production of rat homocytotropic antibodies using low dose, long term oral exposure to penicillin G. Acta Allergologica 30[5], 250-258. 1975.

71. Peterson, S. W. Molecular genetic assessment of relatedness of Penicillium subgenus Penicillium. Reynolds, D. R. and Taylor, J. W. The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. 121-128. 1993. Wallingford, CAB International.

72. Pitt, J. I. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. 1979. London, Academic Press.

73. Pitt, J. I. *Penicillium crustosum* and *Penicillium simplicissimum*, the correct names for two common species producing tremorgenic mycotoxins. Mycologia 71, 1166-1177. 1979.

74. Pitt, J. I. A laboratory guide to common *Penicillium* species. 2000. North Ryde, NSW, Food Science Australia.

75. Pitt, J. I. and Cruickshank, R. H. Speciation and synonymy in *Penicillium* subgenus *Penicillium*- towards a definitive taxonomy. Samson, R. A. and Pitt, J. I. Modern concepts in *Penicillium* and *Aspergillus* classification. [New York], 103-119. 1990. Plenum Press.

76. Planterose, D. N. Antiviral and cytotoxic effects of mycophenolic acid. Journal of General Virology 4[4], 629-630. 1969.

77. Rand, T. G., Flemming, J., Giles, S., Miller, J. D., and Puniani, E. Inflammatory and cytotoxic responses in mouse lungs exposed to purified toxins from building isolated *Penicillium brevicompactum* Dierckx and *P. chrysogenum* Thom. in

press. 2005.

78. Raper, K. B. and Thom, C. A manual of the Penicillia. 1949. Baltimore, Williams and Wilkins.

79. Reshetilova, T. A., Vinokurova, N. G., Khmelenina, V. N., and Kozlovskii, A. G. The role of roquefortine in the synthesis of alkaloids, meleagrine, glandicolines A and B, and oxaline in *Penicillum glandicola* and *P. atramentosum*. Mikrobiologiya 64[1], 36-39. 1995.

80. Richard, J. C., Jarvis, B. B., and Schweikert, M. A. Handbook of Secondary Fungal Metabolites, Vol. I. 172-173. 2003. California, Academic Press.

81. Rundberget, T. and Wilkins, A. L. Determination of *Penicillium* mycotoxins in foods and feeds using liquid chromatography-mass spectrometry. Journal of Chromatography A 964[1-2], 189-197. 2002.

82. Samson, R. A., Seifert, K. A., Kuijpers, A. F. A., Houbraken, J. A. M. P., and Frisvad, J. C. Phylogenetic analysis of Penicillium subgenus Penicillium using partial ß-tubulin sequences. *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. Studies in Mycology 49, 175-200. 2004.

83. Samson, R. A., Stolk, A. C., and Hadlock, R. Revision of the subsection fesciculata of *Penicillium* and some allied species. Studies in Mycology 11, 1-47. 1976. Baarn.

84. Schoch, U., Luthy, J., and Schlatter, C. Subchronic toxicity testing of mold ripened cheese. Z Lebensm Unters Forsch 179, 99-103. 1984.

85. Schoch, U., Luthy, J., and Schlatter, C. Mycotoxins in mold-ripened cheese. Mitt Gebiete Lebensmitteluntersuchung und Hygiene 74, 50-59. 1983.

86. Scott, J., Untereiner, W. A., Wong, B., Straus, N. A., and Malloch, D. Genotypic variation in *Penicillium chrysogenum* from indoor environments. Mycologia 96[5], 1096-1105. 2004.

87. Scott, P. M. Mycotoxins Production, Isolation, Separation and Purification; Roquefortine. Betina, V. 463-468. 1984. Amsterdam, Elsevier.

88. Scott, P. M. and Kennedy, B. P. C. Analysis of blue cheese for roquefortine and other alkaloids from Penicillium roqueforti. Journal of Agricultural and Food Chemistry 24[4], 865-868. 1976.

89. Seifert, K. A. and Frisvad, J. C. *Penicillium* on solid wood products. Integration of modern taxonomic methods for *Penicillium* and *Aspergillus*. 285-298. 2000. Amsterdam, Hardwood academic.

90. Seifert, K. A. and Levesque, C. A. Phylogeny and molecular diagnosis of mycotoxigenic fungi. European Journal of Plant Pathology 110, 449-471. 2004.

91. Smedsgaard, J and Nielsen.J. Metabolite profiling of fungi and yeast: from phenotype to metabolome by MS and informatics. Journal of Experimental Botany 56[410], 273-286. 2005.

92. Steyn, P. S. The biosynthesis of mycotoxins. Revue de Médicine vétérinaire 149[6], 469-478. 1998.

93. Steyn, P. S. and Vleggaar, R. Roquefortine, an intermediate in the biosynthesis of oxaline in cultures of *Penicillium oxalicum*. Journal of the Chemical Society, Chemical Communications , 560-561. 1983.

94. Stockburger, D. W. Multivariate Statistics: Concepts, Models, and Applications. David W.Stockburger. 1996. Missouri.

95. Stolk, A. C., Samson, R. A., Frisvad, J. C., and Filtenborg, O. The systematics of the terverticillate Penicillia. Samson, R. A. and Pitt, J. I. Modern concepts in *Penicillium* and *Aspergillus* classification. 121-136. 1990. New York, Plenum Press.

96. Sumarah, M. Fermentation, biosynthesis and identification of secondary metabolites from Penicillium species. Master of Science Thesis. 2002. Ottawa, Carleton University.

97. Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S., and Fisher, M. C. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31, 21-32. 2000.

98. Thom, C. Cultural studies of species of *Penicillium*. Bulletin Bur Anim Ind US Department of Agriculture 118, 109. 1910.

99. Torssell and K.B.G. Natural product chemistry. 1983. New York, John Wiley and Sons Ltd.

100. Tuller G, Armbruster G, Wiedenmann S, Hanichen T, Schams D, and Bauer J. Occurrence of roquefortine in silage - toxicological relevance to sheep. Journal of Animal Physiology and Animal Nutrition 80, 246-249. 1998.

101. Turner, W. B. and Aldridge, D. C. Fungal metabolites II. 1983. New York, Academic Press.

102. Uchida, K., Watanabe, H., Usui, T., Osada, H., and Kitahara, T. Synthesis and bioactivities of acetophthalidin and its derivatives. Heterocycles 48[10], 2049-2060. 1998.

103. Ueno, Y. and Ueno, I. Toxicology and biochemistry of mycotoxins. Toxicology, Biochemistry and Pathology of Mycotoxins. 1978. Tokyo, Halstead Press.

104. ur Rahman, L., Ikenaga, T., and Kitamura, Y. Penicillin derivatives induce chemical structure-dependent root development and application for plant transformation. Plant cell reports 22, 668-677. 2004.

105. Velluti, A., Marin, S., Bettuci, L., and Ramos, A. J. The effect of fungal competition and on colonization of maize grain by *Fusarium moniliforme*, *F. proliferatum* and *F. graminearum* and on fumonisin B1 and zearalenone formation. International Journal of Food and Microbiology 59, 59-66. 2000.

106. Vesely, D. and Vesela, D. Use of chick embryos for prediction of embryotoxic effects of mycotoxins in mammals. Veterinary Medicine (Praha) 36[3], 175-181. 1991.

107. Wagner, R. E., Davis, N. D., and Diener, U. L. Penitrem A and roquefortine production by *Penicillium commune*. Applied and Environmental Microbiology 39, 882-887. 1980.

108. Wehner, F. C., Thiel, P. G., Van Rensburg, S. J., and Demasius, I. C. Mutagenicity to Salmonella typhimurium of some *Aspergillus* and *Penicillium* mycotoxins. Mutation Research 58[2-3], 193-203. 1978.

109. Wicklow, D. T. The fungal community. Its organization and role in the ecosystem. 1981. New York, Marcel Dekker.

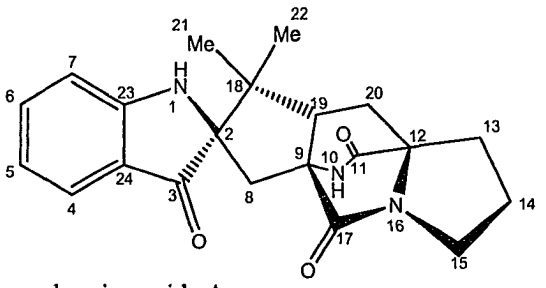
110. Williams, R. M, Stocking, E. M., and Sanz-Cervera, J. F. Biosynthesis of prenylated alkaloids derived from tryptophan. Leeper, F. J. and Vederas, J. C. Topics in Current Chemistry. 209, 97-173. 2000. Berlin, Heidelberg, Springer-Verlag.

111. Williams, R. M., Glinka, T., Kwast, E., Coffman, H., and Stille, J. K. Asymmetric, stereocontrolled total synthesis of (-)-brevianamide B. Journal of the American Chemical Society 112, 808-821. 1990.

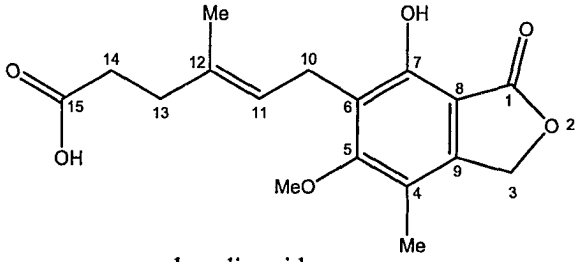
112. Wilson, B. J., Hoekman, T., and Dettbarn, W. D. Effects of a fungus tremorgenic toxin (penitrem A) on transmission on rat phrenic nerve-diaphragm preparations. Brain Research 40, 540-544. 1972.

113. Wilson, B. J., Yang, D. T., and Harris, T. M. Production, isolation and preliminary toxicity studies of brevianamide A from cultures of *Penicillium viridicatum*. Applied Microbiology 26[4], 633-635. 1973.

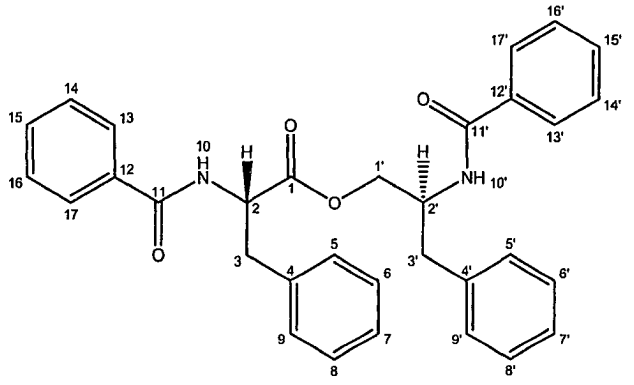
Appendix A
Structures and Spectra



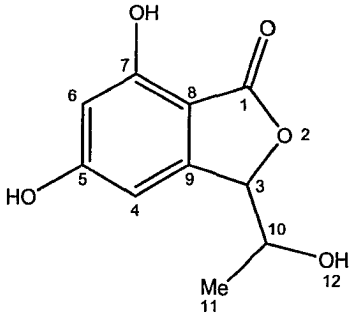
brevianamide A



mycophenolic acid



asperphenamate



Raistrick phenol derivative

Figure A.1 Structures of the secondary metabolites brevianamide A, mycophenolic acid, asperphenamate and a Raistrick phenol derivative, isolated from *P. brevicompactum* strains DAOM 215331 and DAOM 215335.

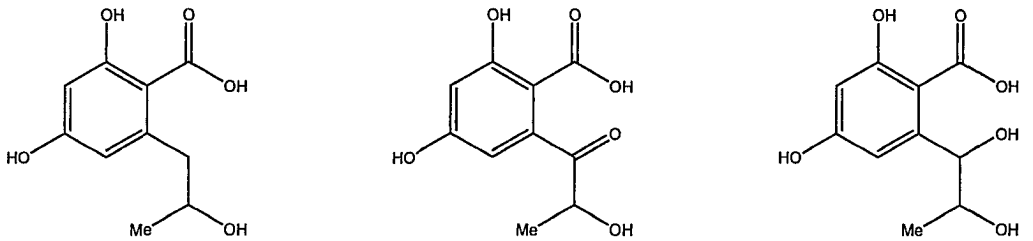
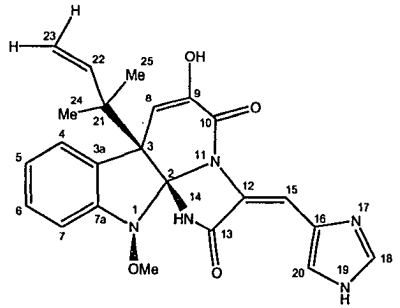
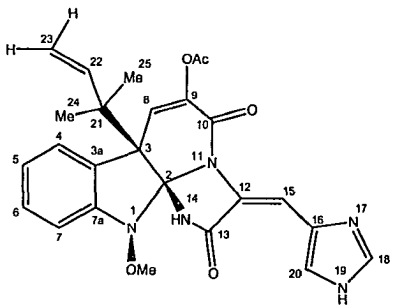


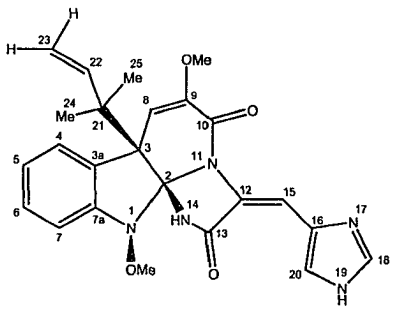
Figure A.2 Structures of the Raistrick phenols3.



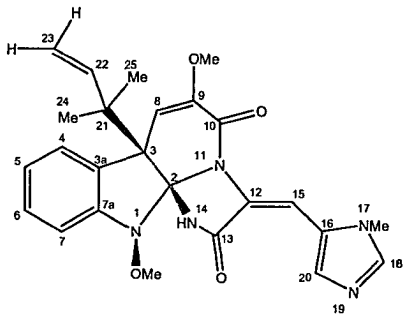
meleagrin



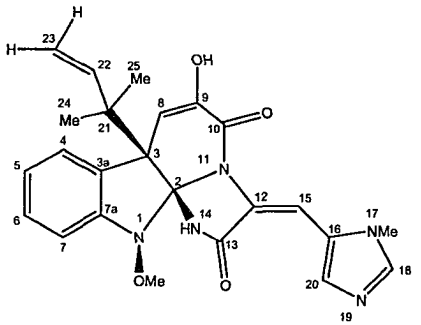
meleagrin acetate



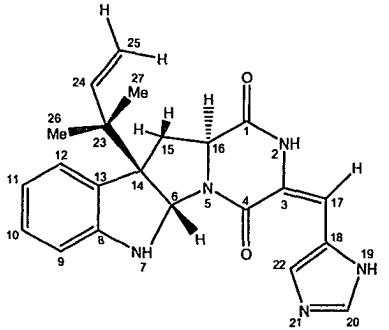
oxaline



N-methyl oxaline



N-methyl meleagrin



roquefortine C

Figure A.3 Structures of the secondary metabolites meleagrin, meleagrin acetate, oxaline, N-methyl oxaline, N-methyl meleagrin, roquefortine C isolated from P. chrysogenum strains DAOM 234065 and DAOM 234067.

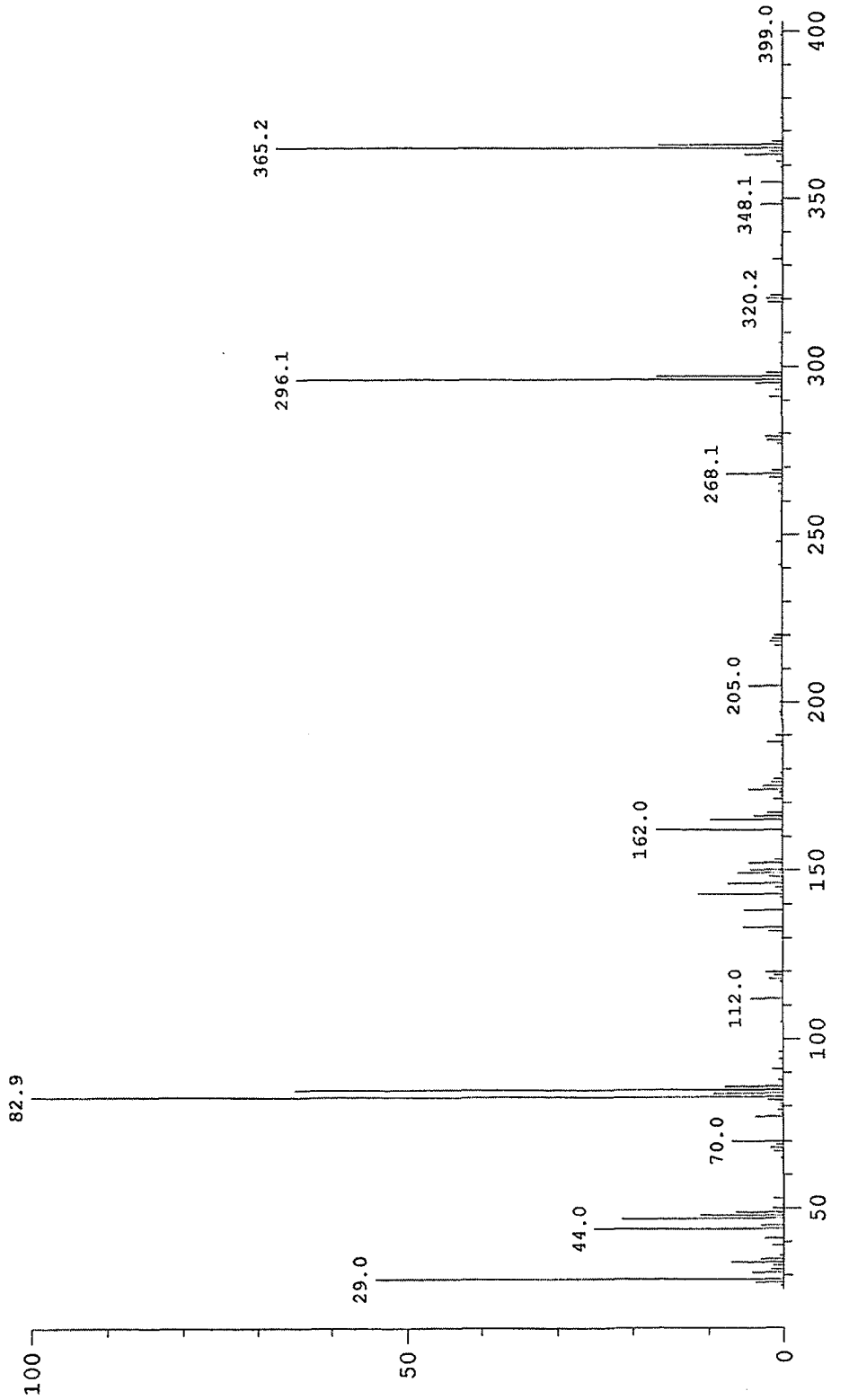


Figure A.4 EIMS/MS of brevianamide A indicating parent 365 (M+1) and daughter ions.

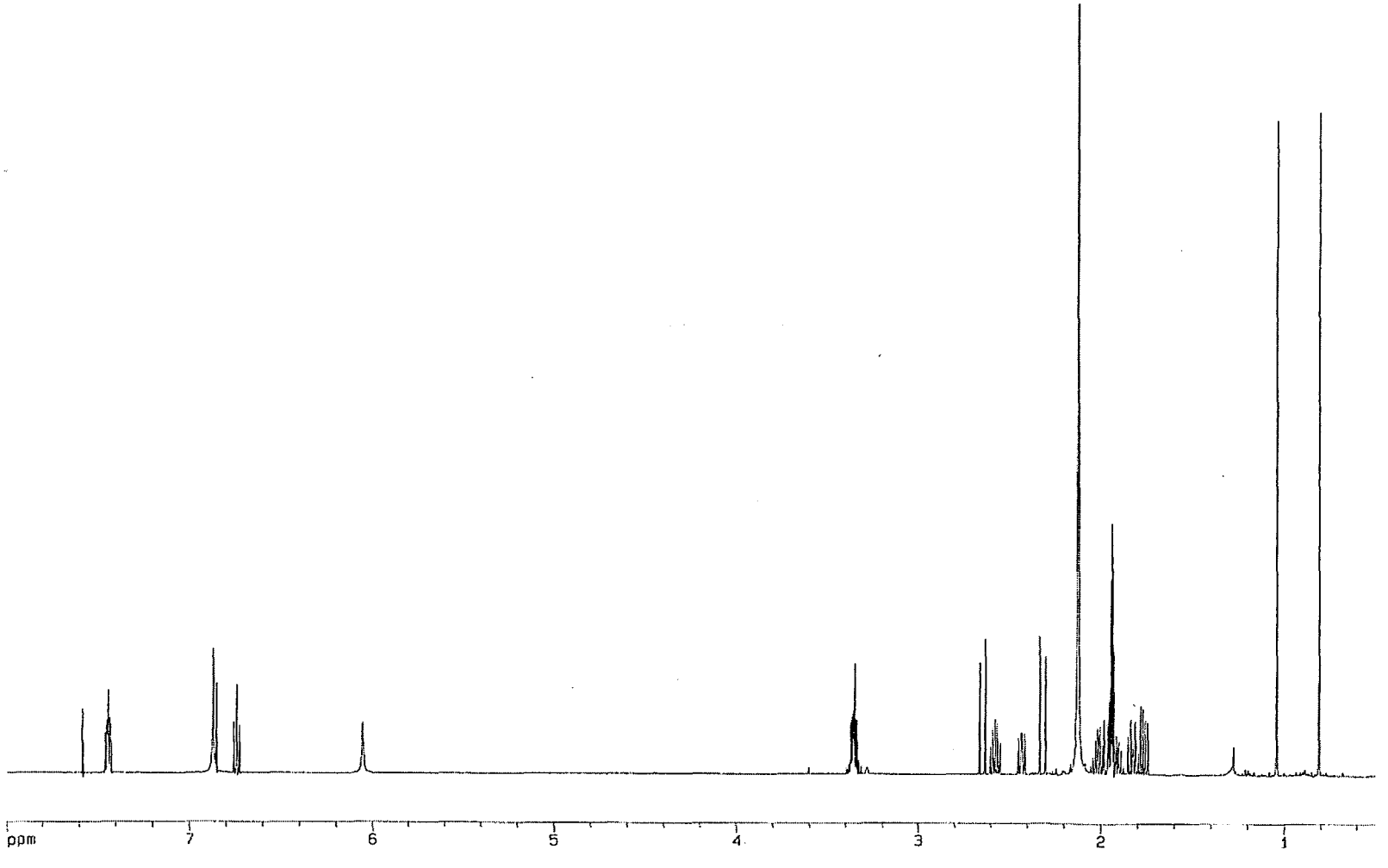


Figure A.5 1H NMR spectrum of brevianamide A in CD3CN (500 MHz).

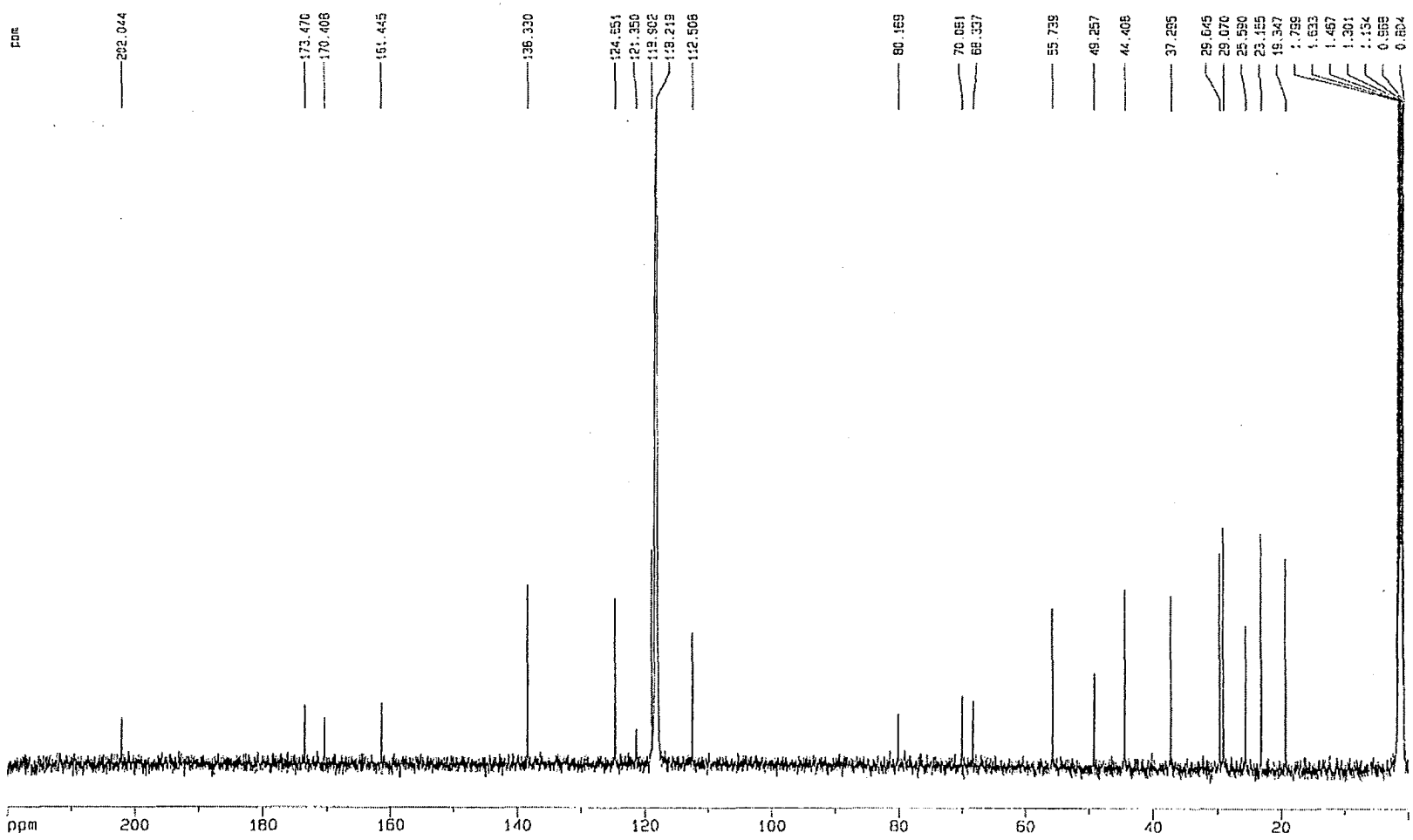


Figure A.6 ^{13}C NMR spectrum of brevianamide A in CD_3CN (500 MHz).

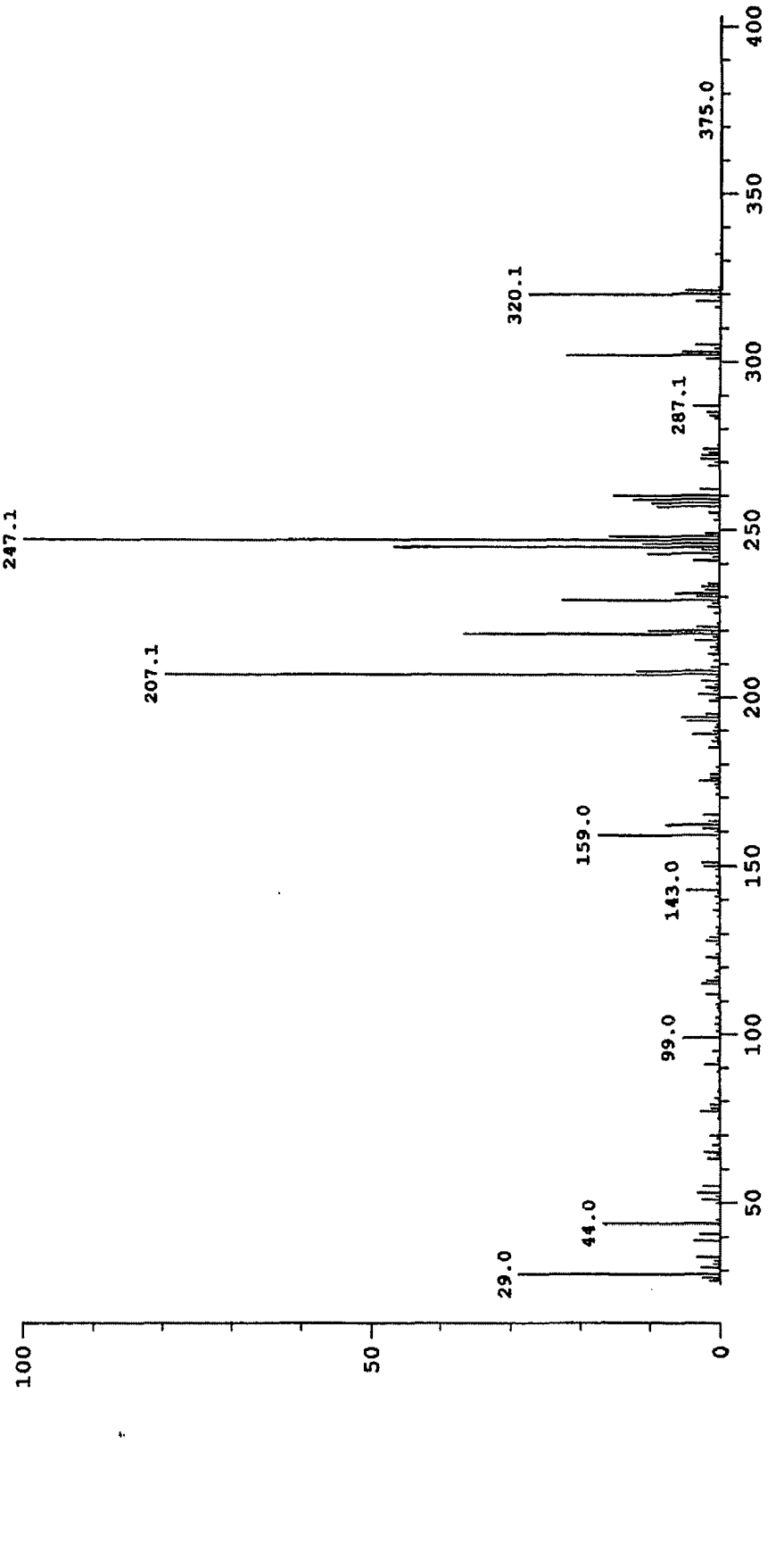


Figure A.7 EIMS/MS of mycophenolic acid indicating parent 320 (M+1) and daughter ions.

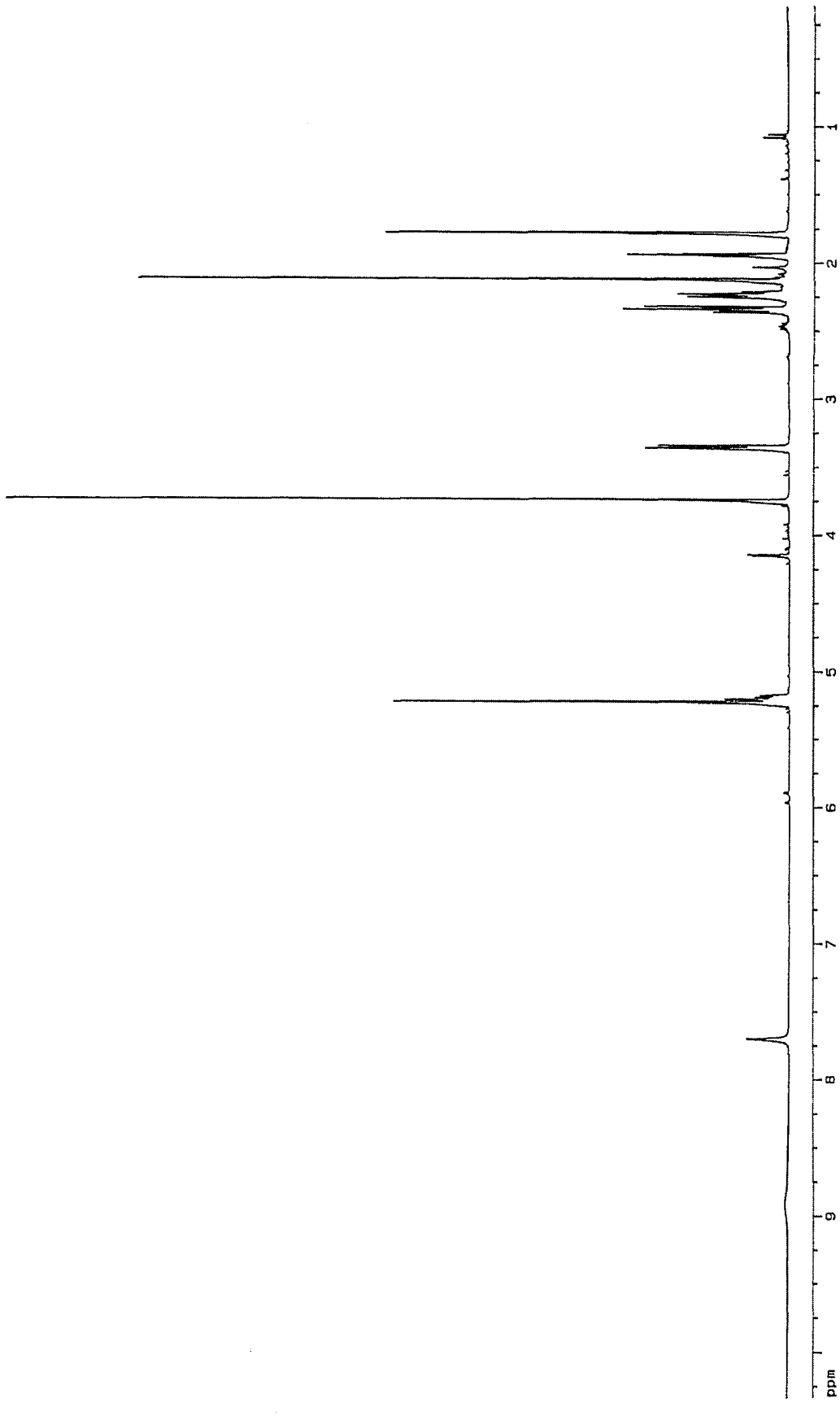


Figure A.8 ^{1}H NMR spectrum of mycophenolic acid in CD_3CN (200 MHz).

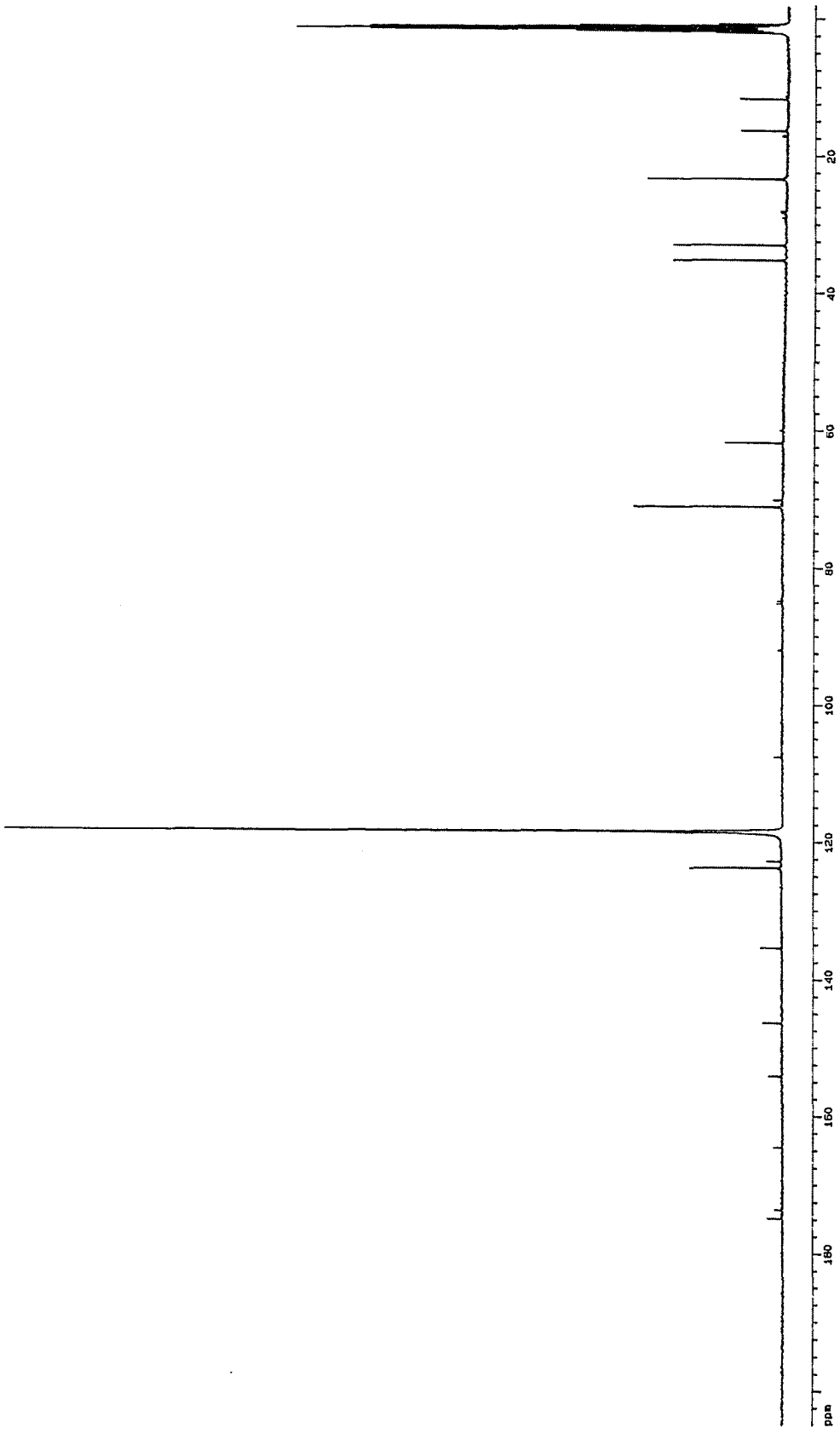


Figure A.9 ^{13}C NMR spectrum for mycophenolic acid in CD_3CN (200 MHz).

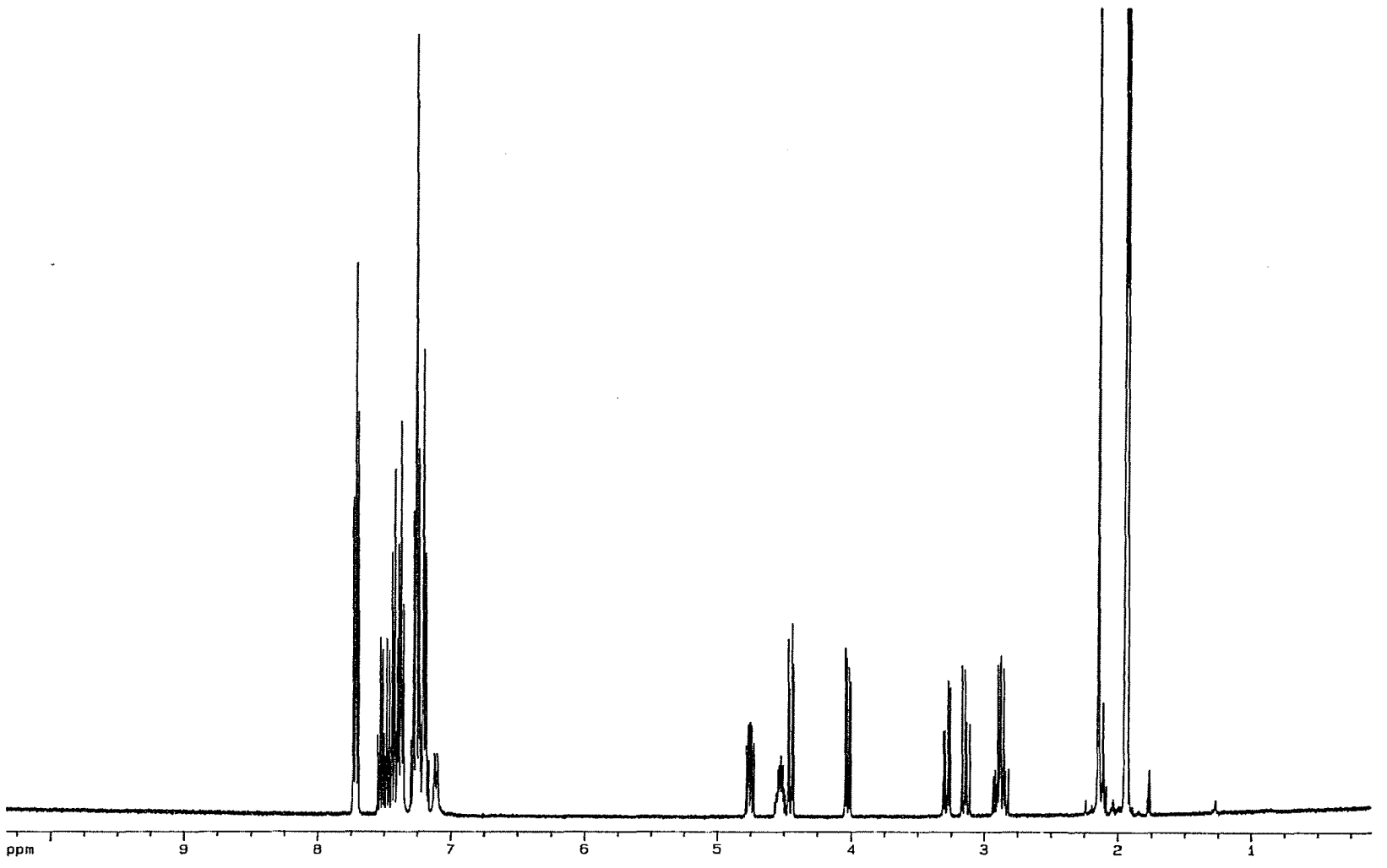


Figure A.10 ^{1}H NMR spectrum of asperphenamate in CD_3CN (200 MHz).

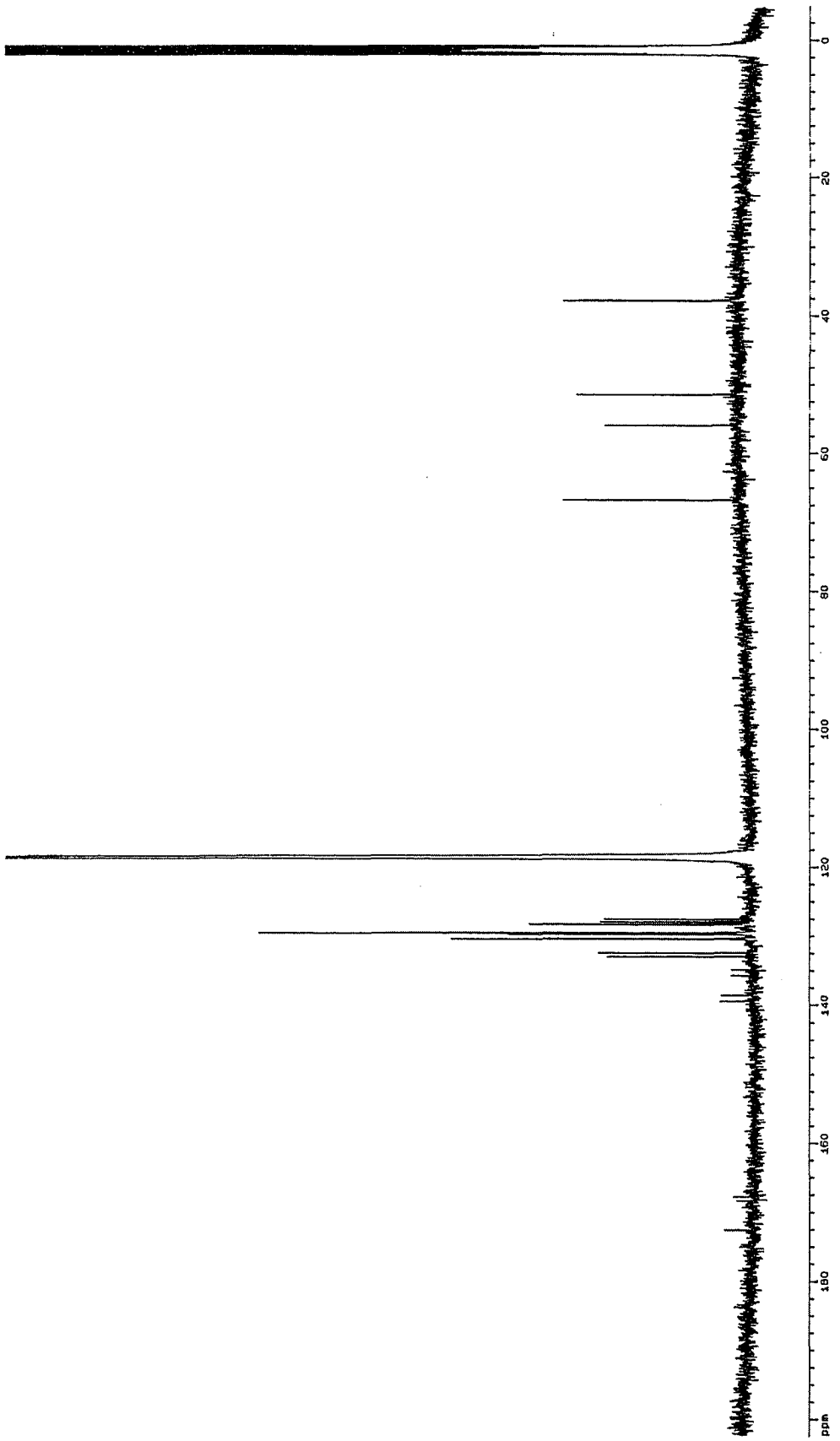


Figure A.11 ^{13}C NMR spectrum of asperphenamate in CD_3CN (200 MHz).

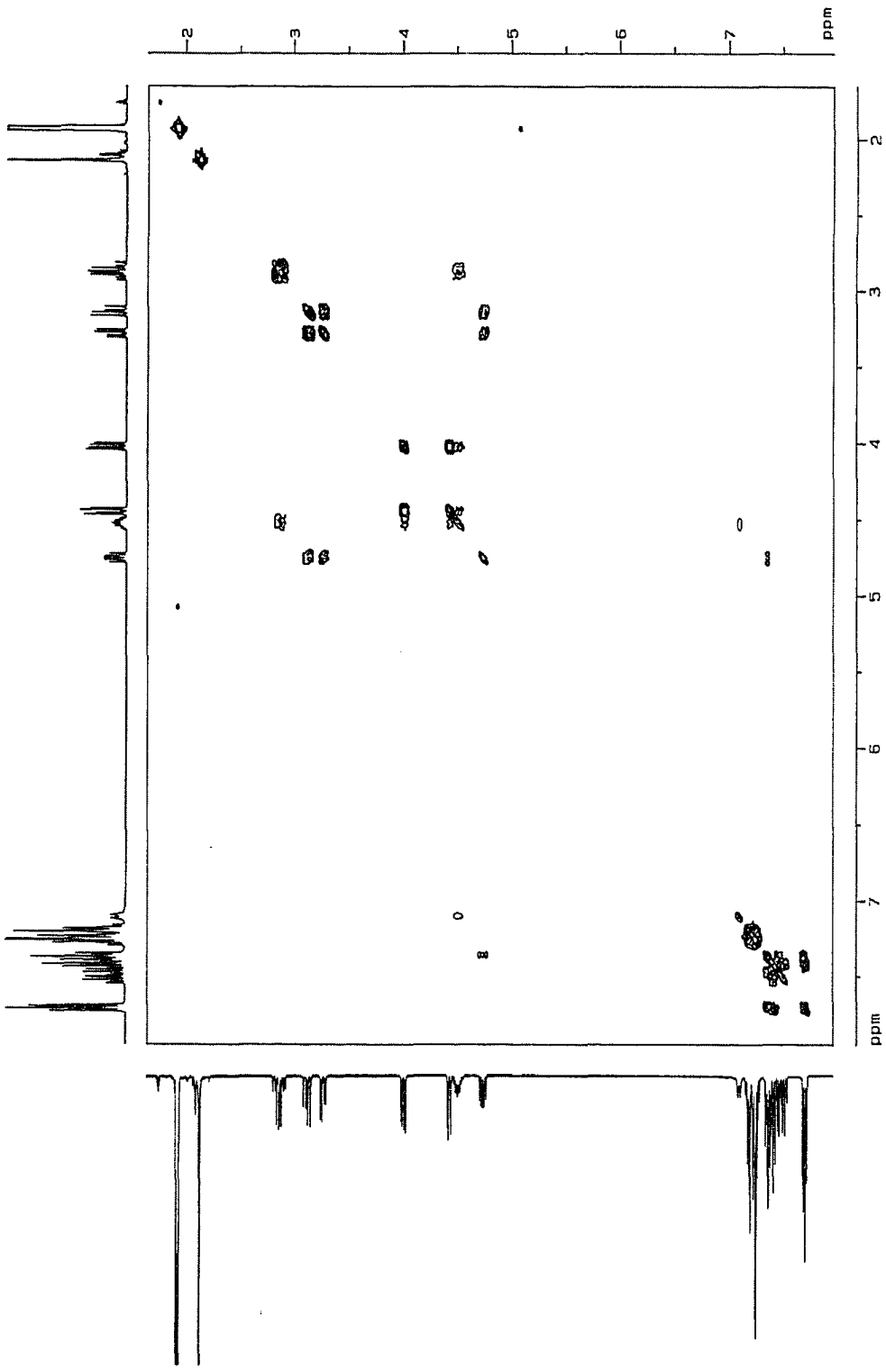


Figure A.12 Gradient DQF-COSY of asperphenamate (200 MHz).

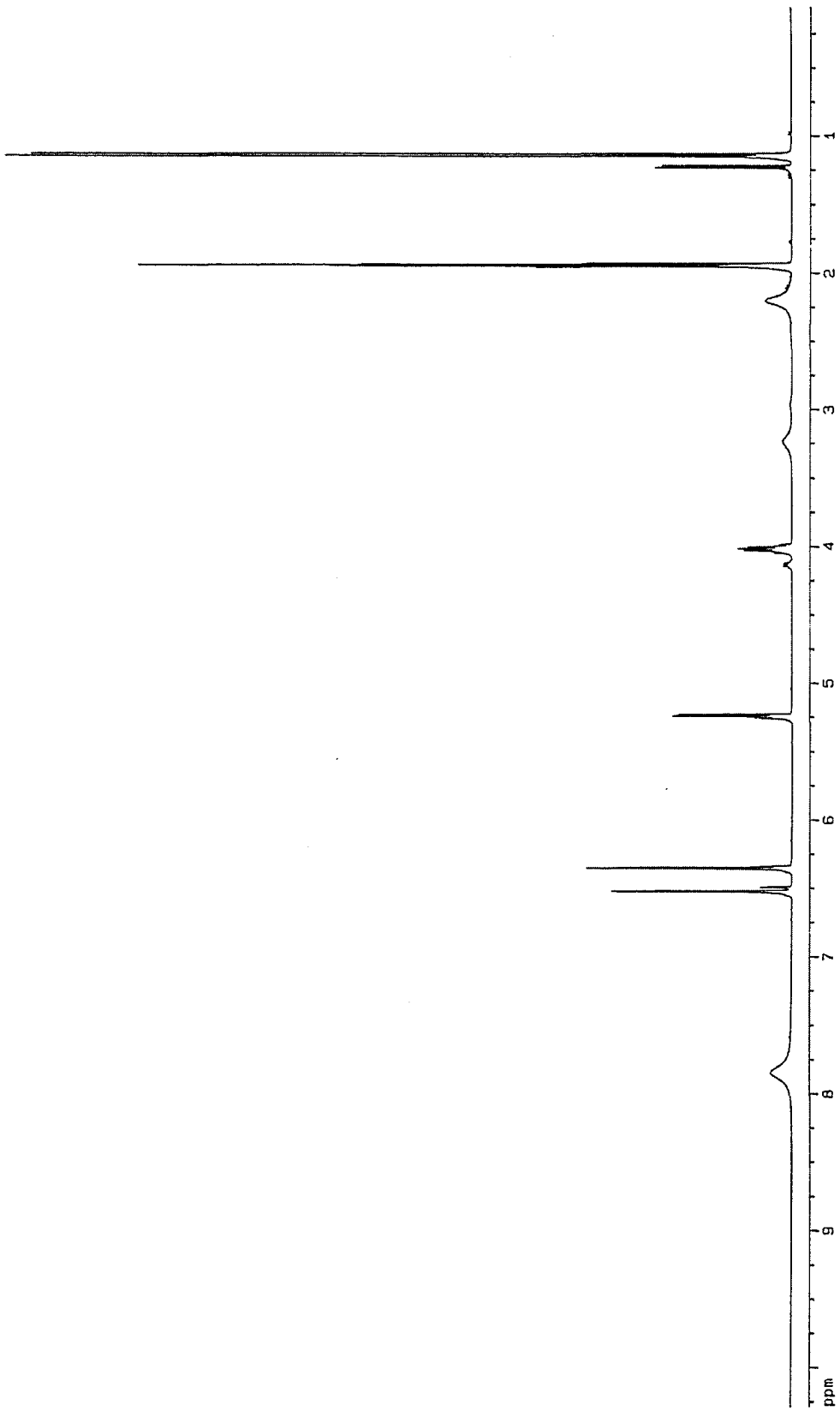


Figure A.13 ^{1}H NMR spectrum of 5, 7-Dihydroxy-3(1-hydroxyethyl)phthalide in CD_3CN (200 MHz).

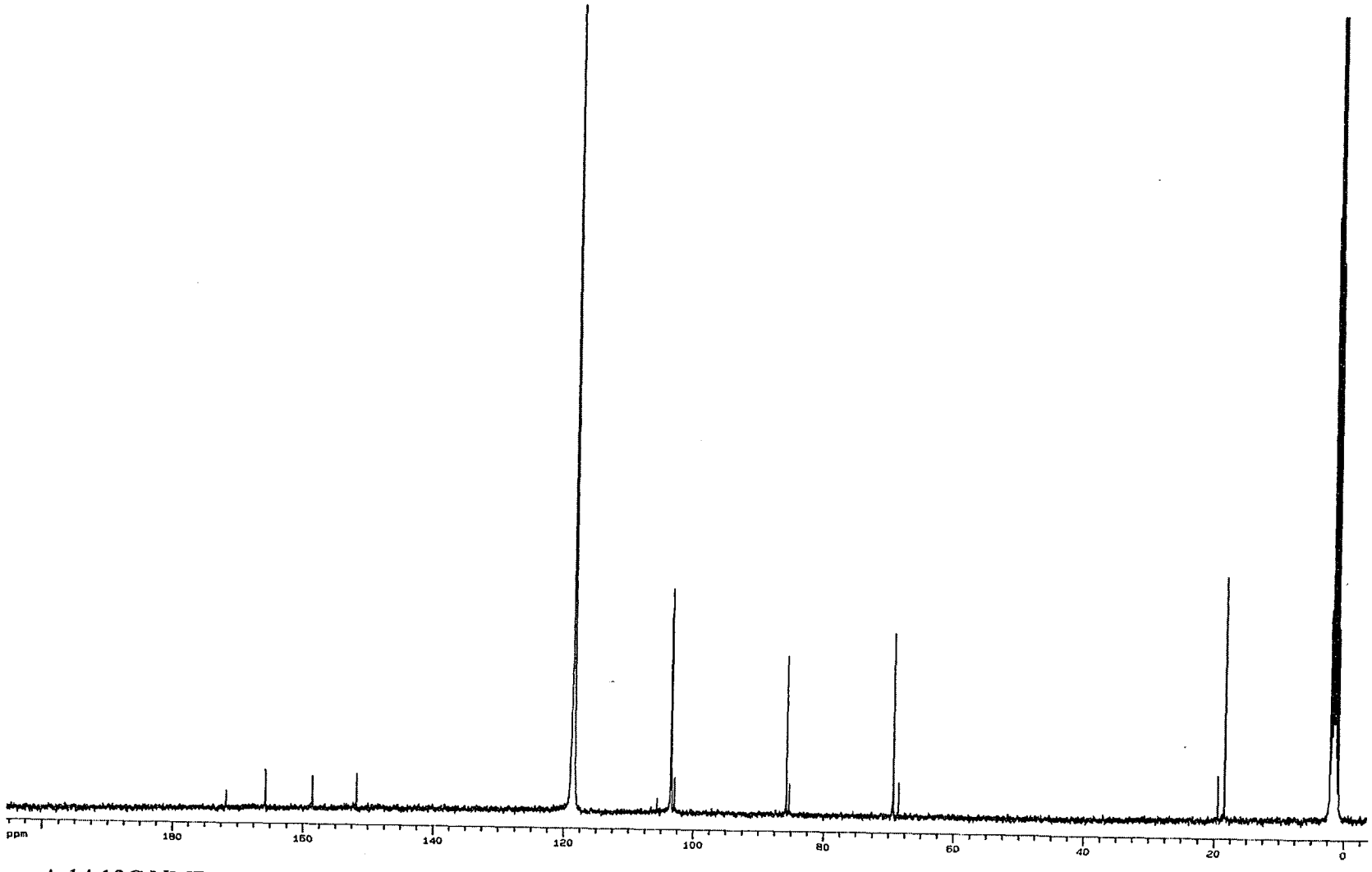


Figure A.14 13C NMR spectrum of 5, 7-Dihydroxy-3(1-hydroxyethyl)phthalide in CD3CN (200 MHz).

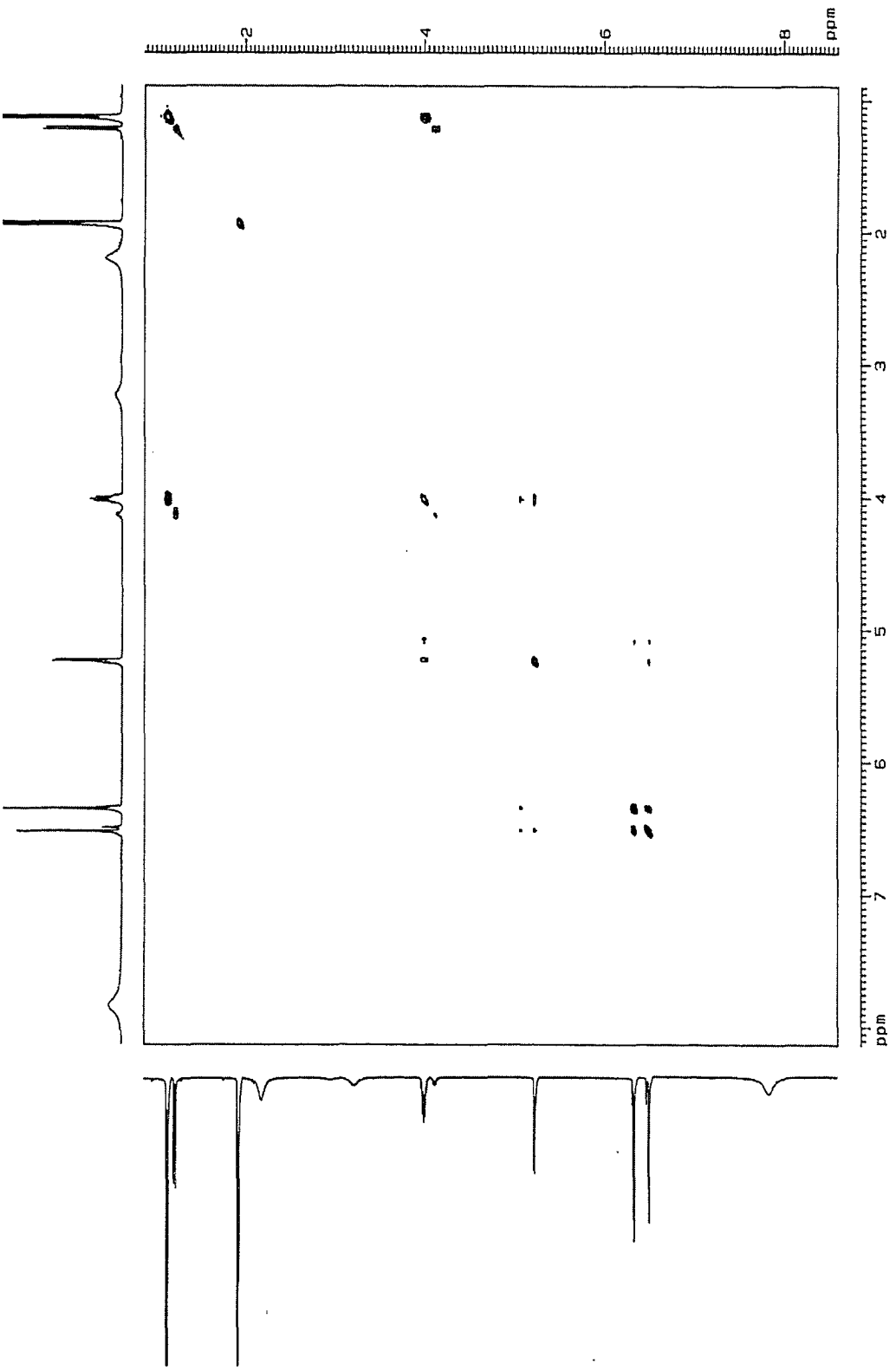


Figure A.15 Gradient DQF-COSY of 5, 7-Dihydroxy-3(1-hydroxyethyl)phthalide in CD_3CN (200 MHz).

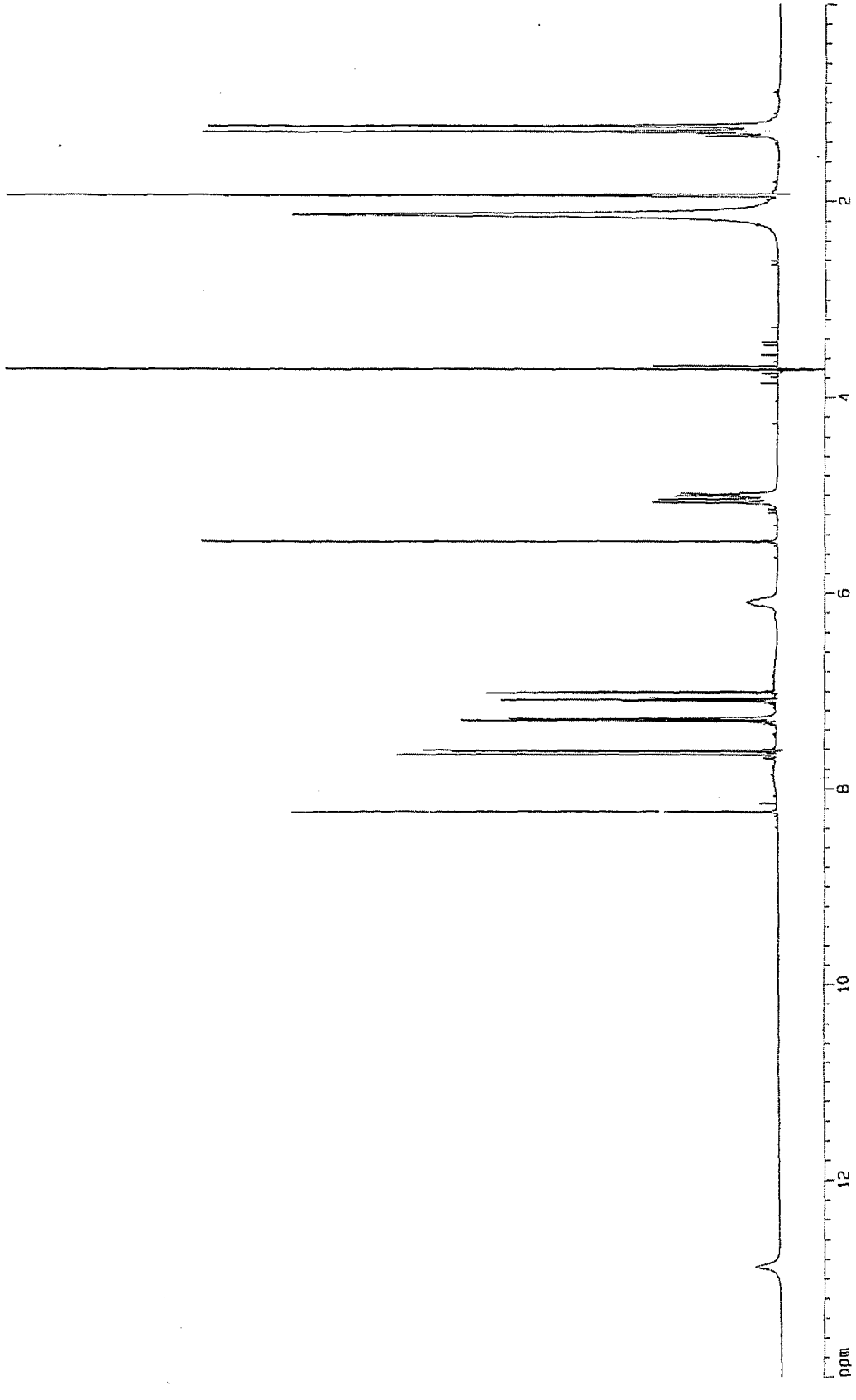


Figure A.16 1H NMR spectrum of meleagrin in CD3CN at 25°C (500 MHz).

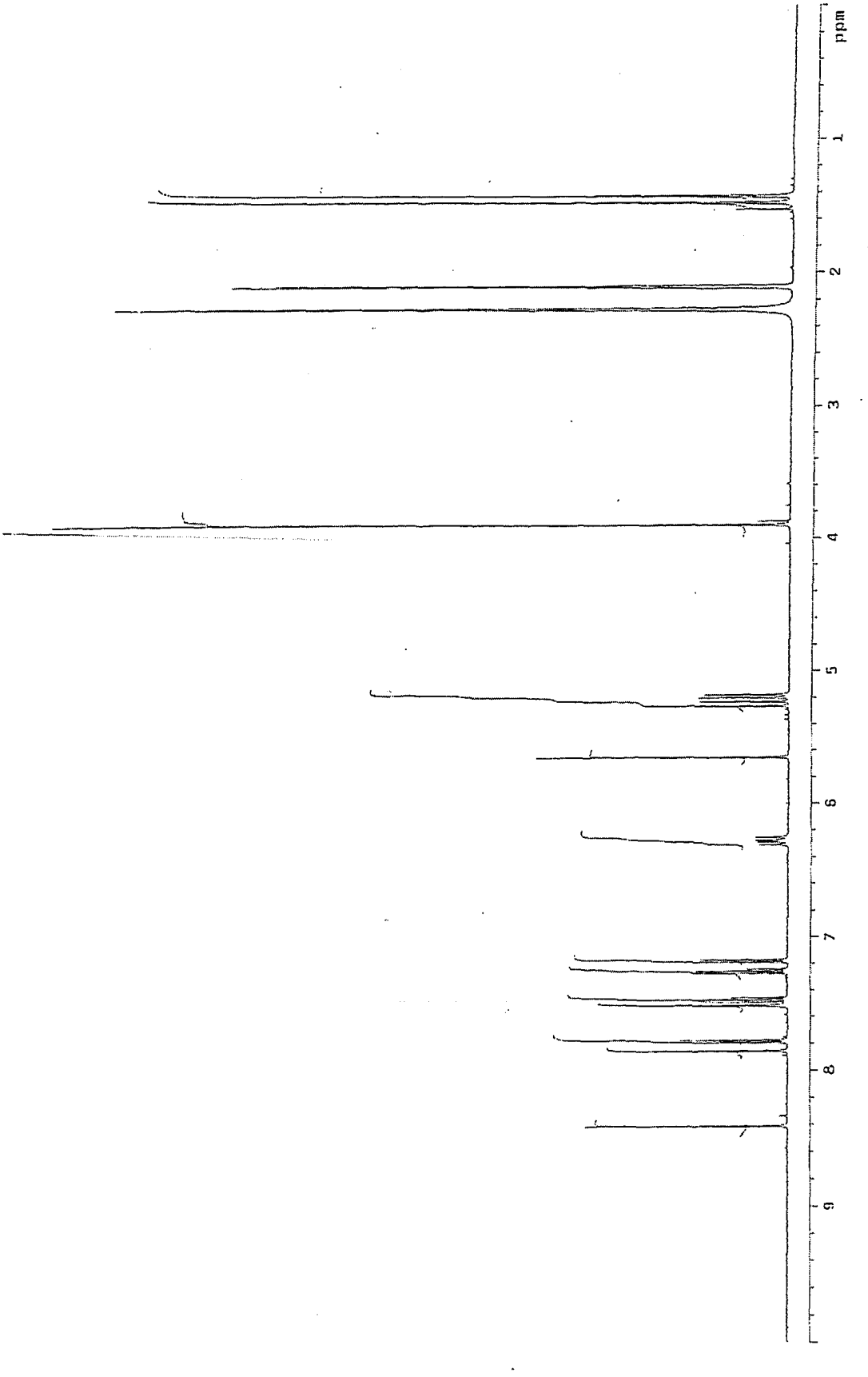


Figure A.17 1H NMR spectrum of meleagrin in CD3CN at 75°C (500 MHz).

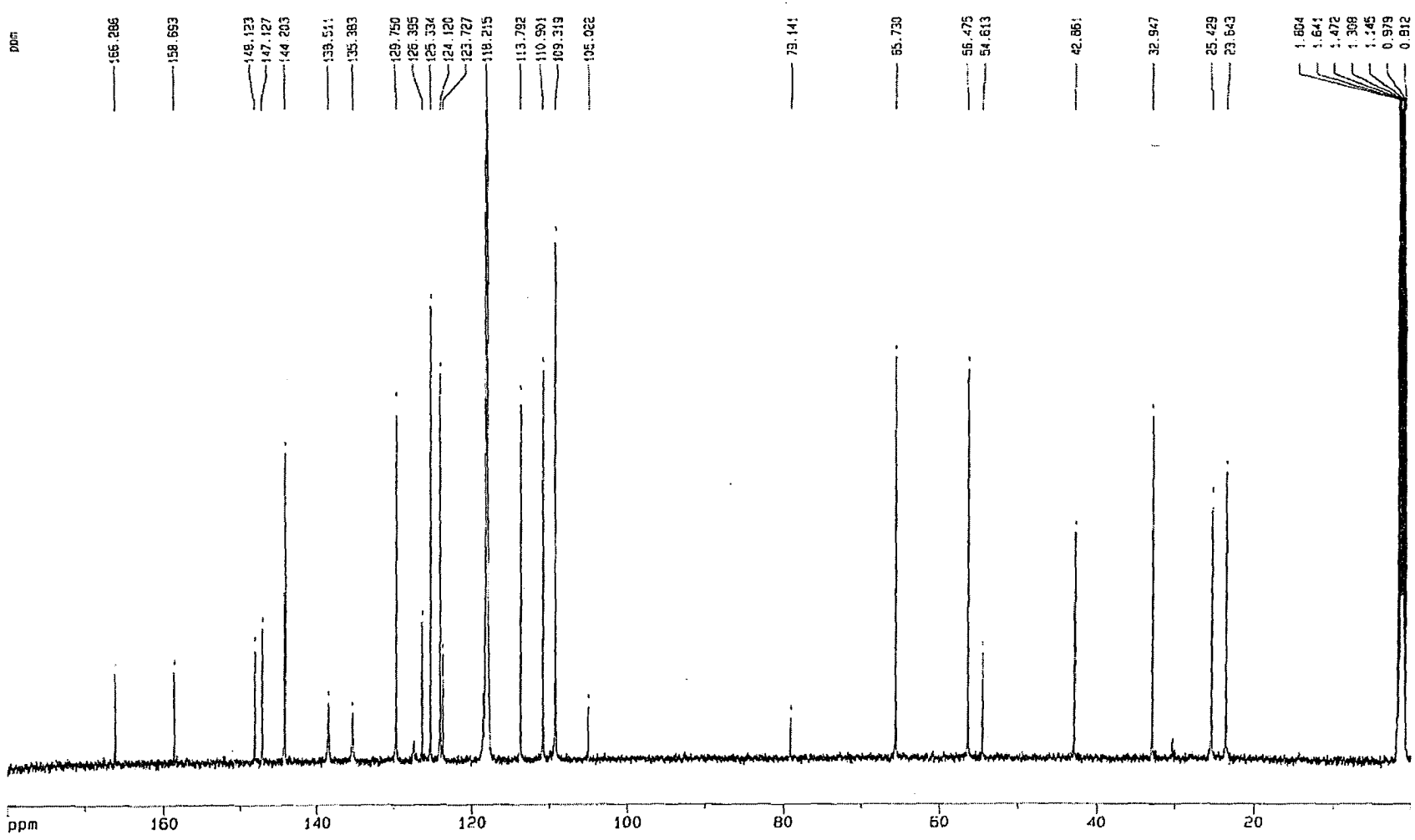


Figure A.18 13C NMR spectrum of meleagrin in CD3CN (500 MHz).

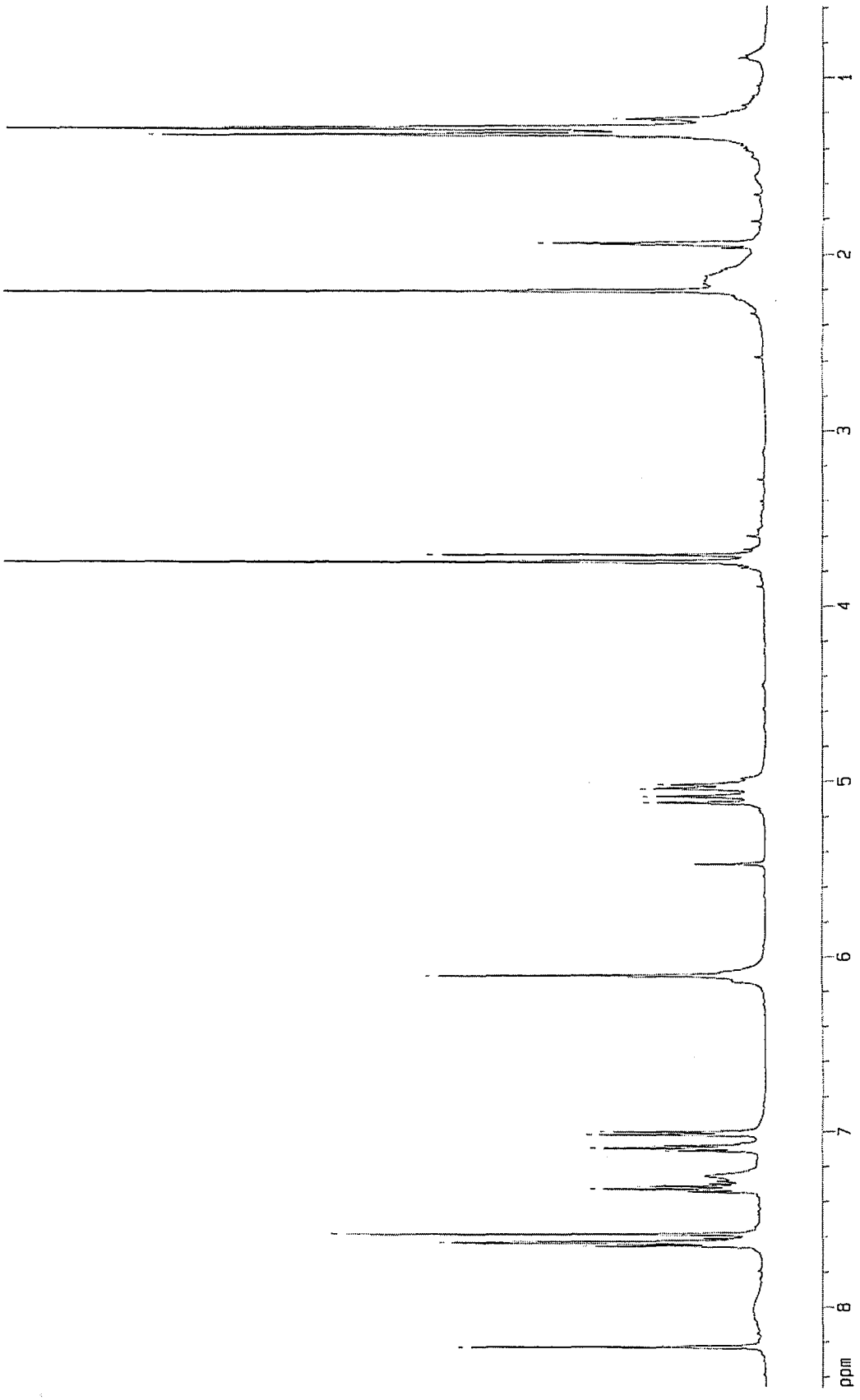


Figure A.19 ^{1}H NMR spectrum of meleagrin acetate in CD_3CN (500 MHz).

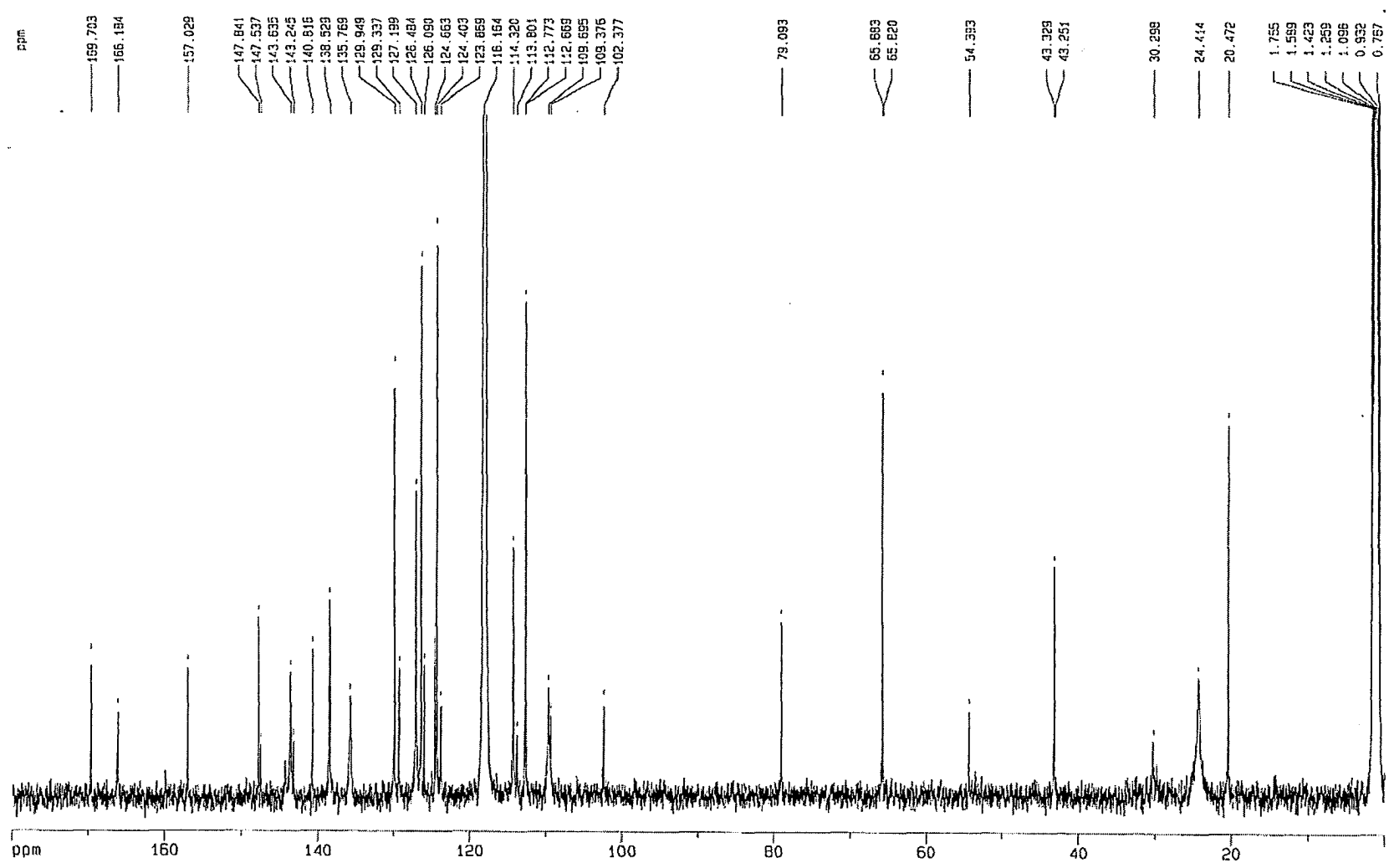


Figure A.20 13C NMR spectrum of meleagrin acetate in CD3CN (500 MHz).

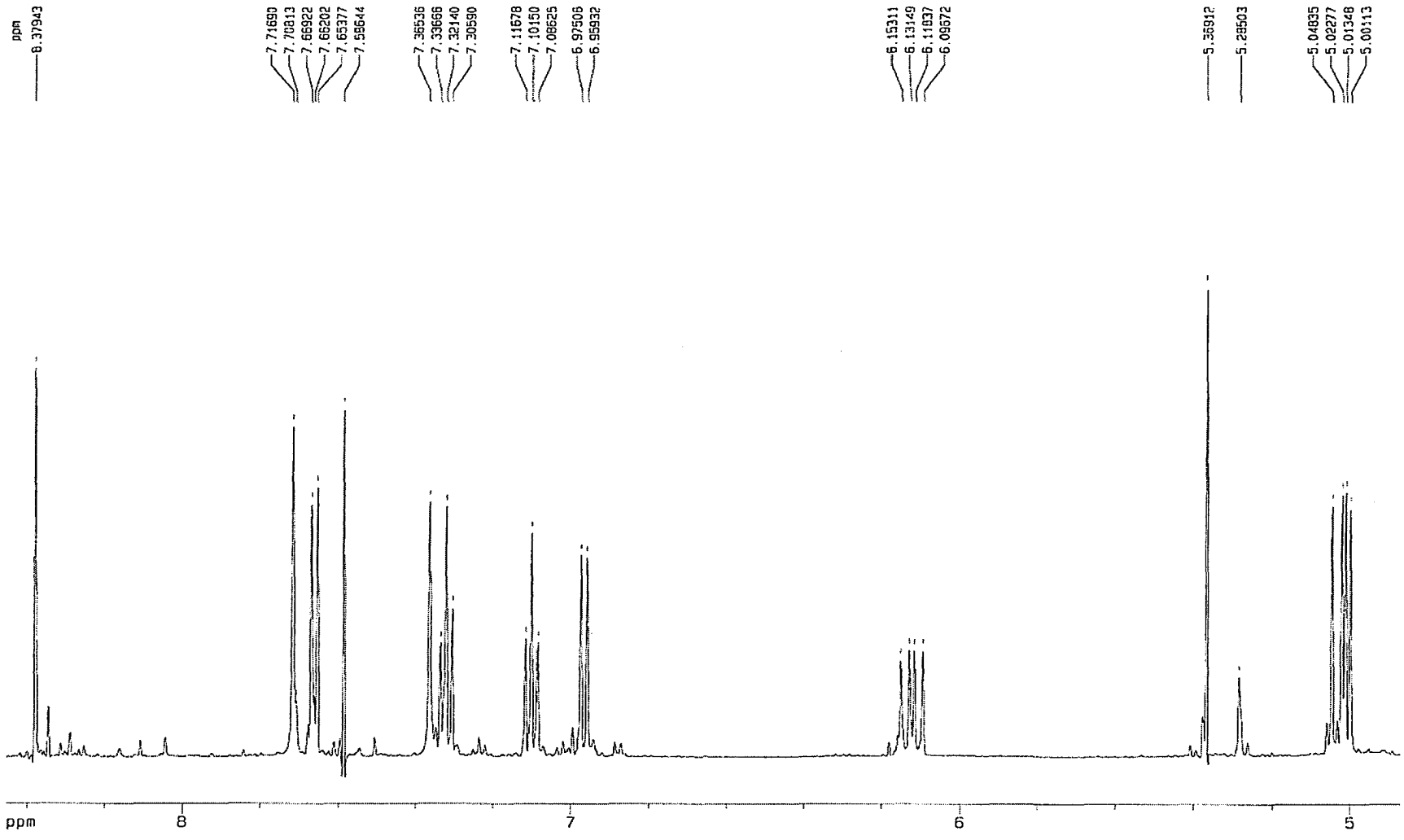


Figure A.21 1H NMR spectrum of N-methyl meleagrin in CD3CN (500 MHz).

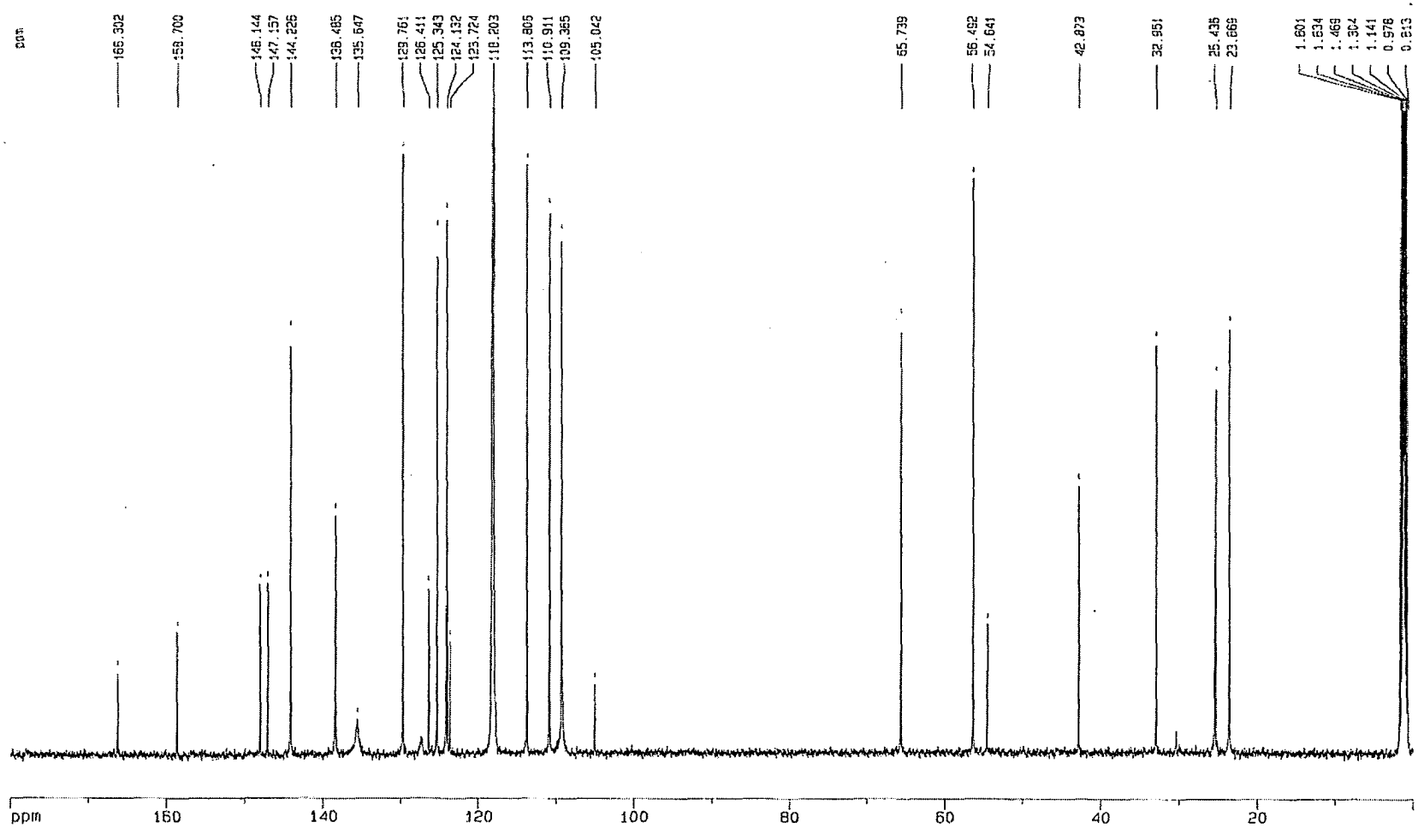


Figure A.22 ^{13}C NMR spectrum of N-methyl meleagrin in CD_3CN (500 MHz).

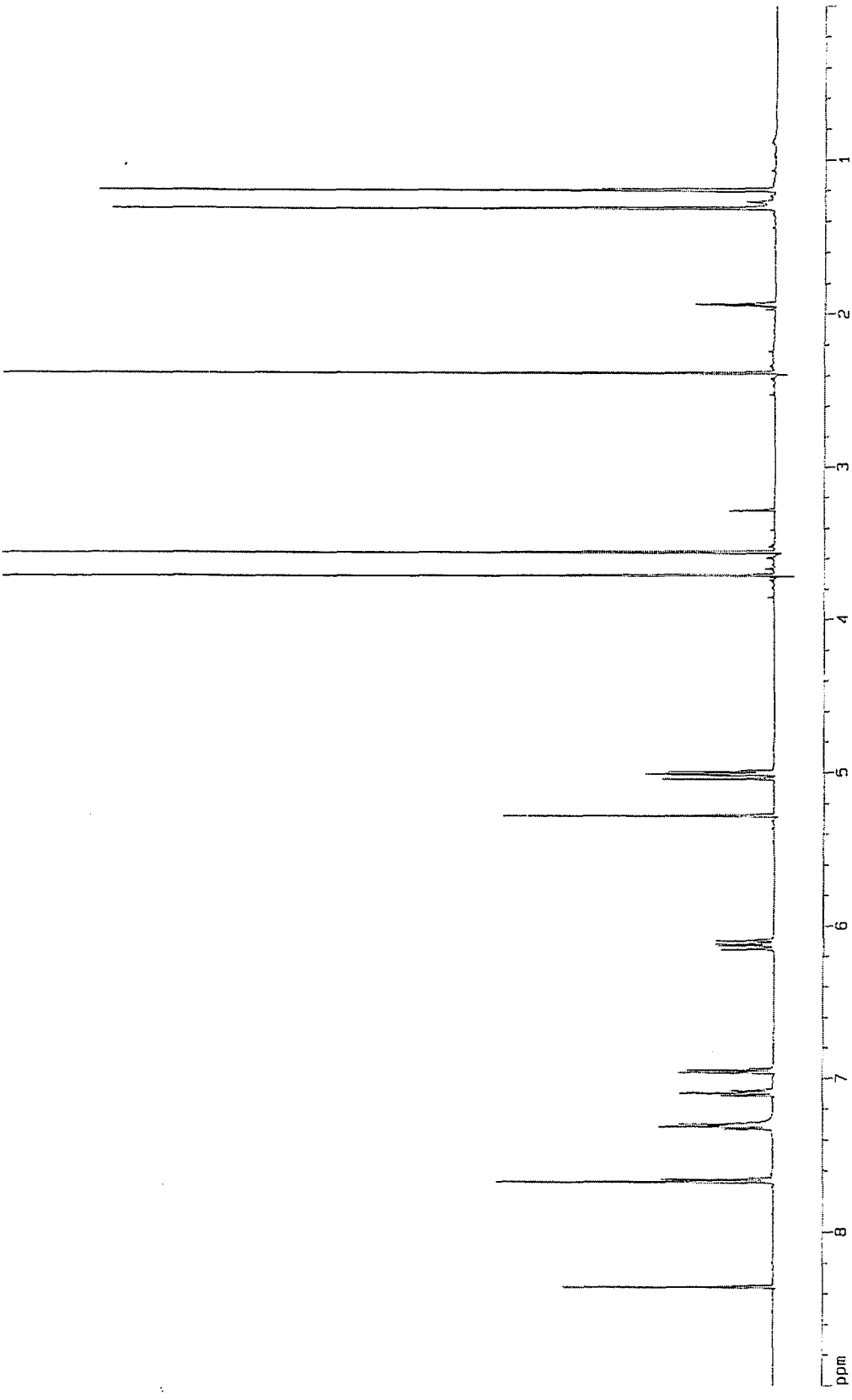


Figure A.23 1H NMR spectrum of N-methyl oxaline in CD3CN (500 MHz).

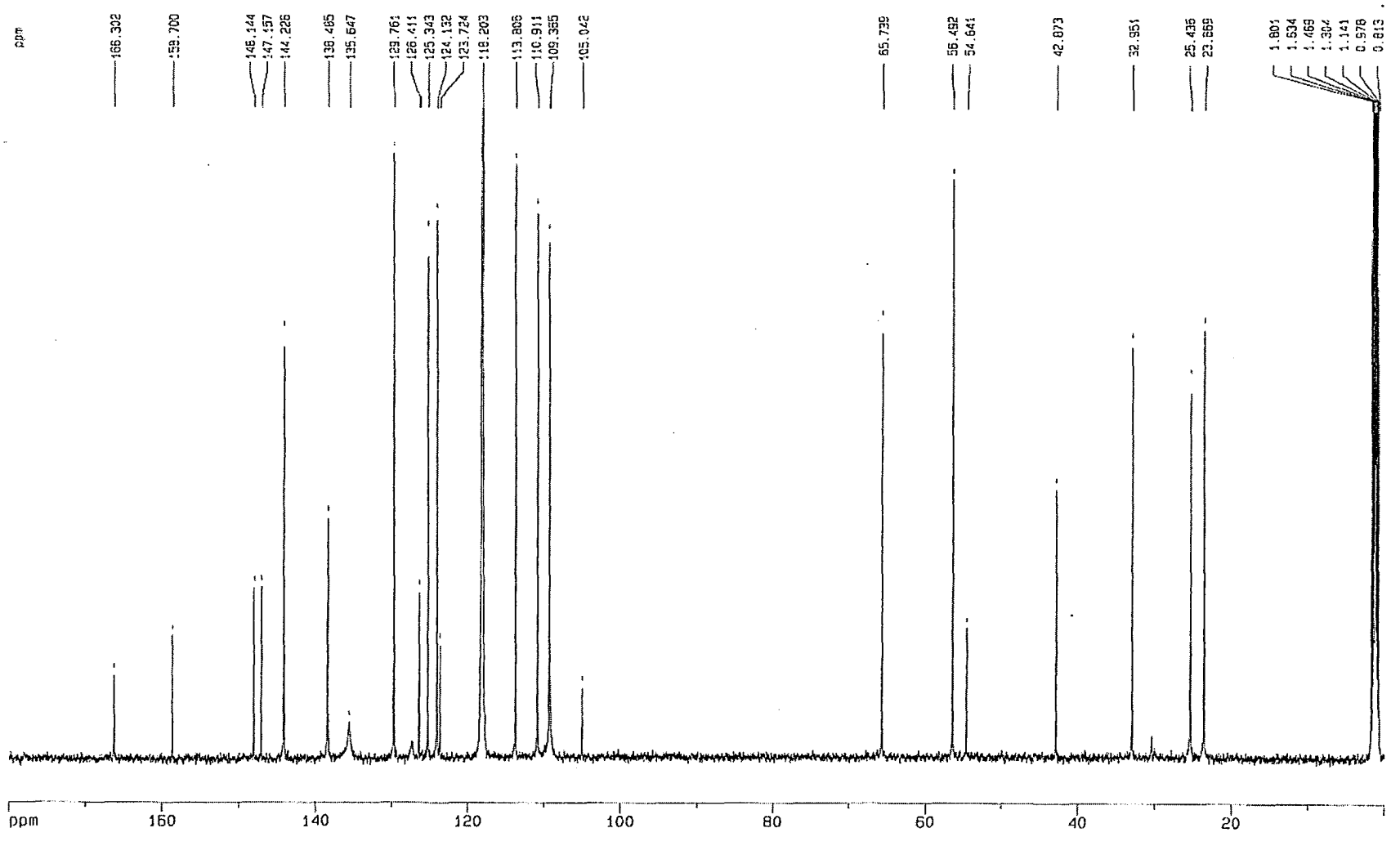


Figure A.24 13C NMR spectrum of N-methyl oxaline in CD3CN (500 MHz).

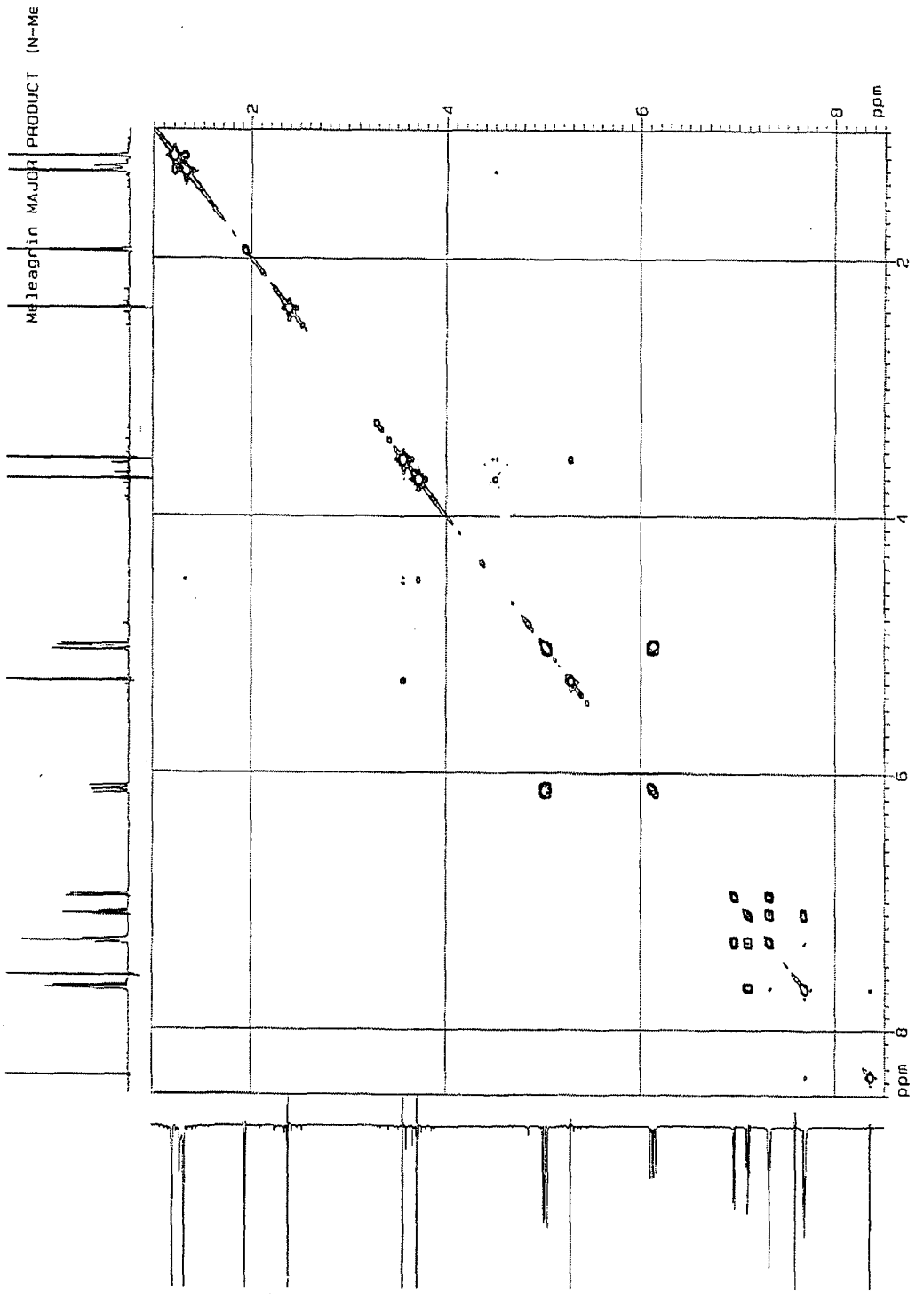


Figure A.25 Gradient DQF-COSY of N-methyl oxaline (500 MHz).

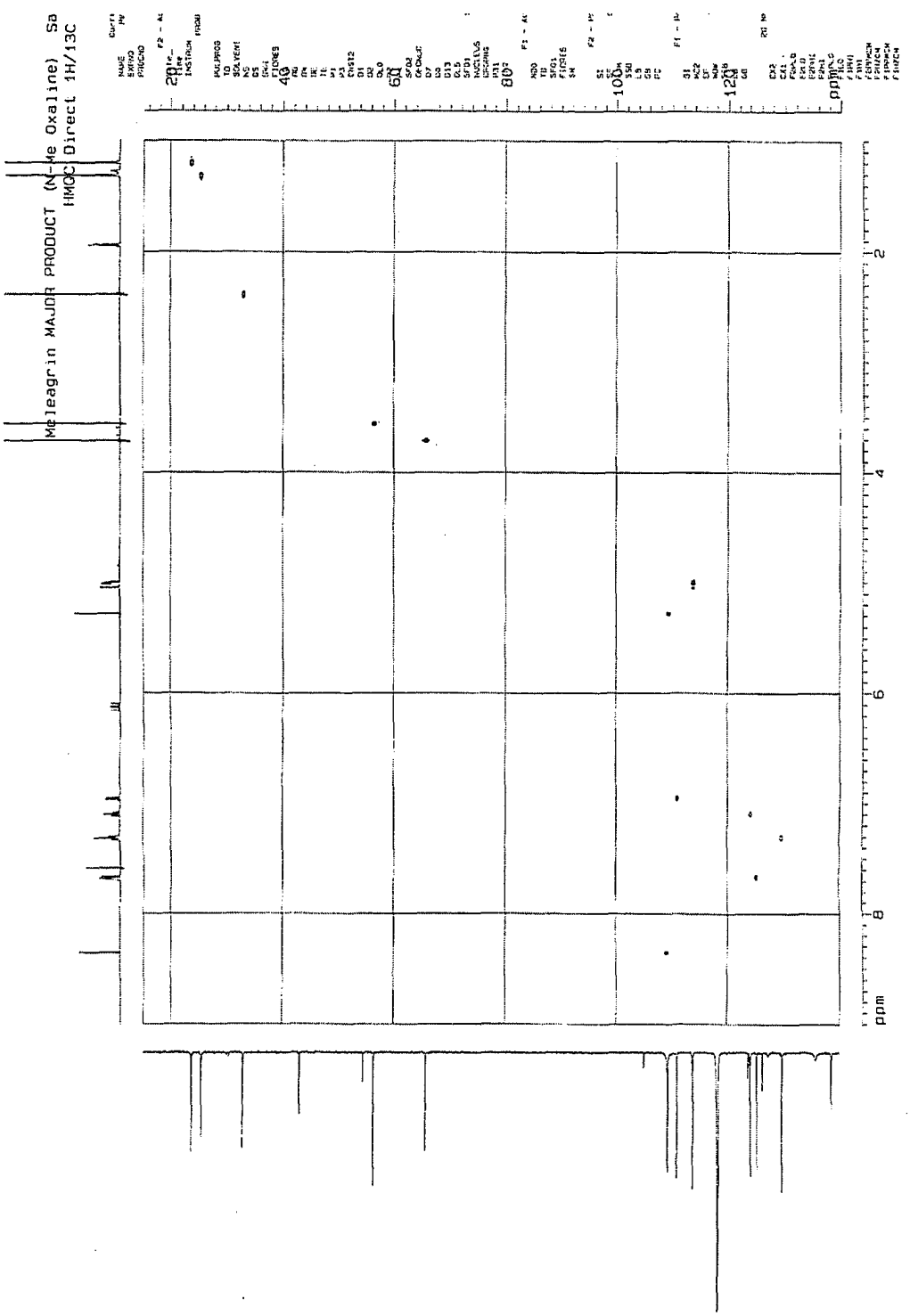


Figure A.26 Gradient HMQC spectrum of N-methyl oxaline (500 MHz).

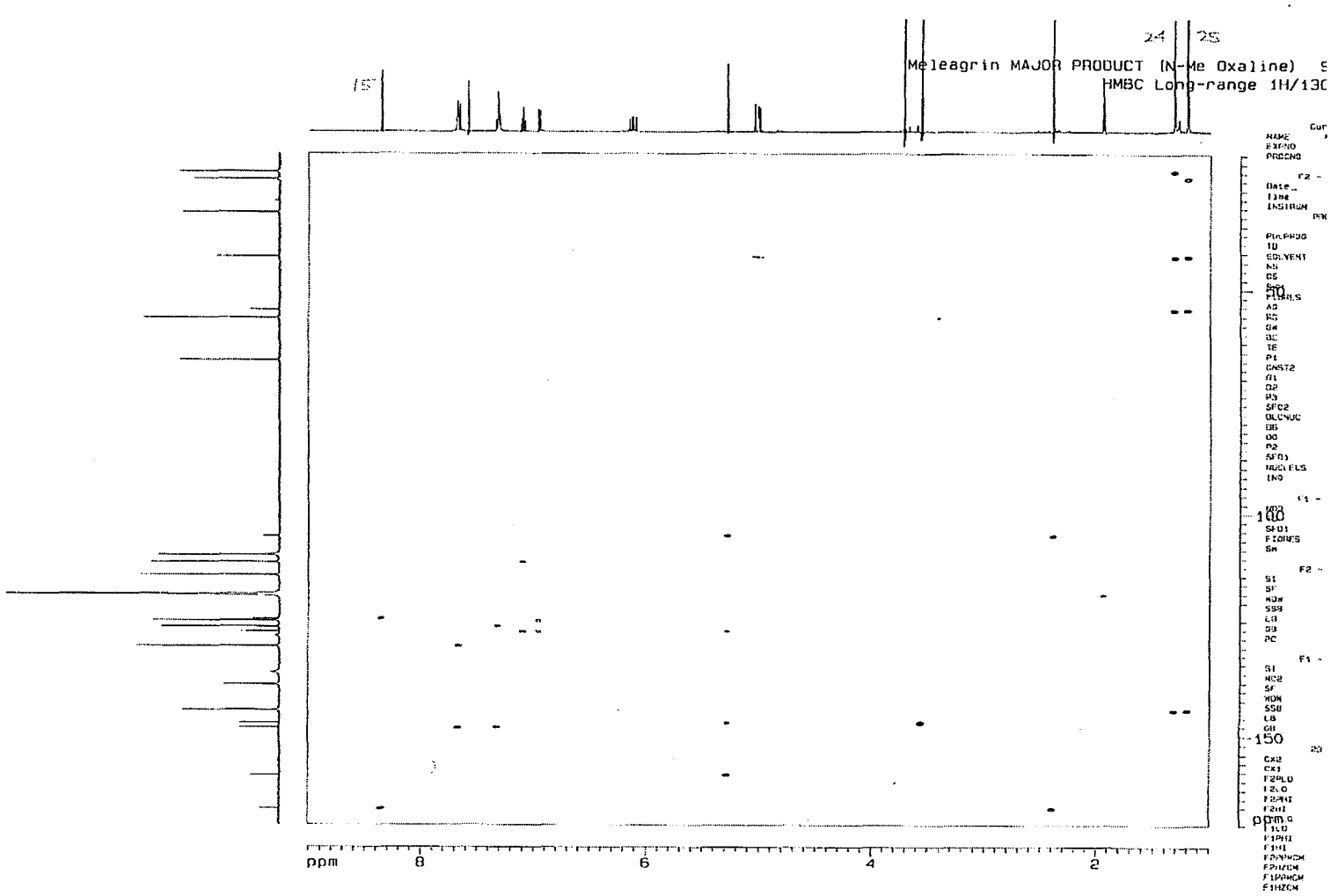


Figure A.27 Gradient HMBC spectrum of N-methyl oxaline (500 MHz).

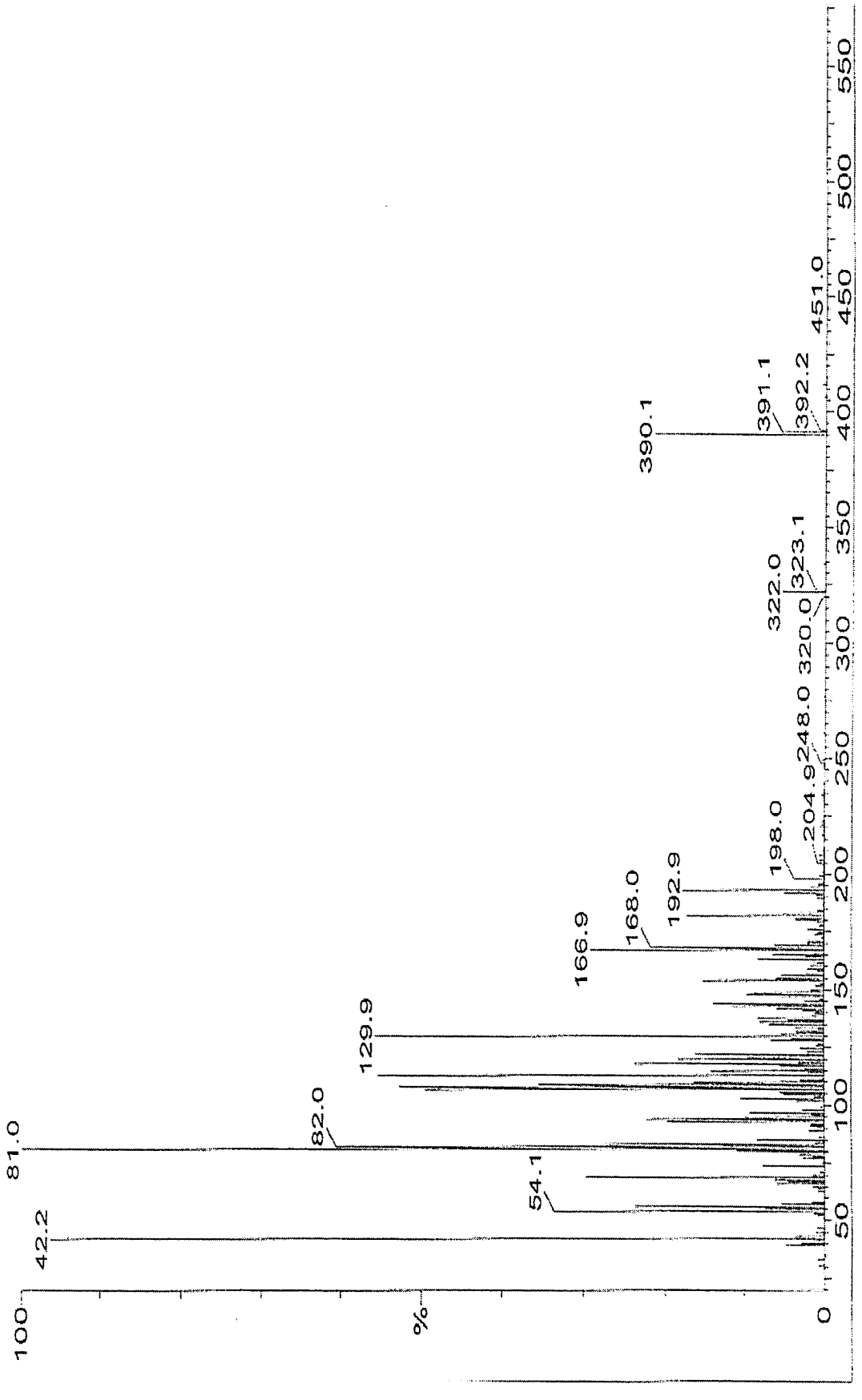


Figure A.28 EIMS of roquefortine C indicating parent (390 M+1) and daughter ions.

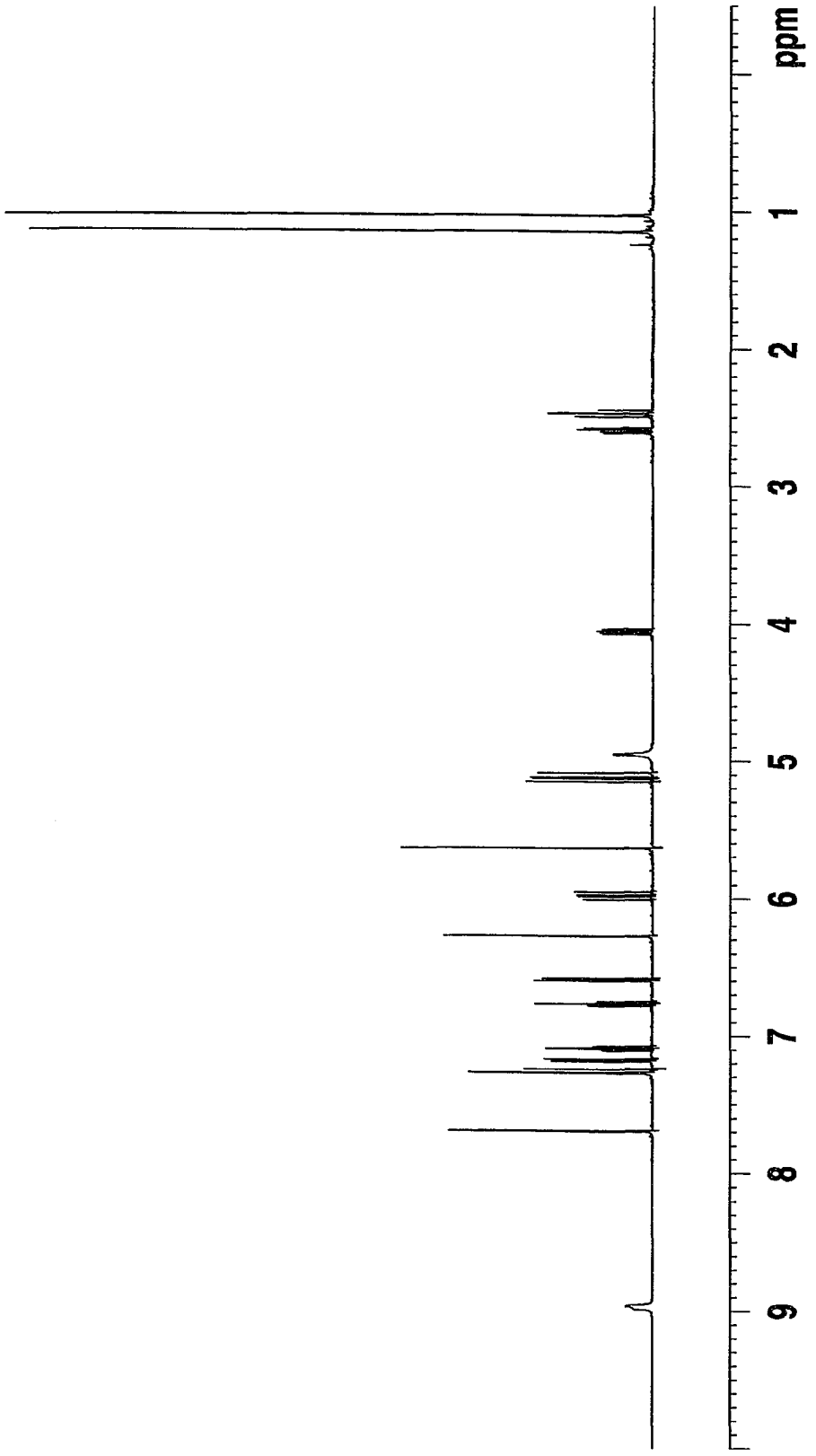


Figure A.29 ^{1}H NMR spectrum of roquefortine in $CDCl_3$ (500 MHz).

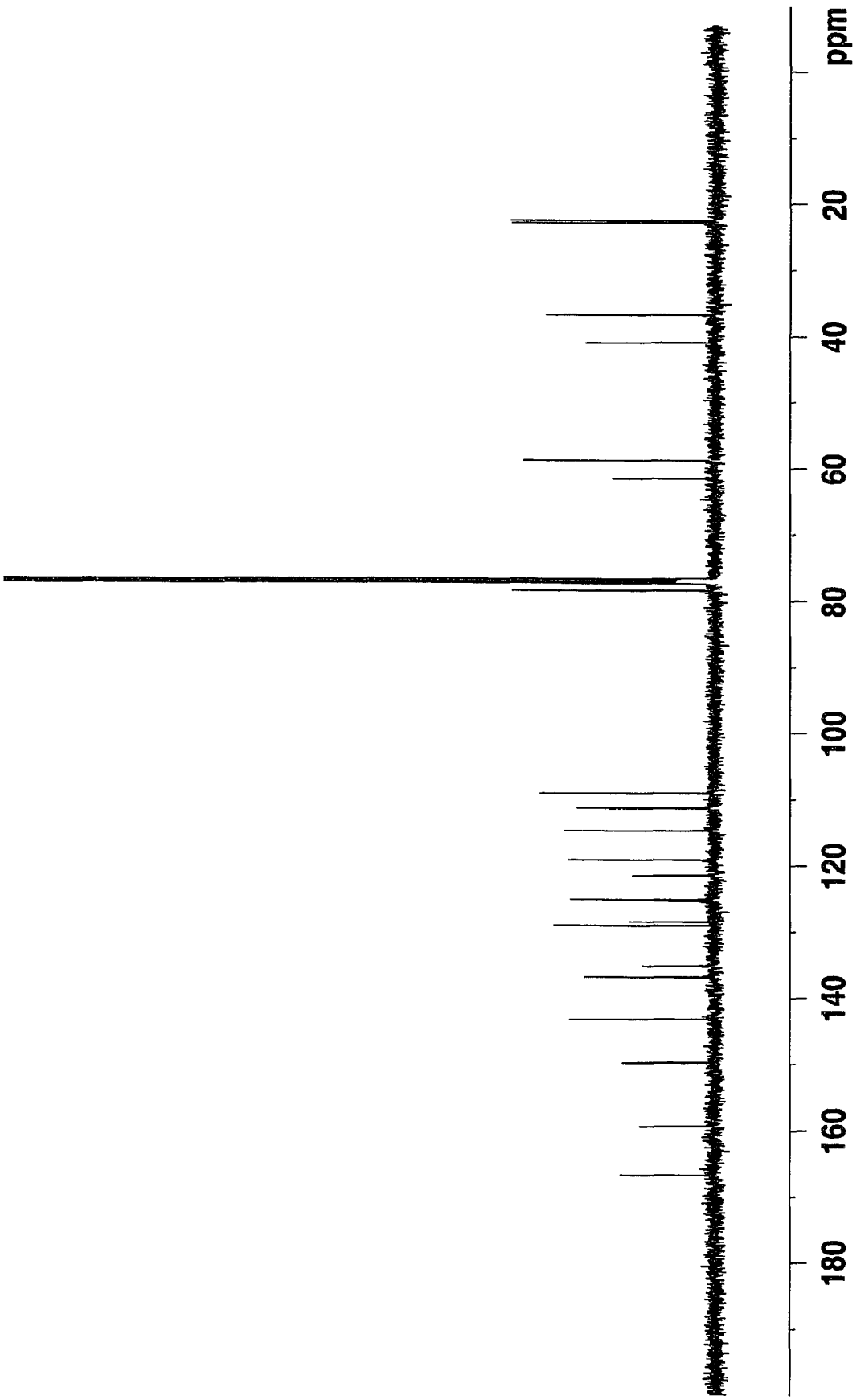


Figure A.30 ^{13}C NMR spectrum of roquefortine in $CDCl_3$ (500 MHz).

Spectroscopic Data

Brevianamide A

Bright yellow crystals, m.p. 200-212°C, (lit. 190-220°C)[14], $[\alpha]_D$ + 413° (EtOH).

^{1}H NMR (500 MHz, CD_3CN): δ (ppm) 7.45 (d, J=7.8 Hz, 1H, H-4), 6.90 (t, J=7.8 Hz, 1H, H-6), 6.75 (t, J=7.8 Hz, 2H, H-5, H-7), 3.35 (br s, 1H, NH), 2.35 (t, J=6.7 Hz, 2H, H-15), 2.45 (m 2H, H-13), 2.60 (m, 2H, H-8), 2.02 (m, 2H, H-14), 2.60 (m, 1H, H-19), 1.80 (m, 3H, H-20), 1.05 (s, 3H, Me-21), 0.80 (s, 3H, Me-22), 7.62 (br s, 1H, NH).

^{13}C NMR (500 MHz, CD_3CN): δ (ppm) 112.2, 202.5, 125.2, 119.8, 139.5, 37.8, 55.0, 171.2, 80.0, 30.0, 26.5, 48.2, 174.8, 45.0, 54.6, 44.8, 24.2, 20.0, 162.2, 122.5.

IRυ_{max} ($CHCl_3$, cm^{-1}): 3317 (HN), 3226 (NH), 1679 (CO), 1669 (C=O), 1620, 1493, 1468, 1396, 1324, 1159, 1134, 1101, 954, 899, 775.

MS (EI) m/z (*rel. int.):* 365 ($[M]^+$, 40), 297 (11), 296 ($[M-C_5H_6]^+$, 41), 268 (6), 162 (6), 87 (16), 85 (82), 83 ($[C_6H_{11}]^+$, 100), 70 (6), 48 (11), 44 (21), 29 (6).

HRMS: *Calculated* for $C_{21}H_{23}N_3O_3$, 365.1741, *found* 365.1750.

Mycophenolic Acid

White crystals, m.p. 141-142°C, (lit. 139-141°C)[35].

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 5.21 (m, 1H, H-11), 5.20 (s, 2H, H-3), 3.76 (s, 3H, OMe), 3.34 (d, J=7.0 Hz, 2 H, H-10), 2.45 (m 2H, H-14), 2.32 (m, 2 H, H-13), 2.15 (s, 3H, Me-16), 1.77 (s, 3H, Me-17), 7.75 (br s, OH).

^{13}C NMR (200 MHz, CD_3CN): δ (ppm) 165.0, 70.0, 118.3, 173.7, 61.7, 107.5, 153.9, 92.5, 146.2, 16.3, 122.3, 135.2, 35.1, 32.9, 174.7, 11.6 (Me), 23.2 (Me).

IRυ_{max} ($CHCl_3$, cm^{-1}): 3417 (OH), 2932 (COOH), 1706 (C=O), 1625, 1447, 1297, 1220, 1002, 861, 828, 709, 761, 650.

MS (EI) m/z (*rel. int.):* 320 ($[M]^+$, 27), 302 (22), 260 (15), 248 (15), 247 ($[M\text{-}CH_2CH_2COOH]^+$, 100), 229 (22), 219 (37), 207 (80), 159 (17), 44 (17), 29 (29).

HRMS: *Calculated* for $C_{17}H_{20}O_6$, 320.1260, *found* 320.1282.

Asperphenamate

White solid, m.p. 205-207°C, (lit. 210°C, lit. 206-207°C, lit. 201-204°C)[57,31], $[\alpha]_D$ -82.3 (EtOH) .

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.00 (J=12.5 Hz, 1H, H-2), 3.12 (J=12.5 Hz, 2H, H-3), 3.27 (H-1'), 4.40 (J=9.0 Hz, 1H, H-2'), 2.85 (J=6.5 Hz, 2H, H-3'), 7.12 (m, 4H, H-5, 5', 9, 9'), 7.20 (m, 4H, H-6, 6', 8, 8'), 7,15 (m, 2H, H-7, 7'), 7.75 (m, 4H, H-13, 13', 17, 17'), 7.44 (m, 4H, H-14, 14', 16, 16'), 7.51 (m, 4H, H-15, 15').

^{13}C NMR (200 MHz, CD_3CN): δ (ppm) 172.4, 55.7, 37.5, 66.5, 51.2, 37.6, 139.2, 138.3, 130.2, 129.4, 129.4, 128.6, 127.5, 168.1, 167.6, 132.7, 128.0, 128.6, 132.2.

IRυ_{max} ($CHCl_3$, cm^{-1}): 3306 (NH), 1751 (C=O), 1734 (C=O), 1638 (CONH), 1532, 1200, 694.

MS (EI) m/z (*rel. int.):* 506 ($[M]^+$, 0.1), 415 ($[M-C_7H_7]^+$, 9), 294 (7), 269 (2), 252 (17), 251 ($[PhCH_2C(NHBz)=C=O]^+$, 3), 225 (7), 224 ($[PhCH_2CHNHBz]^+$, 23), 149 (6), 148 (54), 147 (17), 146 ($PhCH_2C(NHBz)=C=O-105]^+$, 100), 118 (17), 105 ($[C_6H_5CO]^+$, 100), 91 (50), 77 (40).

LC-MS: 507 ($[M+1]^+$).

Raistrick Phenol Derivative or 5,7-Dihydroxy-3(1-hydroxyethyl)phthalide[99].

White solid, m.p. 204-208°C, (lit. 206-208°C)[99], $[\alpha]_D$ – 65.5° (EtOH).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 7.87 (br s, 1H, OH), 8.45 (br s, 1H, OH), 6.37 (d, J=3.0 Hz, H-6), 6.50 (d, J=1.0 Hz, H-4), 5.25 (d, J=4.7 Hz, H-3), 4.00 (d, J=7.0 Hz, OH), 1.81 (m, J=7.0 Hz, 1H, H-10), 1.13 (d, J=6.3 Hz, 3H, Me-11).

^{13}C NMR (200 MHz, CD_3CN): δ (ppm) 172.5, 68.7, 103.7, 165.0, 85.0, 158.7, 118.7, 152.5, 20.0, 18.7.

IRυ_{max} ($CHCl_3$, cm^{-1}): 3329 (OH), 3093, (1710 (C=O), 1611, 1450, 1300, 1221, 1164.

MS (EI) m/z (*rel. int.*): 210 ($[M]^+$, 5), 166 ($[M-CO_2]^+$, 100), 165 (49), 138 (10), 137 (26), 45 (13).

HRMS: *Calculated* for $C_{10}H_{10}O_5$, 210.0528, *found* 210.0525.

Roquefortine C

Very fine feathery solid, m.p. 195-200°C, (lit. 195-200°C)[86], $[\alpha]_D$ -700° ($CHCl_3$).

^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 12.97 (br s, 1H, H-19), 8.97 (br s, NH, H-2), 7.72 (br s, 1H, H-20), 7.29 (br s, 1H, H-22), 7.20 (d, J=7.8 Hz, 1H, H-12), 7.10 (dt, J=7.8 Hz, J=1.3 Hz, 1H, H-10), 6.78 (dt, J=7.8 Hz, J=1.0 Hz, 1H, H-11), 6.60 (d, J=7.8 Hz, 1H, H-9), 6.28 (br s, 1H, H-17), 5.99 (dd, J=11.0 Hz, J=17.2 Hz, 1H, H-24), 5.65 (s, 1H, H-6), 5.16 (dd, J=1.0 Hz, J=10.8 Hz, 1H, H-25), 5.11 (dd, J=0.9 Hz, J=17.4 Hz, 1H, H-25), 4.95 (br s, NH, H-7), 4.07 (dd, J= J=6.0, J=11.5 Hz, 1H, H-16), 2.60 (dd, J=12.4 Hz, J=5.9 Hz, 1H, H-15), 2.50 (dd, J=11.5 Hz, J=12.4 Hz, 1H, H-15), 1.16 (s, 3H, H-26), 1.02 (s, 3H, H-27).

IRυ_{max} ($CHCl_3$, cm^{-1}): 3197 (NH), 2971, 1681 (CONR), 1606, 1483, 1433, 1357, 1316, 1239, 1215, 1102, 921, 751, 643.

MS (EI) m/z *(rel. int.):* 389 ($[M]^+$, 27), 321 (20), 320 (100), 319 (4), 198 (8), 192 (7), 161 (22), 158 (6), 150 (8), 143 (17), 131 (5), 130 (50), 112 (7), 108 (11), 107 (5), 70 (9), 69 (9), 50 (5), 41 (15), 31 (14).

HRMS: *Calculated* for $C_{22}H_{23}N_5O_2$, 389.1854, *found* 389.1861.

Meleagrin

Light yellow solid, m.p. 197-201°C, (lit. 250°C)[49, 65], $[\alpha]_D$ -115° ($CHCl_3$).

^{1}H NMR (75°C, 500 MHz, $CDCl_3$): δ (ppm) 12.75 (br s, NH, H-14), 8.42 (s, 1H, H-15), 7.78 (br s, 1H, OH), 6.70 (br s, NH, H-19), 7.67 (s, 1H, H-18), 7.19 (d, J=7.8 Hz, 1H, H-7), 7.52 (s, 1H, H-20), 7.48 (t, J=7.2 Hz, 1H, H-6), 7.26 (t, J=7.1 Hz, 1H, H-5), 7.79 (d, J=7.1 Hz,1H, H-4), 6.28 (br s, 1H, H-22), 5.66 (s, 1H, H-8), 5.26 (br dt, J=17.2 Hz, J=10.2 Hz, 2H, H-23), 3.90 (s, 3H, N-OMe), 1.48 (s, 3H, H-25), 1.43 (s, 3H, H-24).

^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 165.9 (C=O), 159.7 (C=O), 146.9, 142.3, 137.7 135.5, 129.3, 125.8, 125.5, 124.4, 122.7, 114.9, 112.7, 110.8, 66.0 (N-OMe), 53.0 (C-3), 43.3, 24.0 (Me), 24.1 (Me).

IRυ_{max} ($CHCl_3$, cm^{-1}): 3161 (OH), 3009 (CO_2R), 1696 (CONH), 1668, 1648, 1614, 1439, 1394, 1353, 1310, 1218, 1108, 979, 751.

MS (EI) m/z (*rel. int.):* 433 ($[M]^+$, 8), 365 (38), 364 (50), 318 (17), 277 (9), 86 (70), 84 (100), 47 (19), 41 (11), 31 (9), 29 (8).

HRMS: *Calculated* for $C_{23}H_{23}N_5O_4$, 433.1752, *found* 433.1729

Meleagrin acetate

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 12.72 (s, NH, H-14), 9.54 (br s, NH, H-19), 8.22 (s,1H, H-15), 7.65 (s, 1H, H-18), 7.00 (d, J=7.8 Hz, 1H, H-7), 7.30 (s, 1H, H-20), 7.25 (t, J=7.2 Hz, 1H, H-6), 7.10 (t, J=7.1 Hz, 1H, H-5), 7.60 (d, J=7.1 Hz,1H, H-4), 6.11 (br s, 1H, H-22), 5.45 (s, 1H, H-8), 5.00 (dt, J=17.2 Hz, J=10.2 Hz, 2H, H-23), 3.70 (s, 3H, N-OMe), 2.20 (s, 3H OAc), 1.23 (s, 3H, H-25), 1.28 (s, 3H, H-24).

^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 168.7 (OAc), 166.5 (C=O), 156.1 (C=O), 146.9, 142.2, 136.9, 134.5, 129.1, 125.8, 125.1, 123.5, 122.6, 114.5, 112.0, 110.1, 65.4 (N-OMe), 53.4 (C-3), 42.5, 23.7 (Me), 23.7 (Me), 20.3 (OAc).

IRυ_{max} ($CHCl_3$, cm^{-1}): 3161 (NH), 3009, 2979, 2933, 1699 (CONH), 1668 (CONR), 1648, 1614, 1439, 1394, 1353, 1310, 1218, 1108, 980, 751.

MS (EI) m/z (*rel. int.):* 475 ([M]$^+$, 7), 408 (10), 377 (10), 376 (51), 365 (61), 364 (M^+-C_5H_9, 81), 335 (70), 334 (100), 333 (27), 319 (16), 318 (46), 316 (20), 305 (33), 304 (14), 290 (15), 289 (23), 278 (17), 277 (43), 276 (14), 161 (17), 143 (14), 41 (19), 31 (22).

HRMS: *Calculated* for $C_{25}H_{25}N_5O_5$, 475.1857, *found* 475.1860

N-methyl oxaline

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 13.12 (s, NH, H-14), 8.35 (s,1H, H-15), 7.67 (s, 1H, H-18), 6.97 (d, J=7.7 Hz, 1H, H-7), 7.37 (s, 1H, H-20), 7.30 (t, J=7.2 Hz, 1H, H-6), 7.10 (t, J=7.1 Hz, 1H, H-5), 7.58 (d, J=7.1 Hz,1H, H-4), 6.12 (dd, J=17.3 Hz, J=10.9 Hz, 1H, H-22), 5.28 (s, 1H, H-8), 5.00 (dd, J=17.2 Hz, J=10.2 Hz, 2H, H-23), 3.56 (s, 3H, N-OMe), 3.70 (s, 3H, OMe), 2.38 (s, 3H N-Me), 1.20 (s, 3H, H-25), 1.32 (s, 3H, H-24).

^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 166.3 (C=O), 158.7 (C=O), 147.1, 144.2, 138.5, 135.4, 129.7, 126.3, 125.3, 124.1, 123.7, 113.8, 110.9, 65.7 (N-OMe), 54.6 (OMe), 42.9, 23.6 (Me), 25.3 (Me).

IRυ_{max} ($CHCl_3$, cm^{-1}): 3237 (NH), 1690 (CONH), 1637 (CONR), 1462, 1417, 1360, 1318, 1243, 1105, 1070, 827, 754.

MS (EI) m/z (*rel. int.*): 461 ([M]$^+$, 10), 394 (26), 393 (93), 392 (26), 362 (25), 361 (100), 330 (16), 318 (13), 290 (10), 216 (11), 185 (9), 163 (11), 161 (33), 149 (13), 142 (10), 131, 101 (10), 69 (13), 41 (5), 31 (11).

HRMS: Calculated for $C_{25}H_{27}N_5O_4$, 461.20651, found 461.20798.