MARINE BIOTECHNOLOGY: EVALUATION AND DEVELOPMENT OF METHODS FOR THE DISCOVERY OF NATURAL PRODUCTS FROM FUNGI.

A thesis submitted in fulfillment of the requirements for the Degree of

MASTERS IN SCIENCE (PHARMACY)

of

RHODES UNIVERSITY

by

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DECEMBER 2004

The financial assistance of the Department of Labour (DoL) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the DoL.

ACKNOWLEDGEMENTS

I would like to thank my research supervisor, Dr Denzil Beukes for guiding me through the valley of death and for his guidance, inspiration and encouragement.

I would also like to thank the following people:

- Dr W.D. Leukes
- Special thanks to Dr Edith Beukes for teaching me to become a chemist
- Dr Graeme Bradley for his help with phylogenetics
- Yolisa for all the help with the NMR
- Michael Knott for showing me the lighter side of life
- Henry for all the laughter
- Mr. and Mrs. Morley for their helpfulness and technical assistance
- The Departments of Chemistry; Biochemistry, Biotechnology and Microbiology; Botany; Ichthyology, DNA Sequencing Unit and Electron Microscopy Unit
- Dr D. Hendricks at the University of Cape Town for performing the anti-cancer assays
- NRF and Department of Labour, Erik and Ernest Ethel Bursary, William Francis Barker Bursary and Rhodes University for financial assistance
- Imraan, my husband, for his endless support, encouragement and love, without which I could have never succeeded
- And lastly my family

ABSTRACT

One of the major impediments in the development of marine natural products is the provision of biologically active natural products in sufficient quantity for complete pharmacological evaluation, clinical trials and eventual commercial production. Marine microorganisms show great promise in providing a renewable source of biologically active natural products. The main aim of this study was to develop and evaluate methods for the isolation, identification and cultivation of marine fungi from the South African marine environment for the production of biologically active secondary metabolites.

Twenty-four species of fungi were isolated from marine algae collected from the intertidal zone near Port Alfred, South Africa. The fungi were cultivated in small-scale under static and agitated conditions and their crude intra- and extracellular organic extracts were screened by ^{1}H NMR and a series of bioassays. Using this as a basis, one isolate was selected for further study. By analyses of the ITS1 region of the ribosomal DNA, the fungal isolate was identified as a marine-derived isolate of *Eurotium rubrum* (*Aspergillus ruber*). Although *E. rubrum* has been isolated from the marine environment, no investigations have been undertaken to determine the adaptation of these isolates to the marine environment.

In order to optimise productivity, creativity and incubation time, the fungus was cultivated in small-scale using a variety of carbon (glucose, fructose, lactose, sucrose, mannitol and maltose) and nitrogen sources (ammonium tartrate, urea, peptone and yeast extract). An HPLC-DAD method was developed to assess the metabolic creativity and productivity under different fermentation conditions. Distinctive variations in the range and yield of metabolites produced as well as morphology and growth time were observed.

The crude extracts from all fermentations were combined and six known compounds were isolated by reversed-phase chromatography and their structures elucidated by spectroscopic techniques. The known compounds were flavoglaucin, aspergin, isodihydroauroglaucin, isotetrahydroauroglaucin, neoechinuline A and physcion. Neoechinuline A, isodihydroauroglaucin and isotetrahydroauroglaucin showed activity against oesophageal and cervical cancer cell lines.

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HPLC	High performance liquid chromatography
TSP-LC-MS	Thermospray–liquid chromatography–mass spectrometry
LC-NMR	Liquid chromatography-nuclear magnetic resonance
LC-UV-MS	Liquid chromatography–ultraviolet–mass spectrometry
HPLC-MS	High performance liquid chromatography–mass spectrometry
HPLC-DAD	High performance liquid chromatography diode array detection
ESI-MS	Electrospray ionization-mass spectrometry
FAME	Fatty acid methyl ester
TLC	Thin layer chromatography
GC-MS	Gas chromatography-mass spectrometry
HPLC-ESI	High performance liquid chromatography–electrospray ionisation
SEM	Scanning electron microscope
rDNA	Ribosomal deoxynucleic acid
EtOH	Ethanol
H_2O	Water
EtBr	Ethidium bromide
EtoAc	Ethyl acetate
DMSO-*d6*	Deuterated dimethyl sulfoxide
dddH_2O	Triple distilled water
YPG	Yeast extract, peptone, glucose
ASW	Artificial Sea Water
SWA	Seawater agar
dH_2O	Distilled water
CH_2Cl_3	Chloroform
DMSO	Dimethyl sulfoxide
MeOH	Methanol
ACN	Acetonitrile
$CDCl_3$	Deuterated chloroform
Cmpd	Compound
MW	Molecular weight
^{1}H NMR	Proton nuclear magnetic resonance
ETS	External transcribed region
rRNA	Ribosomal ribonucleic acid

ITS1	Internal transcribed spacer 1
BLAST	Basic local alignment search tool
ITS2	Internal transcribed spacer 2
mtDNA	Mitochondrial DNA
PCR	Polymerase chain reaction
MF	Molecular formula
RP	Reversed-phase
HTS	High through-put
NCI	National Cancer Institute
SIO	Scripps Institute of Oceanography
°C	Degrees Celsius
1D	One Dimensional
2D	Two Dimensional
t	Triplet
sp.	Species
s	Singlet
q	Quartet
m	Multiplet (in connection with NMR data)
br	Broad
d	Doublet
COSY	Correlated Spectroscopy
DEPT	Distortionless Enhancement by Polarisation Transfer
HMBC	Hetero-nuclear Multiple Bond Correlation
HMQC	Hetero-nuclear Multiple Quantum Coherence
Hz	Hertz
J	Spin-spin coupling constant [Hz]
ppm	Parts per million

Chapter 1:
Review of Literature

Microorganisms have rapidly gained resistance to antibiotics due to the inappropriate use of these drugs. Consequently, what was once considered a routine infection can now become fatal. For example, enterococci, traditionally regarded as low-grade pathogens, are now the third most common cause of nosocomial infections in the United States. In 1941, virtually all strains of *Staphylococcus aureus* were sensitive to penicillin G. By 1992, 95% of these strains were antibiotic resistant. Even the primary drug of the last resort for methicillin-resistant *S. aureus*, the glycopeptide vancomycin, has been rendered ineffective in at least three cases (Jensen and Fenical, 2000).

In addition to microbial pathogens, genetic diseases such as cancer continue to be of concern in the 21st century. Currently, the treatment of cancers still involves surgery, radiation and chemotherapy. Chemotherapy has been one of the major therapeutic methods commonly used as treatment for a variety of cancers (Han *et al.*, 1998). However, in many cases, chemotherapy alone cannot achieve a satisfactory therapeutic outcome, namely the complete remission of tumours and prevention of metastasis. In addition, it is difficult to protect healthy cells from the harmful effects of cancer treatment which induces severe side-effects at therapeutically effective doses (Sallager and Lodge, 1998). The side-effects mainly depend on the type and extent of the treatment. Some anticancer drugs can cause side-effects such as loss of fertility, nausea, diarrhea, loss of hair and the reduction of white and red blood cells and platelets. Due to this there is a desperate need for new and improved drugs on the market.

1.1 Drug Discovery and Natural Products Chemistry

There are three main approaches for searching for drugs in a drug discovery programme:

a) Rational Drug Design: Using bioinformatics and molecular modeling drugs are designed to target specific receptors in cells.
b) Synthetic and Combinatorial Chemistry: Manipulating an existing drug or natural product to improve on its pharmacological profile.
c) Natural Product Programme and Combinatorial Biosynthesis: Screening natural products for pharmacological activity and the use of protein/gene engineering to alter biosynthetic pathways thus increasing natural product diversity.

The most successful strategy thus far in finding new drugs has proven to be through natural products research. Natural products, or secondary metabolites, have been exploited by man for a variety of purposes including its use as food, fragrances, pigments, insecticides and

medicines. Historically, plants have served as a major source of medicinally useful natural products, developed from a legacy of folk medicine based on herbal remedies (Carte, 1993). The first hymns of the Sanskrit text *Artharva-veda Samhita*, (~2100 BC) mention destroying "invisible worms" in the body and suggest using a plant growing on the tree (fungus or lichen) to cure infections (Galperin and Koonin, 1990). Since then humans have been utilising various naturally occurring chemical substances with biological activity. Even with the advent of combinatorial chemistry, molecular biology and high throughput screening (HTS), the use of natural products has been the single most successful strategy for the discovery of new medicines (Nisbet and Moore, 1997; Bindseil *et al*, 2001). It is unlikely that rational drug design would have produced many of the complex structures of the therapeutically useful natural products (Lawrence, 1999).

Important natural product derived drugs include cyclosporin, avermectin (antiparasitic), taxol and camptothecin derivatives, (anticancer), erythromycin (antibiotic) and lovastatin derivatives (hypercholesterolaemic agents) (Nisbet and Moore, 1997; Bindseil *et al*, 2001). Towards the end of the 1980's, approximately 75% of the top 20 hospital drugs and 20% of the top 100 most widely prescribed drugs were derived from natural sources. These mostly included antibiotics from microbial fermentation broths and alkaloids from plants (McConell, 1994). Therefore due to their success, natural products are likely to continue as a source of commercially viable drug leads.

Despite the enormous structural diversity, Nature only uses a few basic building blocks. These are acetate, mevalonic acid and shikimic acid. The acetate unit is used in polyketide biosynthesis, while mevalonic acid leads to all terpenoids. However, a mevalonic acid independent pathway - leading to terpenes is well established in bacteria (Eisenreich *et al.*, 1998; Rohmer, 1999; Lichtenthaler, 1999). Shikimic acid leads to the synthesis of aromatic amino acids, cinnamic acids, lignin and lignans. The structural diversity found in natural products results from various enzymes found in each species that introduce new functionalities, such as hydroxyl, epoxy and methoxy groups (Verpoorte, 2000). Table 1.1 shows the major classes of known natural products and clearly illustrates that the terpenoids and the alkaloids are the most abundant. The latter group in particular contains a large number of drugs due to their special characteristic of being water-soluble under acidic conditions and having lipophilic properties under neutral and basic conditions (Verpoorte, 2000).

Table 1.1: Number of Secondary Metabolites from all Organisms in the "Dictionary of Natural Products" (Verpoorte, 2000).

Metabolites	Number
Aliphatics	5 950
Polyketides	2 753
Carbohydrates	3 397
Oxygen Heterocycles	1 484
Simple Aromatics	5 041
Benzofuranoids	444
Benzopyrenoids	2 859
Flavanoids	8 405
Tannins	783
Lignans	1 729
Polycyclic Aromatics	2 621
Terpenoids	30 500
Amino acids, Peptides	4 303
Alkaloids	16 833

While some scientists view natural products as "waste products", others propose that they are produced to provide an advantage to the organism or would have once served a role. Faulkner (2000) hypothesised that organisms that do not contain sophisticated immune systems use natural products or allelochemicals, in response to a variety of ecological, behavioural and physiological factors. In order to survive and reproduce in their environments, sessile organisms must produce compounds that are used to chemically defend themselves and/or to assist in the prevention of overgrowth or fouling because they lack physical defenses. This view was acknowledged by da Rocha *et al.*, (2001) who suggested that organisms evolve due to a selective advantage they obtain as a result of interactions of the natural products with specific receptors in other organisms. Firn and Jones (2003) have recently developed a new model to explain (1) why there is chemical diversity, (2) how it is generated and (3) how it is maintained although there may not be any direct role for the compound. Their hypothesis is that organisms produce a diversity of compounds to increase their chances of producing a compound with potent biological activity. Those organisms which can reduce their metabolic costs (cost of generating and retaining products of no current value) will have an advantage over their competitors. These "no value" compounds are produced by multiple pathways and act as intermediates

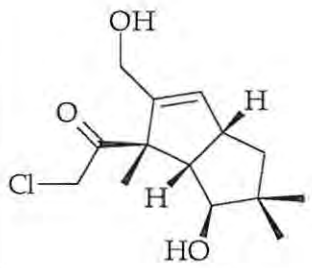
to the final metabolic product, thus increasing the length of the pathway and the metabolic diversity.

Whatever the reasons are for their production, some of these secondary metabolites have therapeutic potential against human diseases because of specific interactions with receptors and enzymes. According to Harvey (2000) specialised cells such as nervous, immune and vascular systems, appeared relatively recently in evolution, while the transmitter molecules they use, may have arisen much earlier in unicellular or simple multi-cellular organisms.

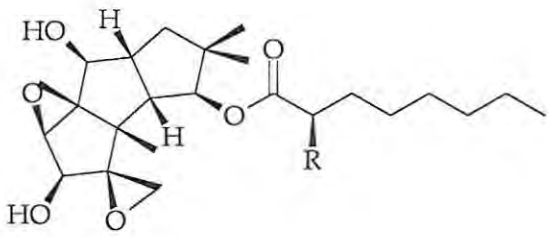
1.2. Marine Natural Products

Traditional bioprospecting sources, i.e. terrestrial plants and soil microbes, have in recent years produced a high percentage of known compounds resulting in a search for new natural product sources. There has been a tremendous level of interest in the marine environment from both academia and industry because of the extensive speciation at all phylogenetic levels, from mammals to microorganisms (Cowan, 1997). This has resulted due to extreme variability of physical conditions such as temperatures of between 1.5°C to 350°C, pressures ranging from 1 to over 1000 atmospheres, nutrient variations ranging from eutrophic to oligotrophic, and extensive photic and non-photic zones.

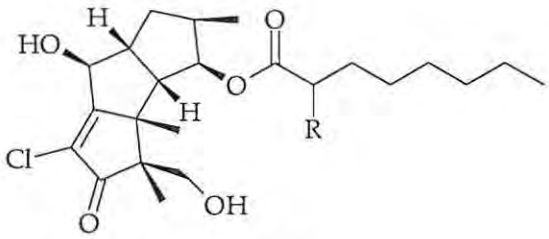
A large number of compounds possessing unique structural features that are not found in terrestrial natural products have been isolated from marine sources (Carte, 1993; Davidson, 1995). One of the most obvious differences between terrestrial and marine natural products is the frequent incorporation of halogens, particularly bromine into the latter. This is due to an adaptation to the marine environment which contains ca. 19g.L^{-1} of chloride and 65mg.L^{-1} bromide (Thompson, 1978). This is exemplified by the isolation of chloriolins A - C (**1.1** - **1.3**), from a marine-derived fungus. These compounds are related to coriolin B (**1.4**) and dihydrocoriolin C (**1.5**), previously isolated from the terrestrial wood rotting fungus *Coriolus consors*. The marine isolates were different in that the marine-derived coriolin derivatives were chlorinated (Davidson, 1995) (Fig. 1.1).



1.1



1.4: R = H
1.5: R = OH



1.2: R = OH
1.3: R = H

Fig. 1.1: Coriolins.
Chloriolin A, B and C isolated from the marine environment.
Coriolin B and dihydrocoriolin C from a terrestrial fungus.

1.2.1. Industrial Applications of Marine Natural Products

Marine natural products have contributed to health-food additives, materials for orthopeaedics, enzymes for molecular biology and bioadhesive materials, all of which are in the market. According to Bongiorni and Pietra (1996), 80% of all patent applications in this area are from the health and health food sector. While drug discovery is dominated by antitumoural and antiviral agents (reflecting the most popular screening systems in use), materials for bone surgery, immunoregulatory agents, anti-inflammatories and antibiotics have also made significant contributions and show potential for commercialism. Table 1.2 shows some of the commercially available marine bioproducts.

Table 1.2: Commercially Available Marine Bioproducts (Bongiorni and Pietra, 1996; McCarthy and Wright, 1997; Pomponi, 1999).

Product	Application	Source
Arabinosyl -A	Antiviral drug	Marine sponge, *Cryptotethya crypta*
Arabinosyl - C	Anticancer drug	*C. crypta*
Okadaic acid	Molecular probe, phosphatase inhibitor	Dinoflagellate
Manoalide	Molecular probe phospholipase A2 inhibitor	Marine sponge, *Luffariella variabilis*
Vent™ DNA polymerase	PCR enzymes	Deep-sea hydrothermal vent bacterium
Resilience (Pseudopterosin C)	Cosmetic by Estee Lauder	*Pseudopterogorgia elisabethae*
Formulaid	Infant food additive	*Crypthecodinium cohnii*
Trans/cis-β-carotene	Food pigment	*Dunaliella* sp.
Cartap hydrochloride	Insecticide	Annelid
Cell-Tak	Bioadhesive	*Mytilus edulis* (mussel)
Green Fluorescent Protein (GFP)	Molecular tool	*Aequorea victoria* (jellyfish)
Formulaid R	Fatty acids, additive in infant nutritional supplement	Marine macroalgae

Initially research targeted organisms such as gorgonians, sea hares, nudibranchs, sponges, molluscs, ascidians, bryozoans, soft corals and algae. Data from the National Cancer Institute (NCI), U.S.A (Fig. 1.2) clearly indicate that marine animals display the highest incidence of significant cytotoxic activity (Munro *et al.*, 1999). However many of the compounds isolated are produced in small quantities and industrial synthesis is hampered because of their complex structures. Thus acquisition of material required for biological evaluation has relied on massive collection efforts, a process which, even when possible, is time-consuming, expensive and potentially damaging to the fragile reef habitat (Davidson, 1995; Borris and Gould, 1999; Jensen and Fenical, 2000). No matter how attractive a biological profile of a compound may be, unless an adequate supply can be generated for testing and thereafter for commercial sales, the compound will not progress on to the next step (Munro *et al.*, 1999). The most famous example of a compound in this situation is Halichondrin B from *Halichondria okadai* (Hirata and Uemura, 1986), which is a potent antimitotic agent. Due to these bottlenecks, research has turned to marine microorganisms

as a source of novel chemicals with therapeutic value as fermentation yields a continuous source of material.

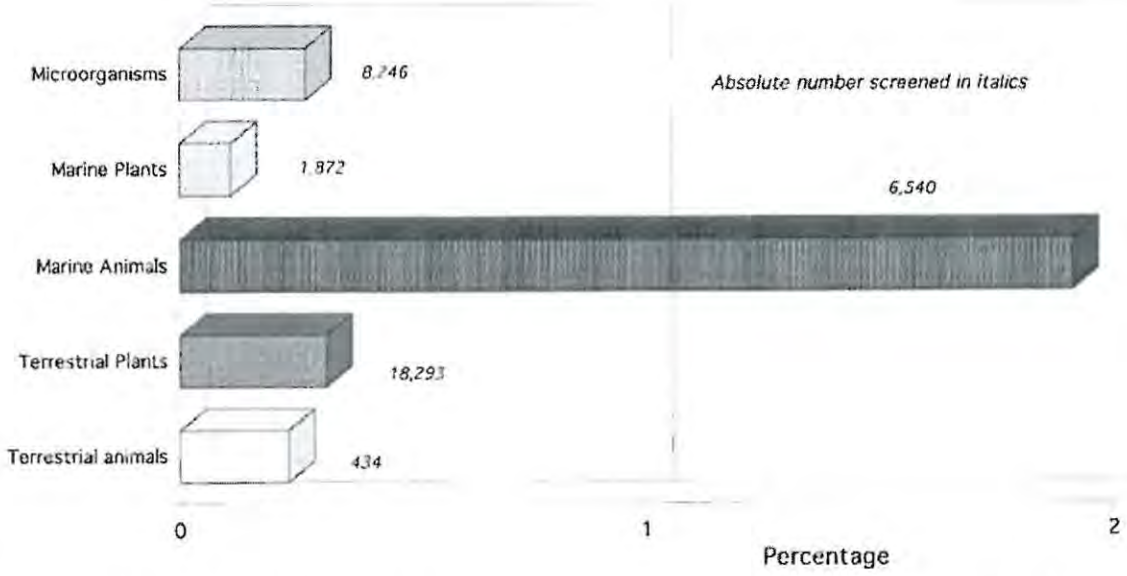


Fig. 1.2: Distribution of Samples with Significant Cytotoxicity at the NCI's Preclinical Screens (Munro *et al.*, 1999).

1.2.2. Marine Microorganisms

Since the discovery of penicillin in 1929, intensive studies have shown that microorganisms are a rich source of structurally unique bioactive substances. Over 50 000 natural products have been discovered from microorganisms of which more than 10 000 of these are biologically active and more than 8 000 are antibiotics and antitumour agents (Betina, 1983; Berdy, 1989). Due to this, marine microorganisms, have received increasing attention over the past decade. These microorganisms have been isolated from marine sediments, water samples, decaying pieces of drift wood, from marine algae (seaweed) and macroorganisms (McConell *et al.*, 1994; Faulkner, 2000). In fact, it has been repeatedly suggested that symbiotic microorganisms isolated from macroorganisms are the true source of these bioactive metabolites (Faulkner, 2000). For example, Cyrindramide isolated from a sponge, is closely related to the cytotoxic alkaloid alteramide A, produced by the sponge-derived *Alteromonas* sp. (Jensen and Fenical, 2000).

1.2.2.1. Marine Fungi

Kohlmeyer and Kohlmeyer (1979), defined obligate marine fungi as those growing and sporulating exclusively in a marine and estuarine habitat. Facultative marine fungi are those from freshwater or terrestrial milieus that are able to grow and possibly sporulate in the marine environment. Marine fungi that do not germinate in the natural marine habitat are not included in this definition. Marine fungi have been poorly investigated. By 1991, only 321 species of marine mycelial fungi worldwide had been identified, represented by 225 Ascomycetes, 6 Basidiomycetes and 60 Deutromycetes (Kohlmeyer and Volkmann-Kohlmeyer, 1991). Now with results from ribosomal RNA (rRNA) sequencing, it has been predicted that less than 1% of the naturally occurring microbial diversity has been cultured. Conservative estimates also suggest that there are likely to be over 1.5 million fungal species (terrestrial and marine), of which less than 5% have been described (Gloer, 1997).

Marine fungi live as saprobes on algae, driftwood, decaying leaves and other dead organic material of plant and animal origin (Davidson, 1995; Libierra and Lindequist, 1995). They may also occur as parasites on mangroves, shells, crabs, sponges, fish and plants and are an important group of pathogens in the marine world (Libierra and Lindequist, 1995). Other species exist as symbionts in invertebrates and plant material. Fungi have also been found on coral reefs (Volkmann-Kohlmeyer and Kohlmeyer, 1992). Among the marine fungi, those living in association with marine algae are a particularly promising source of novel natural products. Fungi commonly thrive in competitive environments, and it is often hypothesised that some of their secondary metabolic capabilities may be influenced by selection pressures exerted by other organisms (Gloer, 1997). The association between fungi and algae seems to be highly developed since nearly one-third of all higher marine fungi described are so-called algicolous or algae-associated organisms (Abdel-Lateff *et al.*, 2002). Fungal representatives from all subdivisions of the Eumycota, i.e. Ascomycotina, Deuteromycotina, Basidiomycotina, Zygomycotina, and Mastigiomycotina, are found growing on algae (Stanley, 1992). The surfaces of marine algae are good nutrient-rich sources of microorganisms e.g. *Penicillium* sp. was found on *Enteromorpha intestinalis* A, which produced the novel polycyclic alkaloids communesins A (**1.6**) and B (**1.7**) (Numata *et al.*, 1993).

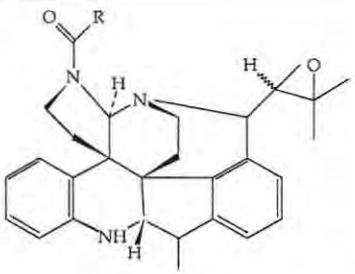


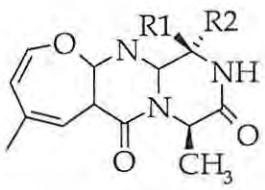
Fig. 1.3: Communesins A and B.
1.6: R= CH_3
1.7: R= CH=CH-CH=CH-CH_3

These nitrogen containing heterocyclic compounds also exhibited moderate to potent cytotoxic activity in the P-388 lymphocytic leukemia test system with ED_{50} values of 3.5 and 0.45μg/mL, respectively (Libierra and Lindequist, 1995). Another algicolous fungus, *Wardomyces anomalus* isolated from *Enteromorpha* sp. showed antimicrobial effects towards *Microbotryum violaceum* and *Eurotium repens*, inhibition of HIV reverse transcriptase and inhibition of $p56^{lck}$ tyrosine kinase (Abdel-Lateff *et al.*, 2003). Hydroquinone derivatives from an algicolous *Acremonium* sp. were also found to have significant antioxidant properties (Abdel-Lateff *et al.*, 2002).

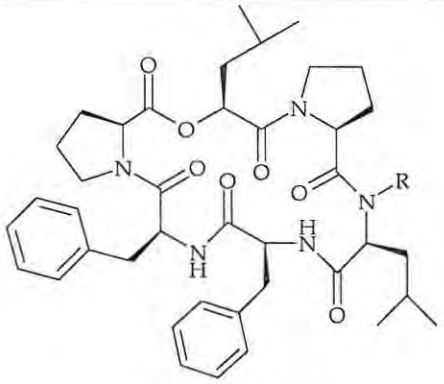
In comparison with secondary metabolites from other marine organisms such as algae, the number of metabolites isolated from marine fungi is small (Libierra and Lindequist, 1995). It has been estimated that less than 1% of potentially useful chemicals from the marine environment have been screened thus far, with microbial products representing approximately 1% of that total number (Colwel, 1993). Of the compounds isolated, the predominant activities, have been compounds with antibacterial, antifungal and cytotoxic activities (Libierra and Lindequist, 1995). Some of the most popular examples are highlighted in Table 1.3.

Table 1.3: Some Metabolites Isolated from Marine Fungi

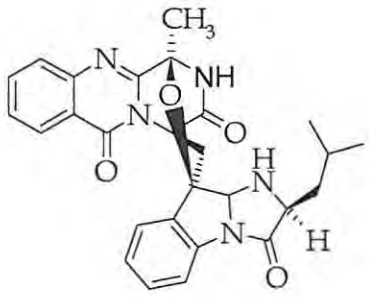
Fungus	Metabolite isolated	Reference
Penicillium sp.	Communesins A (**1.6**) and B (**1.7**)	Davidson, 1995
Acremonium sp.	Oxepinamide A – C (**1.8 – 1.10**), Fumiquinazoline H (**1.11**)	Belofsky *et al.*, 2000
Scytalidium sp.	Exumolidse A (**1.12**) and B (**1.13**)	Jenkins, 1998
Aspergillus sp.	Chlorocarolide A (**1.14**)	Abrell *et al.*, 1996
Spirastrella vagabunda	14,15-secocurvularin (**1.15**)	Abrell *et al.*, 1996
Aspergillus sp.	Aspergilloxide (**1.16**)	Cueto *et al.*, 2002
Ascochyta salicorniae	Ascosalipyrrolidinone A (**1.17**)	Osterhage, 2000
Fusarium sp.	Sansalvamide (**1.18**)	Belofsky *et al.*, 1999



1.8: $R_1 = CH(CH_3)CH_2\text{-}CH_3$; $R_2 = OH$
1.9: $R_1 = OH$; R2 $CH(CH_3)CH_2\text{-}CH_3$
1.10: $R_1 = CH_2CH(CH_3)_2$; R2 = OCH_3



1.12: R = CH_3
1.13: R = H



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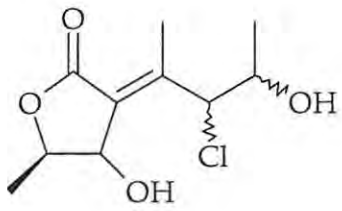

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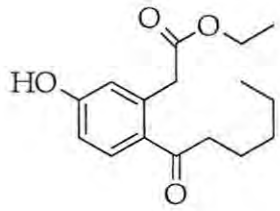
Fig. 1.4: Marine-derived Compounds



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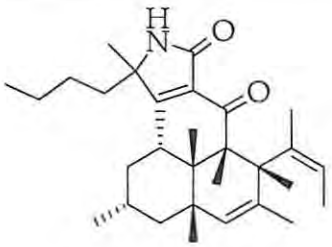

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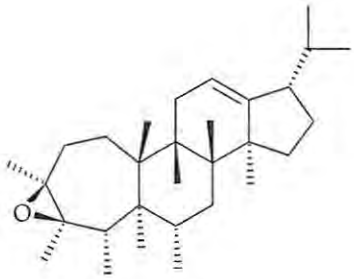

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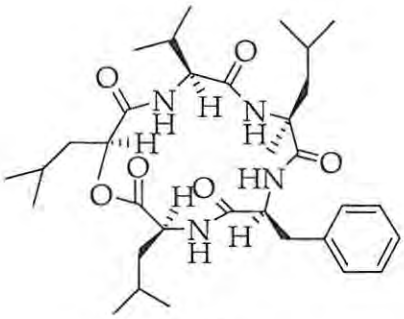


1.18

Fig. 1.4: Marine-derived Compounds CONTD.

1.2.3. South African Marine Natural Products

There are large areas of South Africa's marine environment where biological communities are poorly described at the species level due to a shortage of taxonomists (Pauw and Durham, 2003). In particular, very few studies on marine microorganisms have been undertaken. By 1993, only three research papers on marine fungi collected off the coast of South Africa were published. Gorter (1978), collected ten species from the Atlantic coast; Jones *et al.* (1972), collected three species from the Indian Ocean and Kohlmeyer and Kohlmeyer, (1971), collected nine species from the Indian Ocean, all mainly from the Durban region. Steinke was the only local person researching marine and estuarine fungi up until then. His studies concentrated on lignicolous fungi on driftwood. Currently, Coyne at the University of Cape Town is involved in the identification and characterisation of marine microbes. Thus the southern oceans, in particular the South African marine environment poses a promising frontier for the discovery of new therapeutics.

Like many developing countries, the biodiversity of South Africa provides a very valuable resource (Cowan and Burton, 2002). Given that 99% of existing microbial species in any environment have not yet been cultured, and that the environmental diversity offers a huge

range of microbial habitats, it can be predicted with some confidence that this country harbours millions and possibly tens of millions of new microbial genomes. This has not escaped the attention of international bioprospecting companies, who have already initiated programmes investigating South African biodiversity (Cowan and Burton, 2002).

1.3. Approaches to Natural Products Drug Discovery

Natural product drug discovery can be separated into three categories:

a) Chemically driven: Isolating new chemical entities followed by assessment of biological activities for purified compounds
b) Biologically driven: Bioassay-guided fractionation beginning with crude extracts
c) Combination of chemically and biologically driven approaches

The chemically driven approach has been pursued vigorously, primarily by academic research groups. The number of compounds found in various reviews attest to the success of this approach. Over 1200 pure compounds have been isolated in the laboratories of two pioneers in marine natural products chemistry, William Fenical and D. John Faulkner and although some of these compounds were isolated based on the antibacterial, cytotoxic and/or anti-inflammatory activities of the respective extracts, many were isolated based on purely chemical, chemotaxonomic, or chemical ecological interests (McConell *et al.*, 1994).

Although most of the approaches are directed towards the effective use of new organisms, the most important paradigm shift for natural product chemistry is the general change from activity-guided extract screening (although still widely used) to pure-compound screening. This is the approach MEGAbolite® has adopted and successfully used (Bindseil *et al.*, 2001). The idea to screen extracts from natural sources by neglecting desired biological activities of metabolites in the first stage of screening was postulated by Umezawa in the late 1970's and further developed by Zahner and co-workers (Bindseil *et al.*, 2001). This approach has been mainly adopted by academic institutions because the bioassay-fractionation route requires extensive, time-consuming and costly deconvolution. This results from interfering substances in the crude extracts which cause false positives. Although there are methods to maximise efficiency by dereplication, these require a library of compounds and in-house databases which are expensive. In contrast, the pure compound screening approach is cheaper and permits the isolation of metabolites which can thereafter be screened in various bioassays or the activity predicted by structure-activity-relationships. The disadvantage is

that only those compounds which are produced in abundance (> 1mg for structure elucidation) can be studied.

1.4. Bioprospecting

Bioprospecting is the search for organisms that may potentially serve as a source of natural products. Conventional bioprospecting screening programmes search for these natural products randomly, however nowadays the exploration of metabolites on the basis of ecologically sound observations is gaining support. This method provides a more direct route to the discovery of useful metabolites (de Nys and Steinberg, 2002). The recent efforts of several industrial explorations into the coral reefs i.e. where a large variety of sessile organisms populate (Bongiorni and Pietra, 1996), has confirmed that hypothesis-based studies to be highly lucrative. This type of non-random bioprospecting facilitates dereplication and permits evaluation of a hypothesis.

1.5. Bottlenecks in Marine Natural Products/Biotechnology Industry

There is a need for more effective and rigorous methods of development in marine natural products research especially if it is to successfully make a profound impact on the provision of drug leads. These areas of concern have been highlighted as:

a) Bioprospecting: New niches need to be investigated for new species to be studied
b) Isolation Methods: More effective strategies are needed for the isolation and culturing of marine microorganisms
c) Quick and reliable methods of dereplication of the source organism and of the metabolites isolated
d) Optimisation of production of metabolites
e) Faster and simpler techniques are required for extraction, purification and analyses of metabolites

1.6. Aims and Objectives of this Study

The discussion above has highlighted the need for new drugs and draws attention to some of the main problems associated with the discovery of new drug leads from the marine environment. It is clear that one of the major challenges for Marine Biotechnology is to provide biologically active marine natural products in sufficient quantity for complete pharmacological evaluation, clinical trials and eventual commercial production. Thus, the

main aim of this study was to develop and evaluate methods for the isolation, identification and cultivation of marine fungi from the South African marine environment for the production of biologically active secondary metabolites.

Thus, the specific objectives are:

1. To isolate marine epiphytic and endophytic fungi from seaweed using selective media
2. To identify fungal isolates using DNA analyses and morphology
3. To optimise metabolic creativity and productivity
4. The isolation and structure elucidation of secondary metabolites produced
5. The bioactivity screening of metabolites

Chapter 2:

Isolation, Small-scale Cultivation and Screening of Marine Fungi

2.1. Introduction

A natural products drug discovery programme from microorganisms is essentially an integrated approach of Microbiology, Biotechnology, Natural Product Chemistry and Pharmacology. The first step in the discovery programme is the isolation of the microbe from Nature. This involves selective isolation media to isolate only a certain type of microbe e.g. gram positives, gram negatives, fungi etc. Once isolated and purified, the microbe can be identified via a number of different methods. The biotechnologist optimises fermentation conditions by medium and culture technique development for the production of metabolites and the natural product chemist extracts, purifies, identifies and then screens these metabolites for potential biological activity. A further crucial aspect of drug discovery is the availability of suitable biological assays in order to assess the pharmacological potential of isolated natural products. The primary screening of extracts, although essential for dereplication and the discovery of samples with "interesting chemistry" can be time consuming and costly.

Fungi associated with marine algae have been proven to be rich sources of microorganisms (Burgess *et al.*, 1999) and several bioactive metabolites have already been isolated from algicolous fungi.

The main objectives of the research presented in this chapter were to:
(a) Selectively isolate fungi associated with marine algae,
(b) Cultivate isolated fungi for metabolite production, and
(c) Screen extracts obtained for "interesting chemistry" and biological activity.

2.2. Materials and Methods

2.2.1. General Experimental

All extraction solvents were redistilled. Deuterated chloroform ($CDCl_3$) was used for all ^1H NMR analyses using a Bruker Avance Nuclear Magnetic Resonance Spectrometer (400MHz) at 27°C. All media reagents were obtained from Merck. Seawater was obtained from Port Alfred and filtered before use.

2.2.2. Collection of Marine Macroalgae

Ten species of red marine macroalgae were collected from the intertidal zone at the Three Sisters near Port Alfred on the Eastern Cape coast of South Africa. The seaweeds were stored in separate sterile plastic bags at -20°C before inoculating them onto solid media.

Fig. 2.1: Sampling Site

2.2.3. Isolation of Fungi

Two different media were used for the isolation of fungi. These were composed of (a) 0.5% yeast extract, 0.5% peptone, 1% glucose and 1.7% bacteriological agar made up to 1000mL of natural sea water (YPG Basal medium) and (b) 1.7% bacteriological agar made up to 1000mL sea water (SWA). After autoclavation, the media were supplemented with 75mg/L penicillin (Penilente-Forte) and 75mg/L streptomycin (Novo-Nordisk) by 0.22µm filter sterilisation.

The seaweeds were rinsed in sterile seawater. Both epiphytes and endophytes were selected for, as described by Hunter-Cevera and Belt, (1999). For the selection of epiphytes, the algae were cut into 0.5cm^3 and embedded into the agar. For the selection of endophytes, the surface of the seaweeds were sterilised with 70% ethanol for 5 minutes. The seaweeds were then shredded using a sterile blade to expose the inner tissue and inoculated on agar plates. As a control for contaminating epiphytes, the unshredded surface-sterilised seaweeds were also inoculated. Plates were then incubated at 24°C until fungal growth. All fungi were subcultured to fresh solid media to attain pure cultures.

2.2.4. Growth Kinetics

The growth characteristics of the fungi were studied by measuring the absorbance, as described by Langvad, (1999). Six mycelial circles (five day cultures) of diameter 7mm were cut out from YPG mycelial mats using pasteur pipettes and transferred to 100mL Erlenmeyer flasks containing 30mL sterile seawater. The mycelia were homogenised using a Janke and Kinkel IKA-Werk Ultra-Turrax blender for 3 x 0.5s bursts. 20µL of the homogenate and 180µL of the YPG medium were used to inoculate Costar 3595 (Promex) 96-well microtitre plates. 20µL of seawater was added instead of the homogenate for controls. Plates were inoculated in triplicate for each isolate and incubated at 24°C. The absorbance was measured at 630nm twice daily using a Powerwave X microtitre plate reader. To confirm these results, the broth was extracted at the onset of stationary phase to assay for reducing sugars using the Somogyi-Nelson Glucose Assay (Appendix A).

2.2.5. Cultivation of Fungi

250mL YPG media in 500mL Erlenmeyer flasks were used for static and agitated cultures. 5 x 0.5cm^3 pieces of five day old mycelia maintained on YPG agar were used as inoculum. Agitated cultures were shaken on a Labcon flat bed shaker at 125 rpm at 24°C until the onset of stationary phase. The onset of stationary phase was identified by the production of spores and confirmed by growth kinetic studies.

2.2.6. Metabolite Extraction

At the onset of stationary phase (idiophase), the mycelia and broth were separated by centrifugation at 10 000rpm for 20 minutes. The broth was extracted thrice with 150mL EtOAc and dried over Na_2SO_4 (anhydrous). The mycelia were crushed using liquid nitrogen, extracted with 100mL MeOH for 2 hours then MeOH/dichloromethane (50mL:50mL) overnight and again with MeOH overnight. The broth and mycelial extracts were then dried *in vacuo* using a Buchi rotavapour RE 120. The crude extracts were then analysed by ^{1}H NMR and evaluated using a metabolic creativity index. The assignment of metabolic indices were based on quantitative measurements i.e. peak numbers and their respective chemical shifts. Both the inoculated broth and agar media were used as controls.

2.2.7. Brine-shrimp Assay

Brine-shrimp (*Artemia salina*) eggs were obtained from PRO 100; Ocean Star, International Inc., Utah and hatched in a cone-shaped glass container containing aerated sterile natural seawater at 29°C with artificial light for 24 hours. Phototrophic naupli were collected from the lightened side by pipette.

Extracts were dissolved in absolute EtOH (100µL) with the aid of a sonicator and was used to make up a stock solution of 10mg/mL. 100µL of this solution was added to 650µL of sterile seawater. 150µL of the resulting working solution (1mg/750µL) was used to inoculate row A in microtitre plates (Costar 3595, Promex). Serial dilutions were made to attain final concentrations of 570µg/mL; 285µg/mL; 142µg/mL and 71µg/mL. A suspension of naupli containing 10-15 organisms (100µL) was added to each well. The plates were incubated at 29°C with the lids on for 24 hours. Naupli were then examined with a dissecting microscope (x12) and the numbers of dead naupli in each well recorded. 100µL of MeOH was added to each well to sacrifice the remaining shrimp and after 20 minutes the total number of shrimp in each well was counted. All experiments were done in duplicate with solvent controls.

2.2.8. Anti-cancer Assays

Crystal Violet Cytotoxicity Assay (Appendix A)

The Cytotoxicity Assay was performed at the Department of Medical Biochemistry, University of Cape Town, courtesy of Dr Hendricks. Briefly, compounds were tested at concentrations 50µg/mL, 10µg/mL and 1µg/mL 0.2% DMSO against WHCO1 (an oesophageal cancer cell line) and ME 180 (a cervical cancer cell line) (1500 cells/well) in CellStar 96 well microtitre plates. The plates were incubated at 37°C for 48 hours. Thereafter the media were replaced with MeOH (10 minutes) and then Crystal Violet Solution (20 minutes). The plates were washed for 60 minutes with distilled H_2O and then read at OD_{595} using an Anthos Microtitre Plate Reader. Solvent and media controls were included.

2.2.9. Antibiotic Assays

The antibiotic tests were performed at Tygerberg Hospital/University of Stellenbosch. The surfaces of Mueller Hinton plates were inoculated with 0.5 McFarland turbidity with test

organism solutions (1.5 x 10^8 organisms/mL except for *Candida albicans* which was 1 x 10^6 organisms/mL). These included *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) *C. albicans* (#500312) and *Klebsiella pneumoniae* (#055725). All strains were methicillin resistant except for *S. aureus*. The surfaces were allowed to dry, wells were made and 5 and 10µL of a 100µg/mL of the extract in saline was tested against each organism. Plates were incubated at 28°C. Zones of inhibition were checked for after 24 hours.

2.3. Results and Discussion

2.3.1. Collection and Identification of Seaweeds

The following seaweeds (Table 2.1) were identified by Professor Lubke from the Department of Botany, Rhodes University.

Table 2.1: Provisional Identification of Seaweed Collected from the Three Sisters Beach

Collection no.	Provisional Identification
TS0101	Unidentified
TS0102	*Plocamium* sp.
TS0103	*Gracilaria backeri*
TS0104	*Galaxaura diesingiana*
TS0105	*Plocamium corallorhiza*
TS0106	*Gigortina* sp.
TS0107	Unidentified
TS0108	*Caryophyllum scalare*
TS0109	*Sargassum* sp.
TS0110	*Epymeria* sp.

2.3.2. Isolation of Fungi from Seaweed

The seaweeds were collected and stored separately in sterile bags to ensure that cross contamination of epiphytic fungi or contamination from the terrestrial environment did not occur. Sea Water Agar (SWA) medium, which is a nutrient poor medium, is only composed of seawater, containing trace elements and cell debris, and agar (a carbohydrate source). Therefore this medium permits the growth of fungi which are slower growing. In contrast, YPG medium is a high nutrient medium and supports the growth of fastidious fungi

because these out-compete the slower growing fungi. 17 species were isolated on YPG and 7 on SWA media (Table 2.2).

Table 2.2: Summary of Fungal Isolates.

Seaweed	Medium	Endo/ epiphyte	Colony Morphology	Code*
1.Unidentified (TS0101)	YPG	Epiphyte	Dark orange becoming brown, spore-former.	1Aa
	YPG	Epiphyte	Grey and woolly	1Ab
	YPG	Epiphyte	Grey and woolly	1Ac
	SWA	Epiphyte	White and sparse	S-1Aa
	SWA	Epiphyte	White with black stalks.	S-1Ab
2. *Plocamium* sp. (TS0102)	YPG	Endophyte	Pink and matted producing a pink pigment.	2B
	YPG	Epiphyte	White, dense and aerial with bubble-like formations.	2Aa
3. *Gracilaria beckeri* (TS0103)	YPG	Epiphyte	Orange/pink with stalks.	3A
4. *Galaxaura diesingiana* (TS0104)	YPG	Epiphyte	White and sparse becoming yellow when mature. Aerial and blanket-like	4Aa
	YPG	Epiphyte	Thin and matted. Orange/yellow with white margin.	4Ac
	YPG	Endophyte	Matted. Green with white margins. Produces a bright yellow pigment.	4B
	SWA	Epiphyte	White and transparent becoming dense and aerial on YPG media.	S-4A
5. *Plocamium corallorhiza* (TS0105)	YPG	Endophyte	Yellow and woolly.	5B
	SWA	Endophyte	Green and flat becoming white on YPG media.	S-5B
6. *Gigortina* sp. (TS0106)	YPG	Epiphyte	Green with white margins.	6A
7. Unidentified (TS0107)	YPG	Epiphyte	Velvety and green	7A
	SWA	Epiphyte	Light green and matted.	S-7A
	YPG	Epiphyte	White and wooly. Aerial.	7Ad
8. *Caryophyllum scalare* (TS0108)	YPG	Epiphyte	Velvety dark green with white margin. Matted.	8A
	SWA	Endophyte	Green and aerial.	S-8B
9. *Sargassum* sp. (TS0109)	YPG	Epiphyte	Yellow aerial colonies. Thick. Growth.	9Ab
10. *Epymeria* sp. (TS01010)	YPG	Epiphyte	Dense, grey and matted with white margin.	10Aa
	YPG	Epiphyte	Orange with white margin. Matted.	10Ac
	SWA	Epiphyte	White with black stalks but green on YPG media	S-10A

* Code: Number = seaweed number. A = epiphyte; B = endophyte; S = isolated on SWA medium.

By using both high and low nutrient media, a wider variety and different types of fungi can be isolated. Nineteen epiphytic fungi and five endophytic fungi were attained altogether. In order to live inside the seaweed, the endophytic fungus would have to penetrate the plant, being transferred to the host by spores or through plant vegetative propagules or enter it early on in the growing stages (Moore-Landecker, 1982; Saar *et al.*, 2001). Due to plants having a thick cuticle and epidermal wall that resists penetration, we can expect more epiphytic than endophytic fungi. However, the small number of endophytic fungi could also be due to a long period of surface sterilisation (five minutes) e.g. Hunter-Cevera and Belt, 1999, recommend surface sterilization of one minute. Endophytic fungi have been shown to be preferred by natural product chemists, not only because of the certainty of their origin, but also because their extracts are often biologically active, producing metabolites that are toxic to the pathogens and predators of its host (Wicklow, 1988; Gloer, 1999). However 80% of the highly metabolically productive fungi isolated in this study were epiphytes rather than endophytes. In addition, no fungus was isolated from more than one species of seaweed. It is rather interesting that different fungi were isolated from seaweed grown in the same area. The exact association between fungi and seaweed however requires further investigation.

2.3.3. Growth Kinetics

The growth curves (Fig. 2.2) were typical of fungi with a short lag phase when the fungus adjusts to the environment. This is followed by a comparatively long log phase of exponential growth of the biomass by cell proliferation, culminating in stationary phase (Turner, 1971). In some cultures, diauxic growth occurred (Fig. 2.3). This occurs when the organism switches from utilising one growth-limiting nutrient to another.

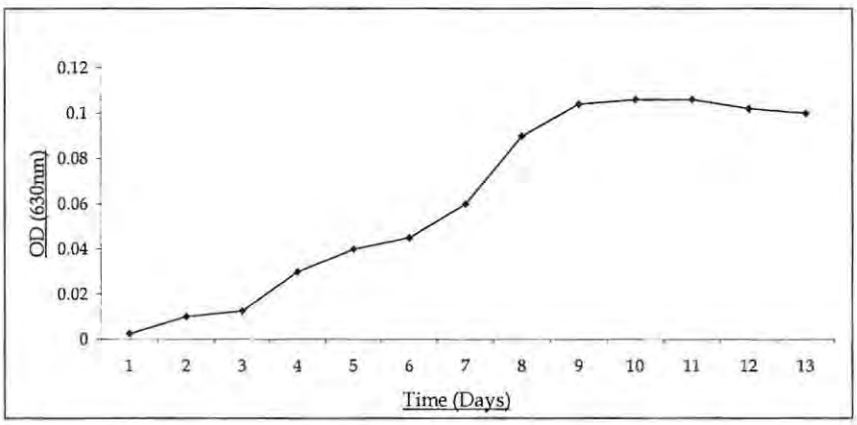


Fig. 2.2: Growth Curve of S-1Ab

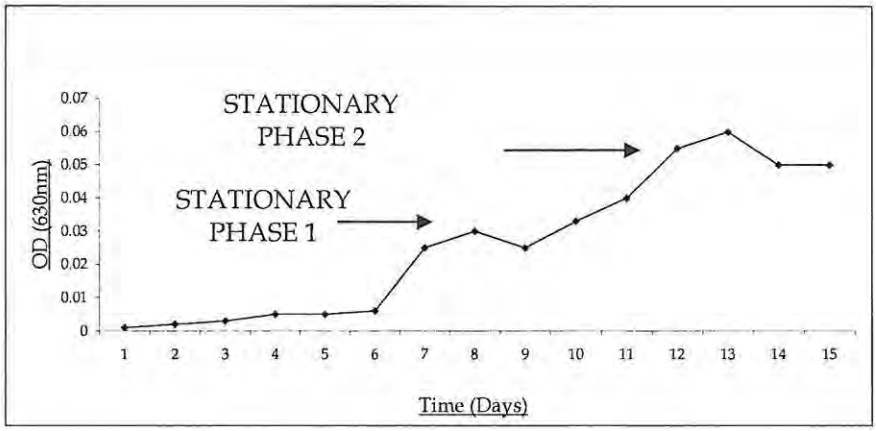


Fig. 2.3: Growth Curve of S-5Ba

Table 2.3: Growth Characteristics of Fungal Isolates on YPG Medium

Isolate	Growth Time (Days)			Glucose (µg/mL)
	Lag phase	Log phase	Stationary phase	
1Aa	1	10	13	0
1Ab	3	15	20	0
1Ac	2	14	18	0
S-1Aa	3	13	15	0
S-1Ab	2.5	10	14	0
2Aa	1.5	5	8	0
3A	3	13	18	0
4B	1.5	6	9	0
4Aa	1.5	6	9	0
S-4A	0.5	6	7	0
4Ac	3	13	15	0
S-5Ba	6	13	21	0
5B	3	15	19.5	0
6A	4	16	20	0
7A	2	18	22	0
S-7A	4	9	14	0
S-7Ad	4	10	14	0
8A	3	13	18	0
S-8B	4	10	19	0
10Aa	1	4	5.5	0
10Ac	2	13	16.5	0
S-10A	3	14	19	0

Although the colour of the some of the fungi influenced the absorbance, the relative values were used as an indication of the initiation of idiophase. Therefore the Somogyi-Nelson Assay was used to confirm the advent of stationary phase by checking the complete utilisation of reducing sugars (glucose) in the microtitre plate (Table 2.3) and the 250mL fermentation, assuming this to be the limiting nutrient. Once the reducing sugar has been

This information is crucial since the fermentation has to be long enough for secondary metabolites to be produced in stationary phase, but for the culture to be harvested before toxic metabolites are produced and the secondary metabolites of interest are degraded. The complete utilisation of glucose in cultures grown in flasks and the onset of idiophase shown by the growth curves of cultures in the microtitre plates show that these results correlate.

2.3.4. Cultivation of Fungi

There are many methods of cultivation and several different types of cultivation vessels. Types of cultivation methods include solid-state, liquid and bioreactors. However, in both industry and academia, small-scale liquid flask cultures are the most frequently used (Hilton, 1999; Buchs, 2001). Although these flask cultures suffer from a variety of limitations, the greatest one being that they are batch cultures and thus never in a steady-state, they are the most favourable because they are simple, cheap and small and therefore allow many fermentations to be run in parallel (Buchs, 2001). Because this was only a primary screening of extracts, flask culture was the most appropriate.

A B

Fig. 2.4: A: Mycelial mat resulting from static culture, B: Pellets resulting from shaken culture

Both static and shaken flasks were used. Although the use of static culture was simple, inexpensive and allowed for many fermentations to be conducted in parallel, the fungi formed an inhomogenous fungal mat (Fig. 2.4). This is because the mat contains mycelia in various stages of growth and in a variety of environments. The surface of the mat is exposed to more aerobic conditions than that which is below the surface of the medium. Thus nutrient uptake is only slowly replaced by diffusion from the lower parts of the medium. Static cultures also suffer from oxygen limitation because of the rate of gas transfer (Turner, 1971). In contrast, shaken cultures are efficient in the uptake of nutrients, resulting in rapid growth and better homogeneity, however, some mycelia may form pellets

aggregates (Fig. 2.4B). This results in the centre of these pellets having a lower nutrient uptake and oxygen level causing nutrient starvation and idiophase.

Because fungi produce both extracellular and intracellular secondary metabolites, both the broth, containing the extracellular metabolites, and the biomass, containing the intracellular metabolites were extracted and analysed for their metabolic creativity.

2.3.5. Screening of Fungal Extracts

Table 2.4 shows that cultivation conditions can have drastic effects on the metabolic creativity of an organism. For example metabolic creativity is much higher in shake flasks, (2Aa mycelium) and (4Ac broth) than static flasks, probably a result of more efficient nutrient and oxygen uptake. With most isolates (65%), the broth contained a larger diversity of metabolites than the biomass.

Table 2.4: Metabolic Creativity Index of Fungi Grown in Static and Agitated Cultures.

Seaweed	Code	Isolation media	Endo/ epiphyte	Culture	Extracted from	Metabolic Creativity (1-5)[1]
1. Unidentified	1Aa1	YPG	Epi	Static	Broth	4
	1Aa2	YPG	Epi	Shaken	Broth	4
	1A2B	YPG	Epi	Shaken	Mycelia	5
	1A1B	YPG	Epi	Static	Mycelia	5
	1Ab1	YPG	Epi	Static	Broth	5
	1Ab2	YPG	Epi	Shaken	Broth	4
	1Ab1B	YPG	Epi	Static	Mycelia	5
	1Ab2B	YPG	Epi	Shaken	Mycelia	2
	1Ac1	YPG	Epi	Static	Broth	2
	1Ac2	YPG	Epi	Shaken	Broth	3
	1Ac2B	YPG	Epi	Shaken	Mycelia	1
	1Ac1B	YPG	Epi	Static	Mycelia	1
	S-1Aa1	SWA	Epi	Static	Broth	1
	S-1Aa2	SWA	Epi	Shaken	Broth	1
	S-1Aa1B	SWA	Epi	Static	Broth	1
	S-1Aa2B	SWA	Epi	Shaken	Mycelia	2
	S-1Ac1	SWA	Epi	Static	Broth	3
	S-1Ac2	SWA	Epi	Shaken	Broth	3
	S-1Ac1B	SWA	Epi	Static	Mycelia	2

[1] 1= very low creativity; 2= low creativity; 3= satisfactory; 4= high creativity; 5= very high creativity

Seaweed	Code	Isolation media	Endo/ epiphyte	Culture	Extracted from	Metabolic Creativity (1-5)
	S-1Ac2B	SWA	Epi	Shaken	Mycelia	1
2. *Plocamium* sp.	2B1	YPG	Endo	Static	Broth	5
	2B2	YPG	Endo	Shaken	Broth	5
	2B1B	YPG	Endo	Static	Mycelia	1
	2B2B	YPG	Epi	Shaken	Mycelia	1
	2Aa1	YPG	Epi	Static	Broth	5
	2Aa2	YPG	Epi	Shaken	Broth	5
	2Aa2B	YPG	Epi	Shaken	Mycelia	4
	2Aa1B	YPG	Epi	Static	Mycelia	1
3. *G. beckeri*	3A1	YPG	Epi	Static	Broth	3
	3A2	YPG	Epi	Shaken	Broth	1
	3A1B	YPG	Epi	Static	Mycelia	1
	3A2B	YPG	Epi	Shaken	Mycelia	1
4. *G. diesingiana*	4Aa1	YPG	Epi	Static	Broth	5
	4Aa2	YPG	Epi	Shaken	Broth	3
	4A1B	YPG	Epi	Static	Mycelia	1
	4A2B	YPG	Epi	Shaken	Mycelia	1
	4Ac1	YPG	Epi	Static	Broth	1
	4Ac2	YPG	Epi	Shaken	Broth	5
	4Ac1B	YPG	Epi	Static	Mycelia	1
	4Ac2B	YPG	Epi	Shaken	Mycelia	1
	4B1	YPG	Endo	Static	Broth	5
	4B2	YPG	Endo	Shaken	Broth	4
	4B1B	YPG	Endo	Static	Mycelia	1
	4B2B	YPG	Endo	Shaken	Mycelia	3
	S-4A1	SWA	Epi	Static	Broth	1
	S-4A2	SWA	Epi	Shaken	Broth	1
	S-4A1B	SWA	Epi	Static	Mycelia	2
	S-4A2B	SWA	Epi	Shaken	Mycelia	2
5. *P. corallorhiza*	5B1	YPG	Endo	Static	Broth	3
	5B2	YPG	Endo	Shaken	Broth	3
	5B1B	YPG	Endo	Static	Mycelia	2
	5B2B	YPG	Endo	Shaken	Mycelia	1
	S-5Ba2B	SWA	Endo	Shaken	Mycelia	1
	S-5Ba1B	SWA	Endo	Static	Mycelia	3
	S-5Ba1	SWA	Endo	Shaken	Broth	1
	S-5Ba2	SWA	Endo	Static	Broth	2

Seaweed	Code	Isolation media	Endo/ epiphyte	Culture	Extracted from	Metabolic Creativity (1-5)
6. *Gigortina* sp.	6A1	YPG	Epi	Static	Broth	2
	6A2	YPG	Epi	Shaken	Broth	2
	6A1B	YPG	Epi	Static	Mycelia	2
	6A2B	YPG	Epi	Shaken	Mycelia	1
7. Unidentified	S-7A1	SWA	Epi	Static	Broth	5
	S-7A2	SWA	Epi	Shaken	Broth	1
	S-7a2B	SWA	Epi	Shaken	Mycelia	1
	S-7Aa1B	SWA	Epi	Static	Mycelia	1
	S-7Ad2B	SWA	Epi	Shaken	Mycelia	1
	S-7Ad2	SWA	Epi	Shaken	Broth	2
	S-7Ad1	SWA	Epi	Static	Broth	2
	S-7A1B	SWA	Epi	Static	Broth	2
	7A1	YPG	Epi	Static	Broth	3
	7A2	YPG	Epi	Shaken	Broth	2
	7A1B	YPG	Epi	Shaken	Mycelia	3
	7A2B	YPG	Epi	Static	Mycelia	3
8. *C. scalare*	8Aa1	YPG	Epi	Static	Broth	3
	8Aa1B	YPG	Epi	Static	Mycelia	1
	8A2	YPG	Epi	Shaken	Broth	2
	8A2B	YPG	Epi	Shaken	Mycelia	1
	S-8B1B	YPG	Endo	Static	Mycelia	1
	S-8B2	YPG	Endo	Shaken	Broth	1
	S-8B2B	YPG	Endo	Shaken	Mycelia	1
	S-8B1	YPG	Endo	Static	Broth	3
9. *Sargassum* sp.	9Ab1	YPG	Epi	Static	Broth	5
	9Ab2	YPG	Epi	Shaken	Broth	2
	9Ab1B	YPG	Epi	Static	Mycelia	2
	9Ab2B	YPG	Epi	Shaken	Mycelia	3
10. *Epymeria* sp.	10Aa1	YPG	Epi	Static	Broth	5
	10Aa2B	YPG	Epi	Shaken	Mycelia	1
	10Aa2	YPG	Epi	Shaken	Broth	4
	10Aa1B	YPG	Epi	Static	Mycelia	2
	S-10A1	SWA	Epi	Static	Broth	3
	S-10A2	SWA	Epi	Shaken	Broth	1
	S-10A1B	SWA	Epi	Shaken	Mycelia	2
	S-10A1B	SWA	Epi	Static	Mycelia	1
	10Ac1B	YPG	Epi	Static	Mycelia	1
	10Ac1	YPG	Epi	Static	Broth	3

In recent years, there have been a number of developments on the chemical screening of crude extracts including high performance liquid chromatography diode array detection (HPLC-DAD) (Frisvad, 1989); HPLC- electrospray ionization (HPLC-ESI) (Smedsgaard and Frisvad, 1996; Julian *et al.*, 1998); liquid chromatography-ultraviolet-mass spectrometry (LC-UV-MS) (Nielsen and Smedsgaard, 2003) and thermospray-liquid chromatography- mass spectrometry (TSP-LC-MS) (Rajakyla *et al.*, 1987). These methods are reliable and provide structural information, however they have to be first optimised for separation before accurate results can be attained. Natural product chemists also use ^{1}H NMR spectroscopy as a simple and fast method of screening crude extracts. This method, which is not as sensitive as HPLC, allows one to infer the types of compounds contained in an extract making this method adequate for primary screening. ^{1}H NMR is also sensitive enough to detect differences in secondary metabolite production under different growth conditions and can be used as a rapid tool for the identification of metabolically productive organisms. An experienced natural products chemist can often very quickly dereplicate extracts containing only simple metabolites such as fatty acids and sugars while at the same time recognising "interesting" structural features of a crude extract. It is also fairly easy to compare the effects of different fermentation conditions on the production of metabolites. However, this method is quite subjective and minor metabolites may easily be overlooked. It is therefore necessary to combine chemical screenings with some form of biological assay. Because this method is subjective, a metabolic creativity index was used to rate the extracts. Fig. 2.5 shows an example of an extract with a low and high metabolic index.

A typical extract of a culture broth will contain secondary metabolites that have some effect on its environment such as antibiotics, mycotoxins, pheromones, insecticides, fungicides and herbicides and the intracellular metabolites may contain chemical defense metabolites against fungivorous insects and arthropods (Gloer, 1997). However it is only advantageous for the organisms to produce these metabolites when required in order to save energy. There must be some cue, whether physical or chemical, to induce production. Since all fermentations consisted of one fungal culture, it was not expected that the broth would contain a greater metabolic diversity than the biomass because there was no other organism to induce production of extracellular metabolites. This discrepancy could be a result of the inhomogenous growth of the fungi, resulting in lysis of cells in death phase, thereby releasing its metabolites into the broth.

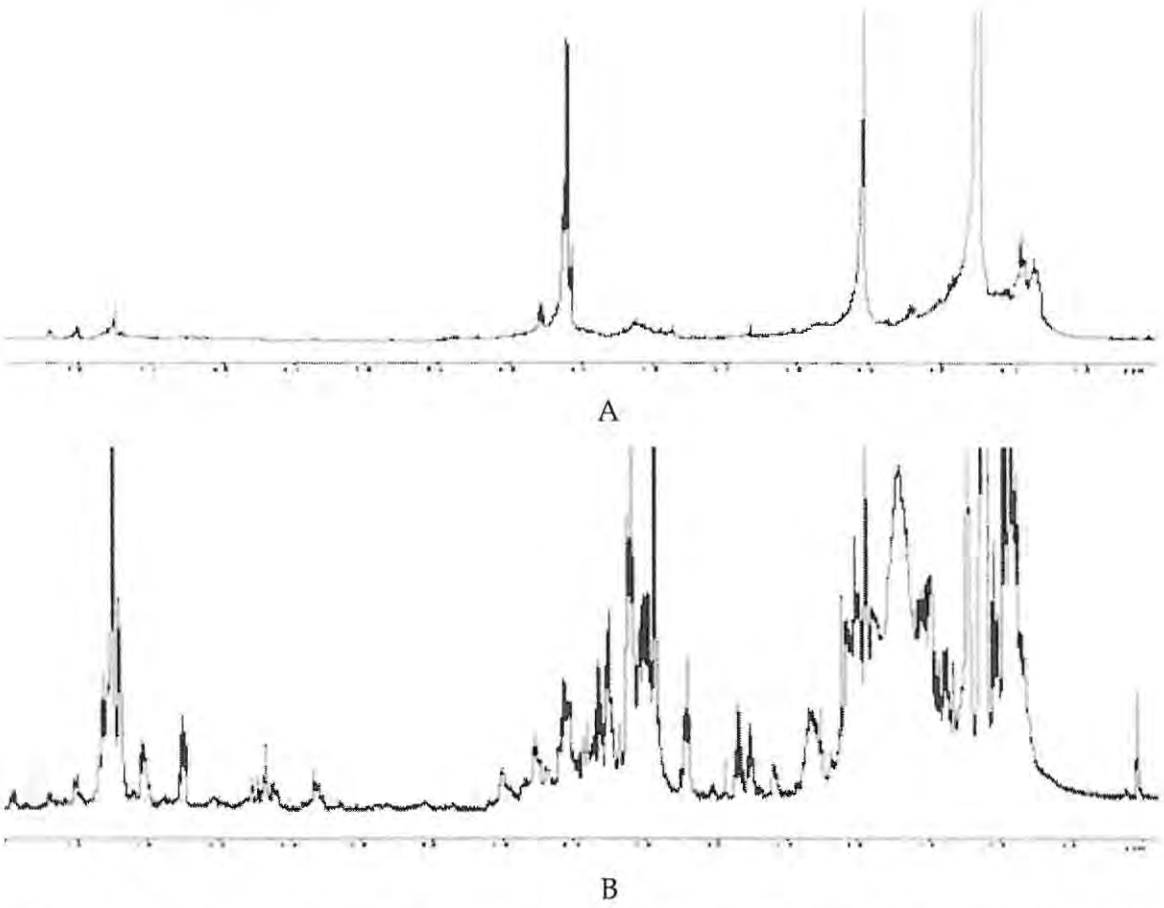


Fig. 2.5: ^{1}H NMR Spectra of a Crude Extract with A: Low Metabolic Creativity and B: High Creativity.

Evidence for this is that several of the intracellular and extracellular spectra are similar. Although hundreds of bioactive metabolites have been isolated from the broth of fungal media, several new compounds have also been isolated by antagonism i.e. the induction of production of bioactive metabolites by introduction of live cells, dead cells or cell extracts of another organism (Burgess *et al.*, 1999).

Of the 24 fungi, only 10 were observed to exhibit interesting metabolic creativity. These were:

- 2Aa (grown under static conditions; broth extract);
- 1Aa (grown under agitated and static conditions, broth extract and static conditions for the mycelia);
- S-7Aa1 (SWA isolation medium, grown under agitated conditions; broth extract);
- 4Aa (grown under static conditions, broth extract);
- 2Ba (grown under static conditions, broth extract);
- 10Aa (grown under static conditions, broth extract);

- 1Ab (grown under static and Shaken conditions, broth extract and static conditions, mycelium);
- 4Ac (grown under Shaken conditions, broth extract);
- 4B (grown under Shaken and static conditions, broth extract);
- 9Ab (grown under static conditions, broth extract)

2.3.6. Bioactivity

2.3.6.1. Cytotoxicity Assays

The cytotoxicity assays show that most of the extracts tested were cytotoxic to brine shrimp at their highest concentration (570µg/mL) (Table 2.5). Most extracts acted in a dose-dependant manner i.e. as the concentration decreased, the percentage mortality decreased. Due to small quantities of extracts from some isolates, only a few extracts could be tested, therefore no comparison can be made regarding the culture techniques employed and bioactivities of the metabolites produced.

Table 2.5: Brine Shrimp Mortality (%)

Isolate	Culture	Extract	Metabolic Creativity	Average Mortality (%)			
				Concentration (µg/mL)			
				570	285	142	71
S-5Ba1B	Static	Mycelia	2	100	100	95	100
4B1	Static	Broth	4	100	100	100	96
4B2	Shaken	Broth	4	100	100	88	72
10Aa	Static	Mycelia	5	100	100	88	70
2Aa1B	Static	Mycelia	1	0	0	0	0
2Ba2B	Shaken	Mycelia	4	100	83	71	50
2Ba2	Shake	Broth	5	100	100	100	100
S-7Ad2	Static	Broth	5	100	90	85	80
1Aa1B	Static	Mycelia	5	100	100	80	60
1Aa2B	Shaken	Mycelia	5	100	50	0	0
3A1	Static	Broth	3	100	90	33	20
S-10Aa1	Static	Broth	3	100	100	100	100
S-5Ba1B	Static	Mycelia	3	100	100	100	100
2-Aa1	Static	Broth	5	100	100	100	100
10Aa1B	Static	Mycelia	2	100	100	100	100
4Aa1B	Static	Mycelia	1	100	100	100	100
S-5B1B	Static	Mycelia	3	100	41	10	10
S-8B1	Shaken	Broth	1	100	100	100	100
3A1B	Static	Mycelia	1	0	0	0	0
S-4A	Shaken	Broth	1	0	0	0	0
10Ac2B	Static	Mycelia	1	100	87	70	66
S-1A2B	Shaken	Mycelia	2	10	6	8	5
5B1	Static	Broth	3	0	0	0	0
8Aa1B	Static	Mycelia	4	100	100	100	84
1Aa1	Static	Broth	5	100	100	100	100

2.3.6.2. Anticancer Assay

The crude extracts which showed some activity against cancer cell lines WHC01 (oesophageal cancer line) and ME180 (cervical cancer line), were:

- S-7AD2
- S-8B1
- 4B1
- 8Aa1B
- 1Aa1B
- 10Ac2B
- 4B2

The extracts that were active against the cancer cell lines were also cytotoxic to brine-shrimp. Therefore a good correlation exists between our assays.

2.3.6.3. Antibiotic Assays

No antibiotic activity was observed for any of the samples tested against methicillin resistant microorganisms. However, no assays against non-resistant microorganisms were investigated.

2.4. Summary and Recommendations

Seaweeds are a good source of fungi, with more epiphytes being isolated than endophytes. Most of the crude broth extracts exhibited a higher metabolic creativity than the biomass extracts, while most isolates produced higher creativity indices when grown under shaken than static growth conditions. Some of these extracts were active against brine-shrimp and cancer cell lines, while none showed antibiotic activity against methicillin resistant bacterial strains.

Although SWA and YPG isolation media were effective in isolating a number of different fungi, a variety of media should be used to assess whether other fungi can be isolated.

Although flask cultures have many disadvantages, their simplicity, cost-effectiveness and the ability of running simultaneous fermentations, makes their use suitable for primary screening of fungal isolates.

The production of spores is a good indication of stationary phase, however where diauxic growth occurs, or when fungi are non-sporulating, other methods of analyses are required. While the use of optical density may not be suitable to coloured fungi, a conjunction of these methods is adequate to acquire information on the growth phases of the cultures.

Extraction of metabolites using solvents is a time-consuming and costly process, therefore alternate methods are recommended such as extraction with reusable resins or the use of high molecular exclusion filters for extraction.

It is also important to note that although ^{1}H spectra of extracts may not be "interesting", these extracts may still possess potent biological activity. It is thus essential that metabolic screening be done in conjunction with bioassays. These methods of screening are quick and inexpensive, however the analyses of spectral data are subjective. If interpreted correctly, this method can be used effectively to screen fungi. .

2.5. Conclusion

Based on the metabolic creativity screening and the bioactivity screening of the crude extracts, isolate 1Aa (RUC0101) was chosen for further study.

Chapter 3:
Identification of RUC0101

3.1. Introduction

An important element of natural product chemistry is dereplication. Dereplication has been defined by Kohlmeyer and Kohlmeyer (1979) as the elimination of replicates or repeats of material already isolated and known in the literature. An initial step in dereplication is the identification of the source organism. The taxonomy of fungi has traditionally been based on morphology, but morphology is only a part of the expression of differentiation in living organisms (Frisvad, 1989).

Other methods for the identification of microorganisms include analyses of: (1) secondary metabolite profiles using liquid chromatography–nuclear magnetic resonance (LC-NMR) (Bobzin *et al.*, 2000); high-performance liquid chromatography–electrospray ionization (HPLC-ESI) (Smedsgaard and Frisvad, 1996; Julian *et al.*, 1998); HPLC with alkylphenone retention indices (Frisvad, 1987); gas chromatography–mass spectrometry (GC-MS) (Frisvad *et al.*, 1989); thin layer chromatography (TLC) (Frisvad *et al.*, 1989) and liquid chromatography–ultraviolet–mass spectrometry (LC-UV-MS) (Nielson and Smedsgaard, 2003), (2) intracellular and extracellular components such as sterols (Van Eijk and Roeymans 1982); long chain fatty acids (Lee *et al.*, 1976); polyols (Pfyffer and Rast, 1980); cell wall carbohydrates (Leal *et al.*, 1984) or GC of whole cells (Kullik and Vincent, 1987). Fatty acid methyl ester (FAME) using GC is also a widely used method that has been successfully applied to the identification of fungal species. However, the concerns with using chemotaxonomy are that (1) compounds are not unique to species e.g. aflatoxins are produced by many species of *Aspergillus* and (2) isolates in a given species may fail to produce one or two of the most diagnostic secondary metabolites (Frisvad, 1989) due to differences in cultivation or strain degeneration. Thus the use of secondary metabolites or intracellular components as taxonomic markers may render results that are questionable.

Since morphology and the production of metabolites are a result of the genetic information of an organism, a direct approach is to identify the organism using their DNA. Phylogenetic studies in eukaryotes utilise the ribosomal DNA (rDNA) (28S, 5.8S and 18S ribosomal sub-units) (Fig. 3.1). There are two internal transcribed spacers, the ITS1 and ITS2, which separate the 18S, 5.8S and 28S genes.

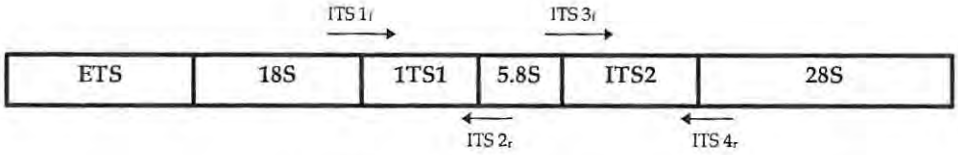


Fig. 3.1: rDNA of Eukaryotes.
ETS: external transcribed region; ITS: internal transcribed spacer.
Arrows indicate direction of respective forward and reverse primers.

Choosing an appropriate region for analysis is of extreme importance. Bruns *et al.*, (1991) identified several factors to consider when selecting a region for sequence analysis:

1) The region should be evolving at an appropriate rate for comparison of interest. Regions that are too conserved will provide too few changes, and regions that are too variable will contain too many inconsistent characters due to multiple substitutions at single positions.
2) The region should be present ideally as a single copy or should at least evolve like a single copy region e.g. rDNA, mitochondrial DNA (mtDNA).
3) The region should have the same function in all taxa because evolution of a new function changes selective pressures and therefore the rate of sequence change.
4) The effect of base composition and codon bias should be examined because both factors can distort estimates of variance.

The 18S, 23S and 5.8S genes evolve at very different rates, thus they are more likely to yield informative data for systematics. Furthermore, the islands of highly conserved sequences are useful for constructing universal primers that can be used for sequencing (Hillis and Dixon, 1991). The most studied rRNA in eukaryotes is the 18S because it is amongst the slowest evolving sequences. Thus it is useful for examining ancient evolutionary events through the Precambrian period. The large subunit, 28S is larger and shows more variation in rates of evolution. It has been used to study events through the Paleozoic and Mesozoic period. The 5.8S rRNA is however too short and its usefulness for phylogenetics is therefore limited (Hillis and Dixon, 1991). Until recently, the spacer regions of the rDNA were not frequently used, however it is now known that these regions can infer phylogeny among closely related taxa (which have diverged within the last 50 million years). These regions can also identify up to strain or species level (Hillis and Dixon, 1991) and have now become popular regions for identification of isolates. Due to this, many sequences have been submitted to the GenBank (an electronic database), and are readily available for sequence comparison.

With the advent of direct sequencing of polymerase chain reaction (PCR) products, the cost and time of sequencing has decreased significantly. The circumvention of cloning (bacterial transformation, plasmid preparation, creation of libraries and fragment purification) aids the number of isolates which can be studied simultaneously.

In this chapter the identification of RUC0101 by direct sequencing of the ITS1 and 2 regions is described.

3.2. Materials and Methods

3.2.1. DNA Analyses

3.2.1.1. Cultivation and Isolation of DNA

DNA was extracted from 0.5cm² of mycelia maintained on YPG plates using QIAgen DNeasy™ Tissue Kit (Southern Cross Biotechnology) following the modified protocol for yeast cells. The mycelia were digested by lyticase (yeast lysis enzyme) 5500U/g, (ICN Biomedicals, Inc) and subsequently suspended in 600µL sorbital buffer (1M sorbital; 100mM EDTA; 14mM β-Mercaptoethanol), followed by centrifugation for 10 minutes at 500 x g using an Eppendorf Centrifuge 5810R. The pellet was resuspended in a further 600µL of sorbital buffer. 200 units (100µL of 2000U/mL stock) of lyticase were added to the mycelia and the samples were incubated at 30°C for 30 minutes. The spheroplasts were pelleted by centrifugation for 10 minutes at 300 x g and the pellet resuspended in 180µL ATL buffer (60-00-4 edetic acid: 2.5-10%; 151-21-3 SDS: 2.5-10%). The protocol used for the isolation of genomic DNA was carried out as described in the QIAgen DNeasy™ Tissue Kit Handbook (1999).

The purity and integrity of the extracted genomic DNA was evaluated using 0.8% agarose gel (molecular grade DI-LE, WhiteSci) containing ethidium bromide (EtBr) (0.5µg/mL) in 0.5X TBE buffer (Appendix A) after electrophoresis at 100V. The DNA was visualized on a UV transilluminator.

The DNA was quantified using the Pharmacia GeneQuant with a 1:100 dilution of the extracted DNA.

3.2.1.2. Amplification of ITS1 and ITS2 regions

Primers for the ITS1 region where chosen according to Brookman *et al.*, (2000) and obtained from Takara (BioInc, Japan). ITS2 region primers were chosen according to White *et al.*, (1990), and were purchased from Inquaba Biotech (Pretoria, South Africa). Using these primers, the sizes of the PCR products were expected to be 300 - 400 bp's. The primers were reconstituted in triple distilled waster ($dddH_2O$) to make a stock solution of 100µM.

Primer	Sequence	Melting Temperature (T_m)
ITS1 forward	5′ TGTACACACCGCCCGTC 3′	58°C
ITS1 reverse	5′ CTGCGTTCTTCATCGAT 3′	50°C
ITS2 forward	5′ GCATCGATGAAGAACGCAGC 3′	62°C
ITS2 reverse	5′ TCCTCCGCTTATTGATATATGC 3′	62°C

A 100µL reaction was prepared according to the Promega PCR Master Mix instruction manual (Promega, USA). The Master Mix consisted of 50U/mL of *Taq* Polymerase (pH=8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM $MgCl_2$

Table 3.1: Reagents and Final Concentrations used in PCR Reactions for the ITS1 and ITS2 Regions.

Reagent	**Amount**	**Final Concentration**
Master Mix (2X)	50µL	1X
Template DNA	3µL	< 250ng
ITS1 primer (10µM)	5µL	0.5µM
ITS2 primer (10µM)	5µL	0.5µM
Water	37µL	
Total	100µL	

The negative control was prepared as above without the template DNA.

The PCR was performed with the PCR Sprint Hybaid using the following cycle parameters:

ITS1 region

- Initial denaturation temperature: 95°C for 2 minutes

30 cycles of:

- Denaturation: 95°C for 1 minute
- Annealing: 45°C for 1 minute
- Extension: 72°C for 1 minute
- Final extension: 72°C for 5 minutes

ITS2 region

- Initial denaturation temperature: 95°C for 2 minutes

30 cycles of:

- Denaturation: 95°C for 1 minute
- Annealing: 57°C for 1 minute
- Extension: 72°C for 1 minute

- Final extension: 72°C for 5 minutes

3.2.1.3. Electrophoresis

Amplified products were visualised in 1% agarose gels (Appendix A) after electrophoresis at 100V. DNA molecular marker XIV (250µg/mL in 10mM Tris-HCl, 1mM EDTA, pH=8) (Roche) was used for the ITS1 region and λ DNA Pst molecular marker (10ng/µL) for the ITS2 region. The DNA was viewed with a UV transilluminator and photographed using a Kodak digital camera. The data was captured using Kodak Digital Science ID v.2 software.

3.2.1.4. DNA Extraction from Gel

The products were excised from the agarose gel and the amplified DNA extracted using the QIAquick Gel Extraction Kit according to the manufacturer's instructions (QIAquick Spin Handbook, 2001). The DNA was quantified using the Pharmacia GeneQuant at 260nm.

3.2.1.5. Cycle Sequencing

Table 3.2: Reagents for Cycle Sequencing of Both the Forward and Reverse Strands.

Reagent	**Amount**
Template DNA	3-10ng
Primer 1/ Primer 2	3.2pmol
Big Dye*	4μL
Dilution Buffer	2μL
Water	made up to 20μL

* Big Dye Terminator v 3.1. Cycle Sequencing Kit.

The GeneAmp PCR system 9700 thermocycler was used with the following parameters:

ITS1

- Initial denaturation: 96°C for 1s

25 cycles of:

- Denaturation: 96°C for 10s
- Annealing: 45°C for 5s
- Extension: 60°C for 4min

ITS2

- Initial denaturation: 96°C for 1s

25 cycles of:

- Denaturation: 96°C for 10s
- Annealing: 50°C for 5s
- Extension: 60°C for 4min

3.2.1.6. DNA Purification

The DNA was purified for sequencing using the DNA Clean & Concentrator Kit™. 100μL of DNA binding buffer was added to each sample. The samples were then loaded onto a Zymo-Spin column placed in 2 ml collection tubes, centrifuged at 10 000 x g for 10 seconds and the flow-through discarded. 200μL of wash buffer was added to each column and the samples centrifuged for 10 seconds at maximum speed. The flow-through was discarded and another 200μL was applied to the column. The samples were centrifuged for 30 seconds at 10 000 x g and the flow-through discarded. To elute the DNA, 8μL of $dddH_2O$ was

applied directly to the column matrix. The Zymo-Spin column was placed in a sterile eppendorf and centrifuged for 10 seconds at 10 000 x g. The DNA was dried at 37°C

3.2.1.7. Electrophoresis

Samples were sequenced using the ABI 3100 Genetic Analyzer (Applied Biosystems Div., Perkin-Elmer Biosystems, U.S.A.).

3.2.1.8. Analyses of Sequence Results

Chromatograms were generated by the ABI PRISM Genetic Analyser Data Collection System 1.0.1 (Applied Biosystems). The resulting sequences were viewed using Chromas v. 2.23 and the sequence output corrected manually. The data were converted into a text file and aligned using BLAST pairwise alignment. The forward sequence was divided into the respective regions i.e. 18S, ITS1, and 5.8S, and the ITS1 sequence was compared to those in the GenBank using BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST). Using these data, BLAST generated a list of the most closely related fungi. The sequences of the fungi homologous to the ITS1 region were aligned using ClustalX v.1.81 alignment algorithm (Thompson *et al.*, 1997) with the default settings. The truncated sequences and non-homologous regions from the sequences were deleted as described by Hall (2001). The length of the ITS1 sequence used for sequence alignment and the construction of the phylogenetic tree was 147 bp's. A non-rooted Neighbour Joining phylogenetic tree (Fig. 3.4) was constructed using ClustalX with the default parameters for bootstrapping. Treeview v.1.61 was used to view the phylogenetic tree.

3.2.2. Microscopic Analyses

Seven-day-old pieces of mycelia (5mm^3) maintained on YPG agar were treated in cold-buffered fixative (2.5% glutaraldehyde in 0.1M phosphate buffer) overnight. The buffer was decanted and replaced twice with cold phosphate buffer for 15 minutes. This was replaced with a series of ethanol concentrations from 30-100% for 15 minutes each. Thereafter the 100% ethanol was replaced with 25%, 50%, 75% and finally 100% amyl acetate: EtOH for 20 minutes each. The specimens were then transferred to the critical point drying apparatus and dried for 2 hours. Thereafter the samples were coated with gold as described by Cross (2001, unpublished manual). A JEOL JSM 840 SEM was used to magnify the specimens.

3.3. Results

3.3.1. DNA analyses

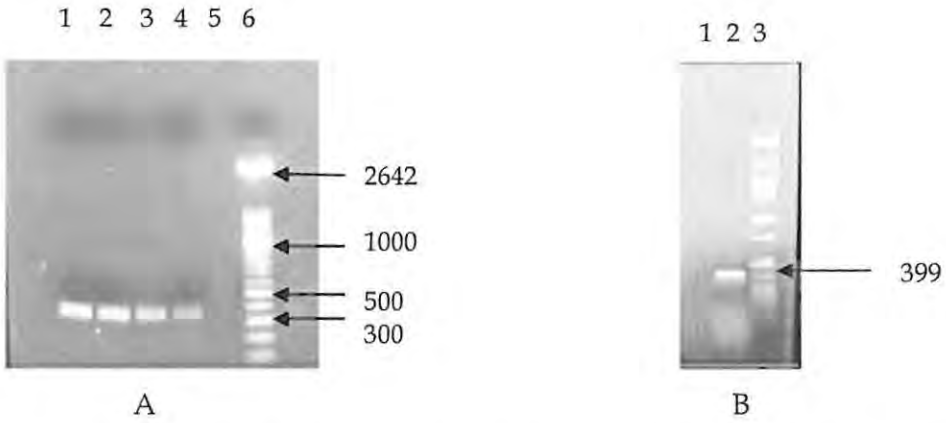


Fig. 3.2: PCR Products of the ITS1 and ITS2 Regions Visualised on a 1% Agarose Gel after Electrophoresis at 100V.

A: ITS1. Lane 1-4 = PCR products. Lane 5 = negative control. Lane 6 = DNA molecular weight marker XIV. B: ITS2. Lane 1 = negative control. Lane 2 = PCR product. Lane 3 = λ DNA Pst marker.

Table 3.3: Most Closely Related Species using the ITS1 Region

Species	Similarity (%)	Score	E-value	Base pairs
Eurotium rubrum strain SRRC 2172	100	291	2e-76	147/147
E. rubrum	100	287	2e-75	145/145
E. niveoglaucum NRRL 137	99	283	4e-74	146/147
Newtonia buchananii wood isolate S35	99	283	4e-74	146/147
E. repens strain ATCC 66457	99	283	4e-74	146/147
Cordyceps sinensis isolate W1023	99	283	4e-74	146/147
E. herbariorum strain NRRL 116	98	280	6e-73	145/147
E. repens isolate W8	99	280	6e-73	146/147

Table 3.4: Most Closely Related Species using the ITS2 Region

Species	Similarity (%)	Score	E-value	Base pairs
E. niveoglaucum NRRL 137	100	331	2e-88	167/167
Newtonia buchananii wood isolate S35	100	331	2e-88	167/167
E. rubrum strain SRRC 2172	100	331	2e-88	167/167
E. repens ATCC 66457	100	331	2e-88	167/167
E. herbariorum strain NRRL 116	100	331	2e-88	167/167
Cordyceps sinensis	100	331	2e-88	167/167
E. rubrum	100	331	2e-88	167/167

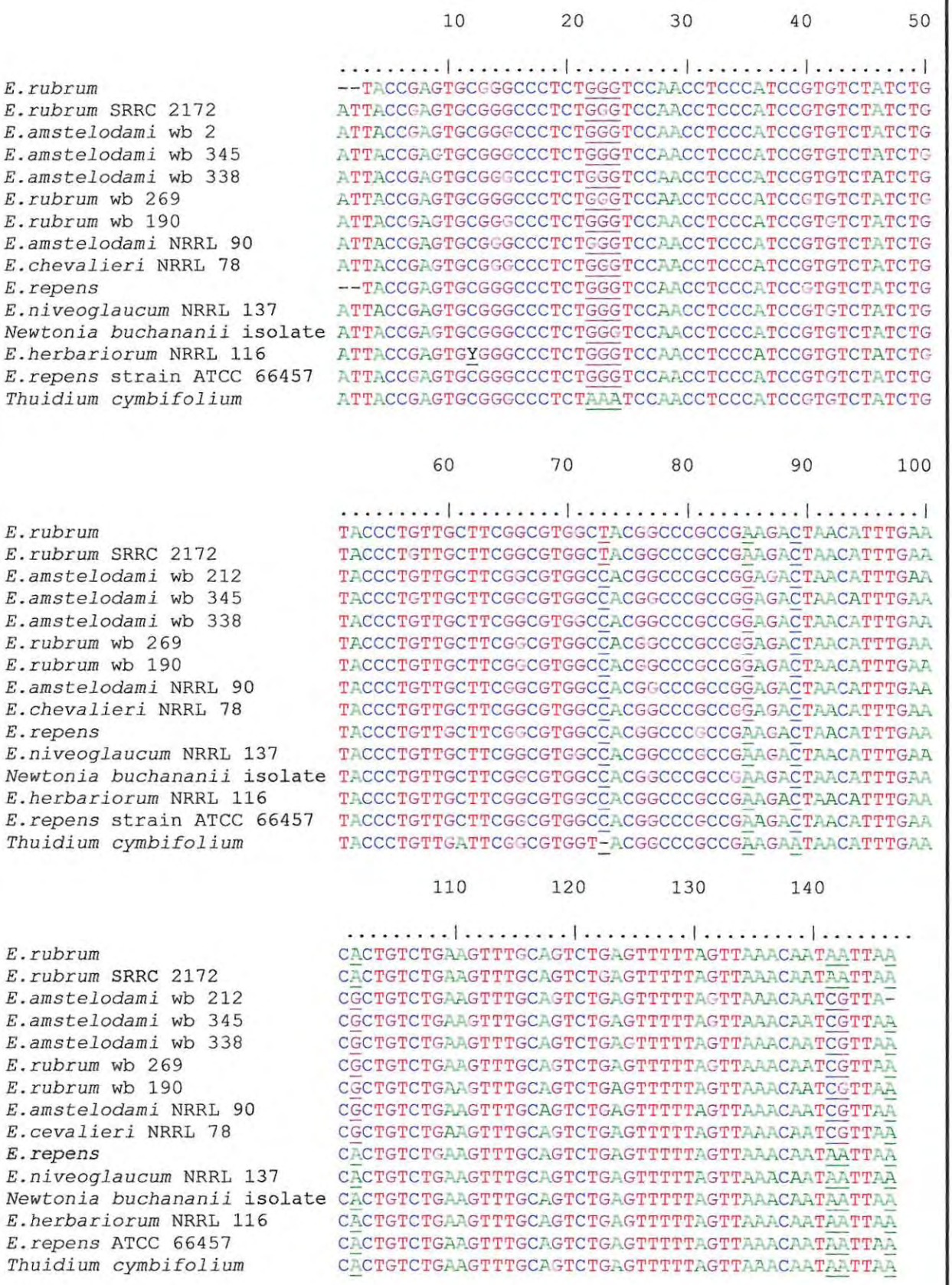


Fig. 3.3: Multi-alignment of Closely Related Species using the ITS1 Region.
Generated by ClustalX using the default parameters for bootstrapping.
Underlined bases indicate low-scoring regions.

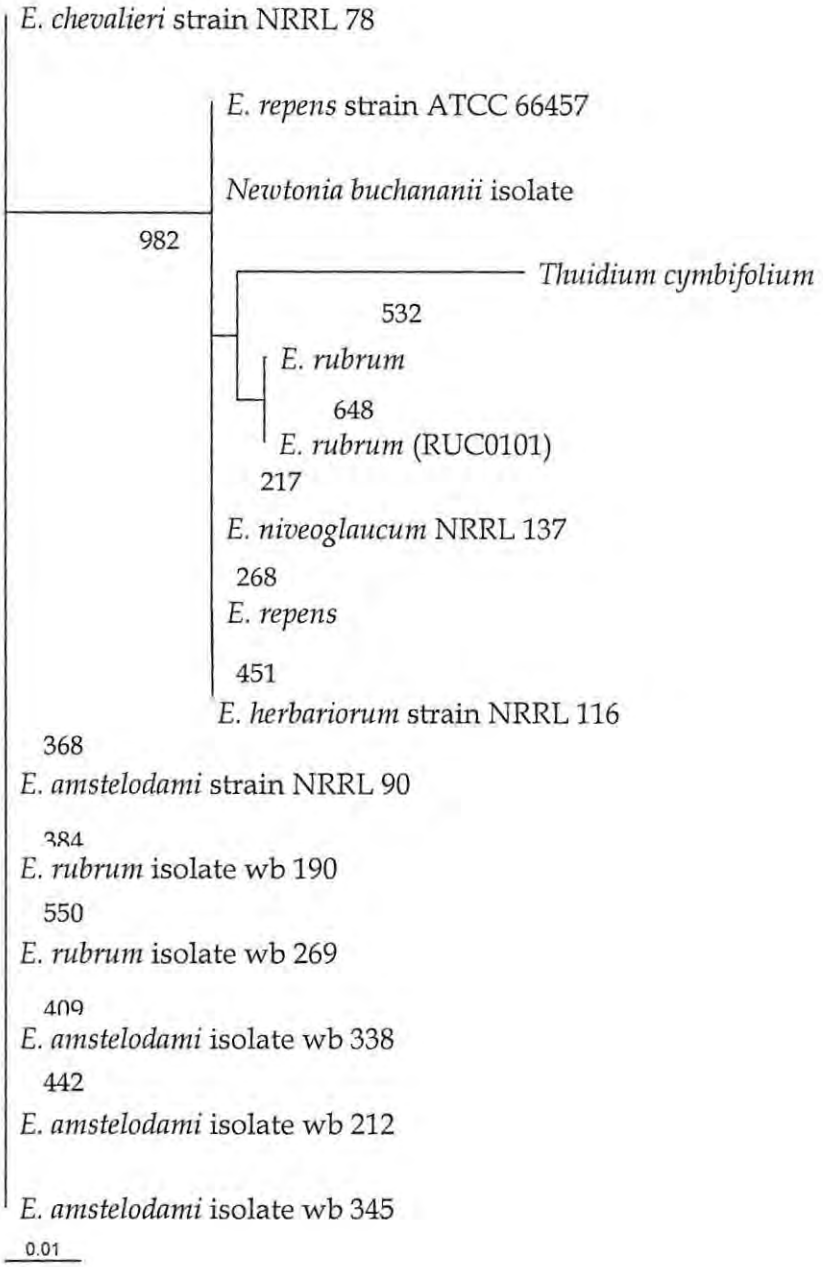
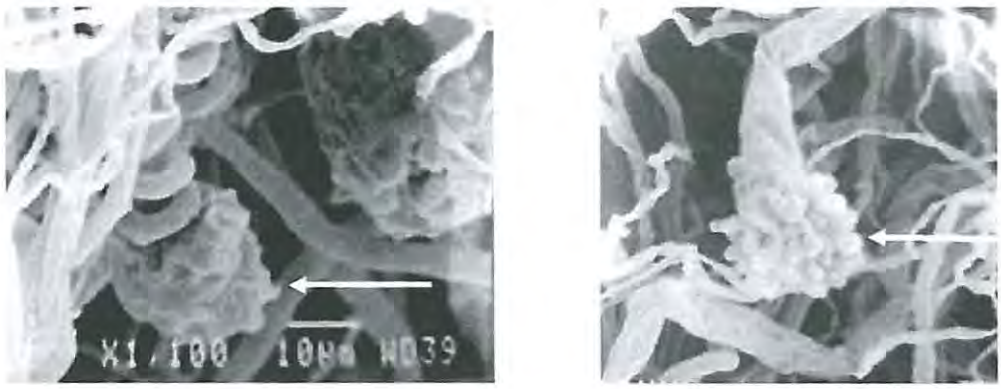


Fig. 3.4: Phylogenetic Tree constructed from the ITS1 region of RUC0101.
Generated from closely related species identified from BLAST search.

Taxonomy: Eukaryota; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiales; Trichocomaceae; Eurotium

3.3.2. Microscopic Analyses



A B

Fig. 3.5: SEM of RUC0101.
A: helical antheridia and ascogonia resulting in the formation of the cleistothecium and ascocarp (yellow) during sexual reproduction (x1100). B: single somatic hyphae terminating in conidiaphores bearing sterigmata (x800).

Fig. 3.6: RUC0101 Maintained on YPG Basal Medium.

3.4. Discussion

The isolation of genomic DNA and the resultant amplification of the ITS1 and ITS2 regions using PCR were successfully accomplished without the need for further optimisation. PCR of the ITS1 and ITS2 (Fig. 3.2) region yielded one fragment each between 300 and 400bp's and this was confirmed by sequencing. The pairwise alignment of the forward and reverse strands for both regions revealed no secondary structures and was subsequently used for phylogenetic studies. The primers chosen also allowed the short 5.8S region (108bps) to be attained because the ITS1 primers permit the PCR of the 5′ end of this region (flanks the 3′ end of the ITS1 region) and the ITS2 primers PCR the 3′ end (flanks the 5′ end of the ITS2 region) (Fig. 3.1).

The results from the ITS2 (Table 3.4) and 5.8S regions of RUC0101 show 100% homology with many species of the genus *Eurotium* while the ITS1 region of RUC0101 shows 100% homology with only *Eurotium rubrum* strain SRRC 2172 (Haugland *et al.*, 2003) (Table 3.3). Although, phylogenetic studies based on the 5.8S region are already known to produce ambiguous results, in this case, the ITS2 region was also too conserved and therefore not suitable for species identification. For the ITS1 region, the high score (291 bits) and a low e-value (2e-76) suggest that these results are of high quality.

Fig. 3.5 clearly shows typical structures of fruiting bodies of the *Eurotium* sp. (Ainsworth *et al.*, 1973), with both the asexual and sexual states being produced (Florian and Manning, 2000). The morphology of RUC0101 is consistent with Raper and Fenell's description of the *Aspergillus glaucus* group as having aerial hyphae encrusted with yellow, orange or red granules. In addition, maximal development and production of the cleistothecia (yellow-orange to brown-red), with limited growth of conidia (green) occurs when grown in a medium high concentrations of sugar and limited nitrogen (Raper and Fenell, 1965) such as YPG basal medium.

Although the DNA sequencing results were of high quality and consistent with that of the microscopic analyses, a full taxonomic analyses which includes physiological testing and possibly chemotaxonomy would be required to be certain that RUC0101 is identical to *Eurotium rubrum* strain SRRC 2172.

The *Aspergillus/ Eurotium* genus is widely distributed from the Poles to the Tropics. They are capable of utilising a variety of substrates for food because of the large number of enzymes they produce (Alexopoulos, 1962). In fact, they are so common that in a recent PhD thesis, a low nutrient isolation medium was specifically used to decrease the chance of isolating these ubiquitous fungi (Holler, 1999).

E. rubrum otherwise known as *Aspergillus ruber* (anamorph) belongs to the *Aspergillus glaucus* group. It is a halotolerant fungus and has been isolated from the Dead Sea (Kis-Papo *et al.*, 2001; Kis-Papo *et al.*, 2003) and the marine environment (Christophersen *et al.*, 1999). However, *E. rubrum* is also a xerophillic fungus and has been commonly isolated as a food spoilage microorganism (Ismail, 2000; Peintner *et al.*, 2000; Weidenbîrner, 2000). The possibility that this isolate is a contaminant is unlikely because stringent collection and isolation procedures were followed. In addition, none of the other plates or the controls contained this fungus. According to the definition by Kohlmeyer and Kohlmeyer, (1979),

RUC0101 is a marine fungus because it is able to grow and sporulate in the marine environment.

From the 1900's to the 1970's, the *Aspergillus glaucus* group was of great interest to researchers. The initial interest was due to the ubiquitous nature of the fungus which arose from its interesting metabolism. This group has been known to produce a number of anthraquinones, indoles and prenylated hydroquinones (Raistrick *et al.*, 1937; Cruickshank *et al.*, 1938; Barbetta *et al.*, 1969; Allen, 1972; Cardillo *et al.*, 1974; Gatti, 1976; Gatti, 1978, Gatti and Fuganti, 1979). The study of this group was pursued because of the types of compounds produced and the biological activity associated with them. The anthraquinones in particular gained interest due to their antitumour and antibiotics activities (Podojil *et al.*, 1978; Anke *et al.*, 1980a; Anke *et al.*, 1980b; Arai *et al.*, 1989; Manojlovic *et al.*, 2000; Yen *et al.*, 2003).

3.5. Conclusion

Using DNA analyses of the ITS1 region, RUC0101 has been identified as a close relative of *Eurotium rubrum*. Although *E. rubrum* has been isolated from the marine environment, no investigations have been undertaken to determine the adaptation of these isolates to the marine environment. Thus one of the objectives of this study is to determine if any new metabolites are produced by RUC0101. Furthermore because this group has been well studied, the results from our investigation can be used to evaluate the methods employed.

Chapter 4:

Preliminary Optimisation of Culture Conditions

4.1. Introduction

4.1.1. Optimisation

It is well recognised that small changes in growth media and fermentation conditions can cause the expression of different secondary metabolites in microorganisms (Bu'Lock, 1975). By varying the fermentation conditions, the optimum parameters for the production of (1) a single high-value metabolite or (2) a range of metabolites (metabolic creativity) can be determined. With the large number of known metabolites being isolated from microorganisms it is essential to optimise the fermentation conditions in order to increase the chances of finding new metabolites. However, it is not feasible for an academic research group, interested in discovering new chemical entities, to optimise culture conditions for each new microorganism that is isolated. In this study we wished to evaluate whether our standard fermentation protocol allowed us access to all the major metabolites of our marine isolate of *E. rubrum*. A commonly encountered strategy for optimisation studies is the "one-factor-at-a-time" method. This method involves optimising one factor first (e.g. carbon source), then using this optimised carbon source to optimise the next factor (e.g. nitrogen source) etc. The two main disadvantages of this method are: (1) it is laborious and time-consuming, and (2) it does not take into consideration interactions between components of the media.

4.1.2. Composition of Fermentation Media

Fungi require carbon, nitrogen, phosphorus, sulphur, minerals, vitamins and other growth factors. They may also require specific nutrients that they are unable to synthesise, such as amino acids and vitamins (Greasham and Herber, 1997). They are sensitive to hydrogen ion concentrations (pH), temperature, and oxygen/carbon dioxide in the environment. The major challenge is to provide the fungus with conditions that allow for the expression of secondary metabolites and the accumulation of unusual products (Pearce, 1997). Nutrients have to be supplied to first establish growth and then to maximise secondary product formation. In addition, the cost, consistency in composition and availability also need to be taken into consideration (Dahod, 1999).

Required nutrients may be supplied via either chemically defined or complex media. Chemically defined media consist of both organic and inorganic ingredients that are chemically defined (e.g. glucose; glycerol), while complex media consists of one or more

nutritional components that cannot be easily defined (e.g. soybean flour; corn steep liquor; peptone). When a complex medium contains several chemically defined nutrients and only one complex component, it may be referred to as a semi-defined medium.

(1) *Chemically defined media*: At small-scale production, these types of media are routinely used to develop a fundamental understanding of the nutritional requirements of the isolate, which is impossible with complex media. Traditionally chemically defined media have been unattractive at commercial scales for the production of secondary metabolites because of their relatively high costs and support of low productivities (Dahod, 1999). However they are slowly gaining popularity with the production of recombinant proteins at levels equal to or greater than those achieved with complex media. Furthermore they have the inherent characteristic of supporting consistent fermentation performance. Fermentations developed with chemically defined media often lead to rapid scale-up and are less sensitive to large-scale sterilisation (Dahod, 1999).

(2) *Complex media*: Most industrial fermentations are complex formulations. These ingredients are cost-effective and support faster and increased cell mass (Dahod, 1999), however as mentioned previously, consistency is a problem because these types of media are by-products and waste materials from the agricultural and food industries or extracts of biological materials (Greasham and Herber, 1997).

Microorganisms utilise carbon compounds for the production of cell mass and secondary metabolites, as well as for an energy source. The concentration of the C-source is also an important factor for growth and production of metabolites. For fungi, carbon and nitrogen constitute 48 and 6% of the dry weight, respectively (Greasham and Herber, 1997). If the concentration is too high, the microorganism may experience dehydration. Glucose, usually an excellent carbon source for growth, interferes with the biosynthesis of many secondary metabolites (Demain *et al.*, 1992). Also required is nitrogen to support the biosynthesis of nitrogenous metabolites, both primary (purines, pyrimidines and amino acids) and secondary. Both organic and inorganic nitrogen sources have been used in the culture media for other members of the *Aspergillus glaucus* group. Gould and Raistrick (1934) reported that with *A. novus* (a closely related fungus to *E. rubrum*), di-ammonium tartrate produced higher yields of metabolite than with sodium nitrate as a nitrogen source and *A. umbrosus*, another relative, grew better on ammonium than nitrate nitrogen (Carbone and Johnson, 1964). Although glucose affects biosynthesis, it is still a common approach when developing media

to add glucose because it is a rapidly utilisable sugar, in addition to a slowly utilisable sugar such as mannitol together with a nitrogen source and minerals. This frequently results in rapid growth of the fungus due to the glucose and then a slower growing phase favouring secondary metabolism.

Ammonium and phosphate levels also control secondary metabolism and secondary metabolites are accumulated when these nutrients become depleted. Sulphate, potassium, calcium, magnesium, manganese, zinc and ferric ions are also needed by microorganisms for growth and to provide the required osmotic potential. Organic phosphate plays a role in intermediary metabolism, especially as a component in "high energy" compounds such as adenosine triphosphate, adenosine diphosphate and arginine phosphate. Phosphate is also a constituent of phospholipids and nucleic acids acts as a regulator of certain enzymes in primary and secondary metabolism (Greasham and Herber, 1997). Sulphur is required for the biosynthesis of sulphur containing amino acids and certain secondary metabolites e.g. β-lactam antibiotics (Greasham and Herber, 1997). The remaining ions are trace elements and are required as co-factors and activators for many enzyme systems and as biosynthetic regulators of several secondary metabolites (Greasham and Herber, 1997). For marine microorganisms, chlorides and bromides are particularly important for metabolite production, and may also serve to increase the osmotic potential required for their growth. For example, *A. umbrosus* grew better in media that contained NaCl, however, for *A. cristatum*, very high salt concentration (2M) caused a decrease in growth and pigment production (Anke *et al.*, 1980a).

4.1.3. Metabolic Profiling

The main reason for carrying out optimisation studies is to determine the conditions for optimum metabolite production. The development of suitable analytical procedures to detect and quantify metabolites is therefore essential to this process. Metabolic profiling is a commonly used term to describe the methods of determining what and how many compounds are contained in an extract. Instruments which offer accuracy and precision are required for the detection and analyses of secondary metabolites. Time and cost are also important. Over the years, a number of research groups have developed methods for metabolic profiling. One group in particular, lead by Frisvad has done a great deal of work on fungal secondary metabolites profiling. Some of the methods include thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), high

performance liquid chromatography–diode array detection (HPLC-DAD) and electrospray ionization mass spectrometry (ESI-MS).

Although optimisation studies have been performed on *E. rubrum* in terms of growth (Gould and Raistrick, 1934), media optimisation for metabolic creativity and metabolite productivity have not been investigated. The main aim of this study was to assess the affect of different carbon and nitrogen sources and the availability of vitamins on the metabolite production of our marine isolate of *E. rubrum* (RUC0101).

The objectives for this study were to:

(a) Simultaneously optimise

(1) production of metabolites,

(2) incubation time and

(3) metabolic creativity.

Concentration data were only obtained for the two standards, flavoglaucin and physcion.

(b) Develop an HPLC-DAD method for the metabolic profiling of *E. rubrum* (RUC0101).

4.2. Materials and Methods

4.2.1. General Experimental

HPLC solvents were of HPLC grade (Merck) while all extraction solvents were redistilled before use. Media reagents (glucose, lactose, urea, peptone, ammonium tartrate, yeast extract, fructose, sucrose, maltose, and mannitol) were also obtained from Merck. Seawater was obtained from the coastal waters of Port Alfred and was filtered before use. Fermentation media were autoclaved for 20 minutes at 121°C.

4.2.2. Optimisation of Temperature and pH

The optimum pH and temperature was determined by using growth as an indicator. The method used is described by Langvad (1999). A seed culture was grown by inoculating 50mL of YPG basal medium (yeast extract, peptone, and glucose) in a 500mL Erlenmeyer flask with six circles (7mm diameter) of mycelial mat grown on YPG agar. The culture was grown at 24°C at 180rpm until mid-log phase (3 days). Upon harvest, the inoculum was centrifuged at 13 000rpm for 25 minutes. The biomass was resuspended and washed with sterile seawater (50mL) and centrifuged again. The biomass was thereafter resuspended in

30mL of sterile seawater, adjusted with either NaOH or H_2SO_4 to the relevant pH (pH 3 - 8), homogenised for 3 x 0.5 second bursts and used to inoculate 96-well microtitre plates (Costar 3595). Each plate contained 4 replicates of 180µL YPG basal medium adjusted to pH's 3-8 with Universal Buffer (Britton and Robinson, 1931) (Appendix A) and 4 replicates adjusted without buffer. 20µL of homogenate was used to inoculate each well. The plates were sealed with parafilm and the four plates incubated at 20°C; 24°C; 28°C and 37°C. The optical density at 630nm was measured twice daily using a Powerwave X microtitre plate reader.

4.2.3. Media Optimisation

4.2.3.1. Experimental Design

Six carbon sources (glucose, sucrose, maltose, lactose, mannitol and fructose), four nitrogen sources (peptone, yeast extract, urea and ammonium tartrate) and the absence/presence of vitamin solution (Appendix A) were used to determine the optimum medium for the production of secondary metabolites and metabolic creativity. Since this was a preliminary screening experiment, the optimisation of the concentrations of nutrients was not determined. All combinations of the three factors were investigated resulting in 48 experiments, which were done in duplicate.

4.2.3.2. Preparation of Inoculum and Cultivation

An inoculum was prepared as described in 4.2.2. The biomass obtained from the seed culture was washed with sterile H_2O and thereafter resuspended in 100mL of sterile sea water adjusted to pH = 5 with buffer. 1mL of the homogenate was used as inoculum for each medium. The media were made up follows:

Nutrient	Concentration
C-source	0.5M
N-source	5g/L
Vitamin solution	4mL
Buffer (Teorell and Stenhagen, 1938)	50mL
Seawater made to	250mL

The pH was adjusted with buffer to 5 and the solution autoclaved. Vitamin solution and urea were added by filter sterilisation (0.22µm) to the relevant flasks and glucose was

autoclaved separately and added to the relevant sterile media. The cultures were incubated at 24°C under static conditions due to the higher creativity of the fungus under these conditions (Chapter 2). This is consistent with results obtained by Carbone and Johnson (1964) who reported *A. umbrosus* to have better growth and more pigment per gram of mycelium when grown under static conditions. By inspection of the mycelial growth, the end of stationary phase was determined whereupon the mycelial mats were separated from the broth by centrifugation and each stored separately at -20°C until extraction.

4.2.4. Preparation of Fungal Extracts for Analysis

The mycelia were frozen using liquid nitrogen and crushed using a mortar and pestle. Thereafter the mycelia were extracted sequentially with MeOH, MeOH/DCM (1:1, v/v) and again with MeOH. The extracts were dried *in vacuo* and weighed. A stock solution containing 1mg/mL of crude extract was prepared in acetonitrile, for each experiment, and passed through a 0.45µm filter (Millipore, U.S.A) before analysis by HPLC.

4.2.5. Preparation of Standards

The standards used in this section were isolated from crude extracts of RUC0101 (Chapter 5). Two standards were chosen: physcion (compound **1**) and flavoglaucin (compound **7**) and were used as representatives of compounds produced in each class i.e. anthraquinones and auroglaucin analogues respectively.

A series of dilutions ranging from 50 - 1000µg/mL and 10 - 1000µg/mL respectively, for compounds **1** and **7**, were prepared and injected in duplicate using the parameters discussed in section 4.2.6. These concentrations were chosen in order to cover the range of peak areas that were produced by these compounds in the optimisation experiments. The linearity of both compounds was determined over the designated concentration range by linear regression analyses.

4.2.6. Instrumentation and HPLC Analysis

All analyses were done on an Agilent HP 1100 Liquid Chromatograph equipped with quaternary gradient pump, 1100 PDA detector, C_{18} µBondupak reversed phase column (4.6 x 250mm) attached to a guard column (Security Guard) and a rheodyne injector (20µL sample loop). 20µL of a 1mg/mL sample solution were injected and the results captured on Agilent

ChemStation software package. The analysis was done at a flow rate of 0.5mL/min with a water-acetonitrile gradient starting at acetonitrile-water (40:60) for 15 minutes followed by a linear profile from 15 - 45 minutes to acetonitrile-water (90:10) and finally a step to 100% acetonitrile at which it was maintained from 45 - 55 minutes. The system was then equilibrated for 10 min with the starting conditions. Almost all the metabolites isolated from the *A. glaucus* group are either aromatic or have conjugated chromophores thus making their detection by ultraviolet spectroscopy possible. Metabolites were therefore detected at wavelengths of 254 and 230nm.

4.2.7. Analysis of Results

The metabolic creativity was calculated as described by Monhaghan *et al.* (1994) as the number of peak areas that were greater than 1% of the total area produced by all peaks. The productivity was calculated from the peak areas at 254nm using a standard curve for each compound.

4.3. Results and Discussion

4.3.1. Development of HPLC Method

Although several solvent combinations and gradients were evaluated, a mobile phase containing acetonitrile-water with 0.05% trifloroacetic acid gave optimum results for the marine isolate RUC0101. This is in accordance with results obtained by Frisvad and Thrane (1987) for the analysis of a variety of mycotoxins. Peak purity was assessed by checking the UV spectra of the upslope, apex and downslope of each peak. The UV spectra of physcion (1[1]), isodihydroauroglaucin (**2**), isotetrahydroauroglaucin (**3**), aspergin (**6**), flavoglaucin (**7**) and neoechinuline A (**18**) were slightly different to reported values (Fig. 4.3) but this was expected as the absorption wavelength and maxima are influenced by the actual gradient composition at the moment of detection, especially in the presence of TFA (Frisvad, 1989). Significant variations in peak areas were observed when duplicate analyses were done on different days. However, percentage peak areas showed very little variation.

[1] Numbers refer to that in the isolation scheme (Scheme 5.1)

4.3.2. Analysis of Standards

One of the main objectives of this study was to assess how the fermentation conditions affect the metabolic creativity (number of compounds in the crude extract) of the fungus. In addition to this we also evaluated the effect of different fermentation conditions on the amount of flavoglaucin (**7**) and physcion (**1**) produced. These two compounds were selected because they were isolated in a sufficiently pure state and gave well-resolved peaks in the pure (Fig. 4.1 and 4.3) and crude HPLC chromatograms. These two compounds also represent two different classes of secondary metabolites produced by this fungus. Unfortunately neoechinuline A (**18**) did not give a well resolved HPLC peak under the conditions used and was not tested. Both flavoglaucin (**7**) and physcion (**1**) exhibited a linear relationship between area and concentration up to 1000µg/mL (Fig. 4.2 and 4.4). The linear regression equations are $y = 145.65 + 27.68x$ with correlation coefficient, $r^2 = 0.9999$ for flavoglaucin and $y = 9.36x - 379.17$ with $r^2 = 0.9855$ for physcion. For areas which correlated to concentrations below the range of the standard curve a result of <10µg/mL or <50µg/mL for flavoglaucin and physcion respectively were reported.

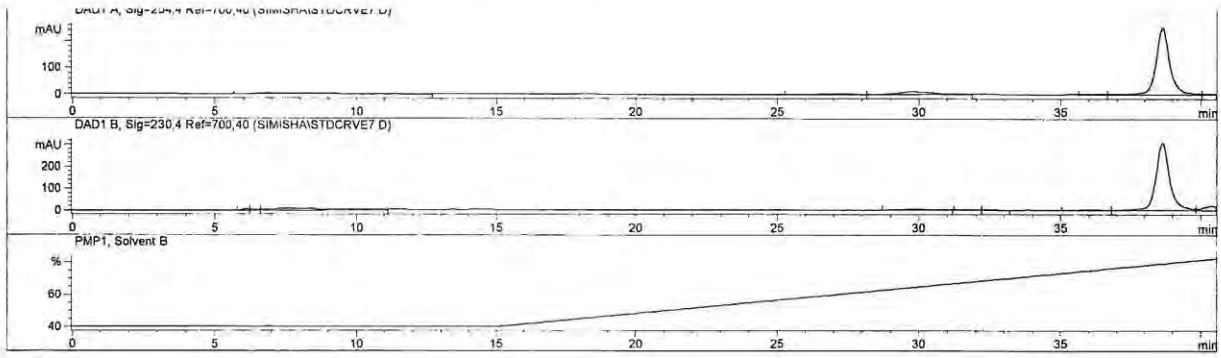


Fig. 4.1: HPLC Chromatogram of Physcion (1)

Table 4.1: Range of Linearity of Physcion

Concentration (µg/mL)	Mean Area (mAU*s)	% RSD
50	344.5	0.0764
100	695.9	0.0983
200	1667.5	0.0739
400	3068.9	0.0893
500	3636.4	0.0360
1000	9371.8	0.0873

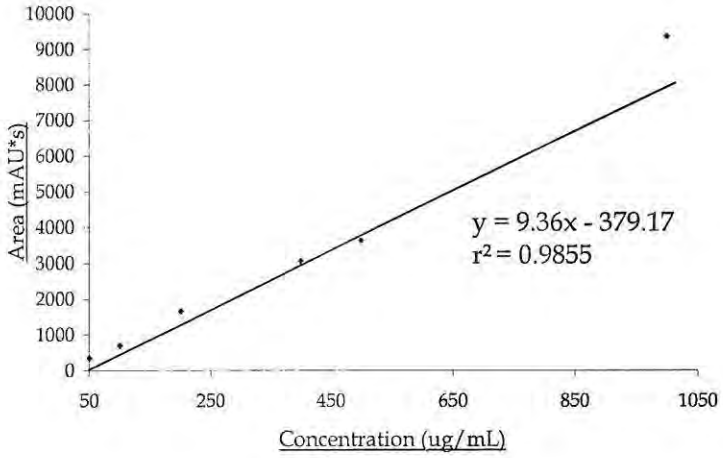


Fig. 4.2: Standard Curve of Physcion

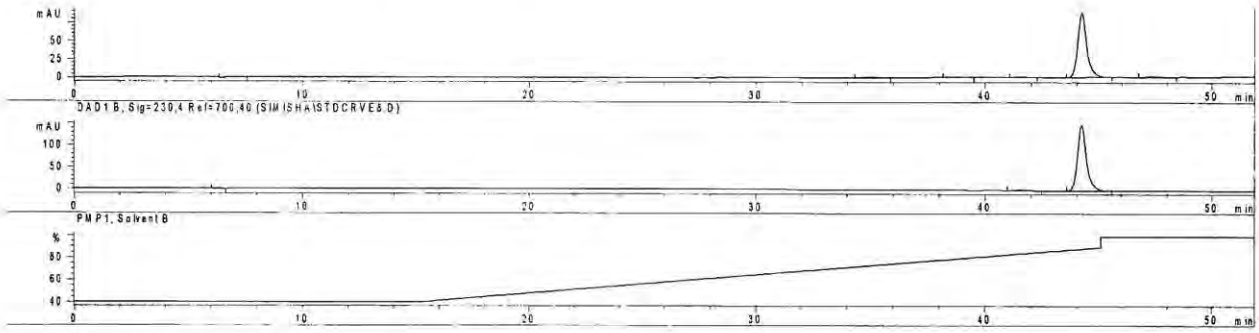


Fig. 4.3: HPLC Chromatogram of Flavoglaucin (7).

Table 4.2: Range of Linearity of Flavoglaucin.

Concentration (µg/mL)	Mean Area (mAU*s)	% RSD
10	314.8	0.003
40	1203.8	0.0266
80	2426.7	0.0096
120	3552.6	0.0087
240	6814.6	0.0027
1000	27804.6	0.0067

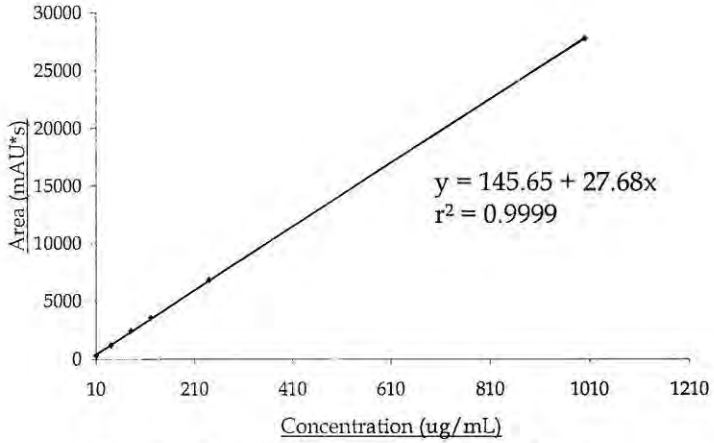


Fig. 4.4: Standard Curve of Flavoglaucin

Table 4.3: Retention Time and UV Maxima of Standards

COMPOUND NAME	RETENTION TIME (min)	UV MAXIMA (nm)	REPORTED UV MAXIMA (nm)
Physcion (1)	38.6	190; 225; 270; 290; 440	224; 257; 268; 288; 436
Flavoglaucin (7)	44.2	210; 250; 275; 400	240; 276; 388

4.3.3. Optimisation of Media

4.3.3.1. Influence of pH and Temperature on the Growth of RUC0101

Growth was used to determine the optimum temperature and pH in order to detect which physical parameters caused a time decrease in the lag phase resulting in production of idiolites (secondary metabolites) in stationary phase. However, although these physical parameters may increase biomass and result in shorter incubation periods, they may affect the production of certain metabolites. A good way of using growth as an indicator of an optimum medium would therefore be to analyse the biomass by HPLC to determine if there is a significant effect on metabolite productivity. However due to a number of experimental trials, this was not feasible.

The results obtained by using absorbance as a measurement of growth were inconsistent with actual onset of stationary phase. The optical density measurement is in the wavelength range of the visible spectrum (630nm). Therefore increases in the thickness of the mycelia cause an increase in the absorbance measurements. The discrepancy in the results is due to the production of pigments (secondary metabolites), which cause the mycelia to change from white to yellow to orange to red to brown. This affects the absorbance measurements by causing it to increase when it is actually in stationary phase. Therefore instead of optical density, visual inspection was used i.e. production of spores and production of reddish pigments known to be produced during secondary metabolism in the *Aspergillus glaucus* group (Raper and Fenell, 1965) (Fig.4.5). This method was better suited for RUC0101 than optical density, although it is more subjective. However, because growth of the fungus is not homogenous, different regions will be in different stages of growth. Thus the conversion of a metabolite of interest may have already taken place in some regions while in another it is still being produced.

The fungus grew best at 24°C - 27°C, pH 4 - 5 with buffer. No growth occurred at 37°C and growth at 20°C was slow (Table 4.4). The addition of the buffering agent increased the growth rate at all temperatures and ionic concentrations. These results are consistent with previous literature from Gould and Raistrick (1934), who found that the optimum pH for growth for members of the *Aspergillus glaucus* group was in the vicinity of neutrality with little or no growth above pH 8 and below pH 4 and the optimum temperature was between 18 and 27°C with the best growth for most species at 24°C. According to Raper and Fenell (1965) *E. rubrum* grew on Czapek's solution at room temperature (24 - 26°C), and *Aspergillus*

umbrosus, which according to Blaser (1974) is catergorised into the same group as *E. rubrum* grew best at pH 5.5 at 24°C. These conditions enabled *A. umbrosus* to produce the optimum amounts of crude secondary metabolite extracts (Carbone and Johnson, 1964).

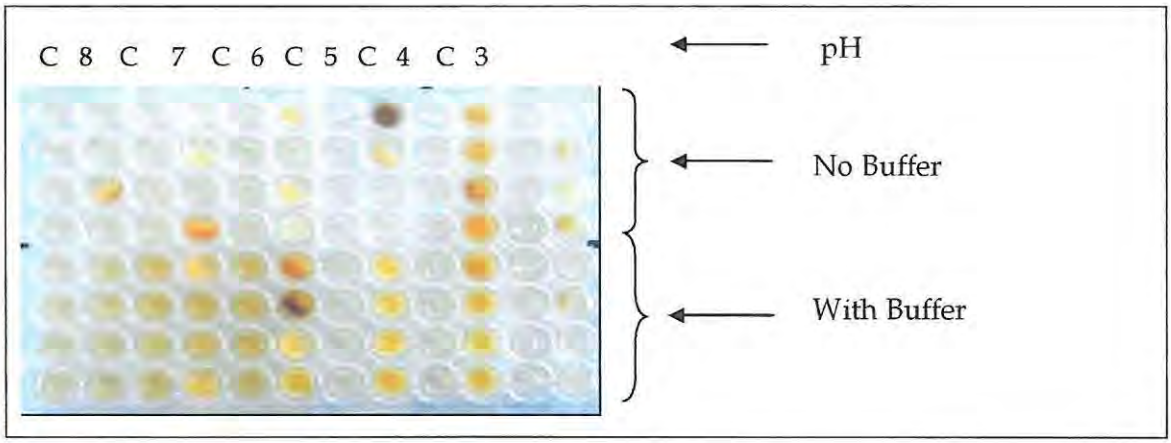


Fig. 4.5: RUC0101 grown at 27°C with YPG Basal medium; pH range 3-8.
C= control/blanks.

Table 4.4: Time Taken for RUC0101 to Reach Stationary Growth Phase under the Various Conditions

Time (Days)				
pH	**Buffer**			
	20°C	**24°C**	**27°C**	**37°C**
8	>8	>8	>8	No growth
7	>8	>8	>8	No growth
6	>8	5	5	No growth
5	>8	5	5	No growth
4	8	5	4	No growth
3	>8	6	6	No growth
No Buffer				
8	>8	No growth	>8	No growth
7	>8	No growth	>8	No growth
6	>8	7	7	No growth
5	>8	7	7	No growth
4	>8	7	6	No growth
3	>8	>8	8	No growth

4.3.3.2. Optimisation of Media

4.3.3.2.1. Growth Time

With regard to incubation time and the total amount of secondary metabolites produced, GP (glucose, peptone) supported the fastest growth (eight days) producing 307mg/250mL of crude extract as expected. In members of the *A. glaucus* group, this combination produced abundant growth but consisted of more conidia than perithecia (Gould and Raistrick, 1934). This was not the case here because when grown in media containing a higher carbon than

nitrogen content the fungus reproduces sexually and thus more perithecia than conidia (asexual) are formed. SYEV (sucrose, yeast extract, vitamins) supported the highest production of metabolites (650mg/250mL) after 19 days of incubation. Although YPG basal medium still produced one of the highest amounts of crude extract (622mg/250mL), the culture only reached stationary phase after approximately 25 days. With most other media, growth was between 11 and 13 days but these did not support high amounts of metabolite production, except for MYEV (453mg/250mL). Of the nitrogen sources, yeast extract and peptone produced higher yields than urea and ammonium tartrate, most probably due to these complex nutrients containing a small amount of carbohydrates, vitamins and other trace elements in addition to nitrogen. All carbon sources were comparable, except for mannitol which produced low yields. This was expected because mannitol is a slow-utilisable sugar.

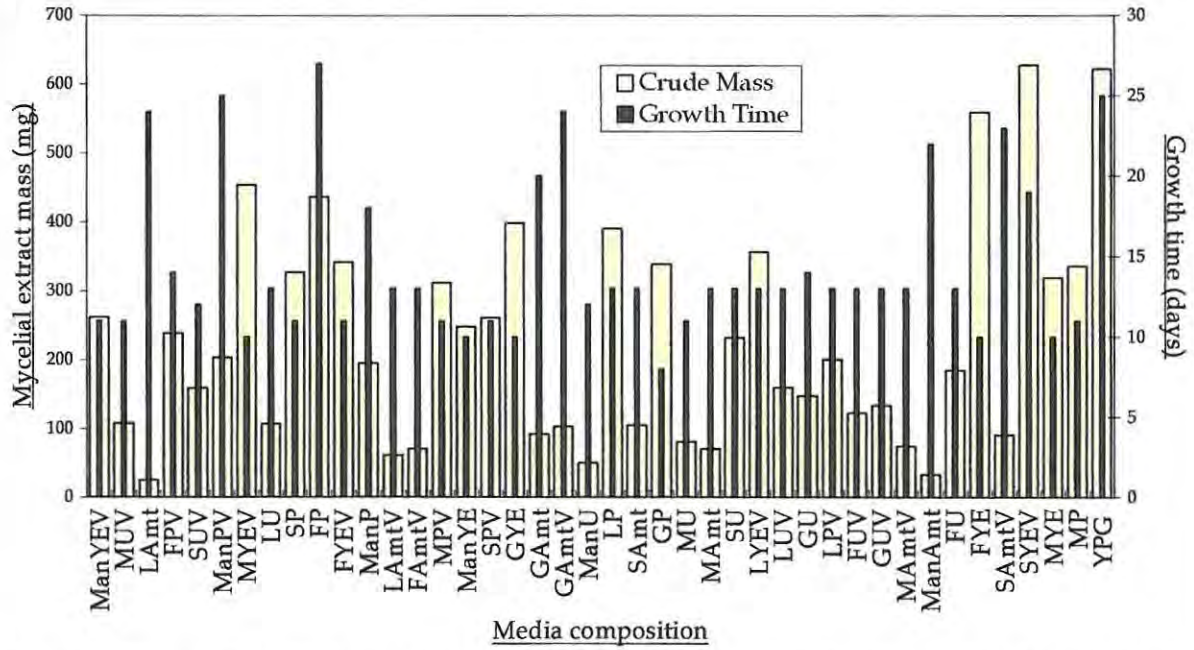


Fig. 4.6: Effect of Media Composition on Growth and Intracellular Metabolite Production.
G= glucose; L= lactose; M= maltose; F= fructose; Man= mannitol; S= sucrose; P= peptone; Amt= ammonium tartrate; YE= yeast extract; U= urea; V= vitamins.

4.3.3.2.2. Analysis of Crude Extracts

The HPLC chromatogram of RUC0101 grown on YPG Basal Medium shows the compounds of interest (Fig. 4.8 - A). Although these compounds were produced when RUC0101 was cultivated on most of the media used, their amounts differed considerably. Therefore, the metabolic creativity was calculated as the number of compounds produced that elicited a response of more than 1% of the total area produced by all the compounds in that extract.

Using this, ManP (mannitol, peptone), MAmt (maltose, ammonium tartrate) produced the most number of compounds (Fig. 4.7). Although flavoglaucin (7) aspergin (6), physcion (1) and isodihydroauroglaucin (2) were produced by the fungus in most of the media, their amounts were significantly different in different media. In addition a number of other compounds (that were not isolated) were produced in one or a few of the media.

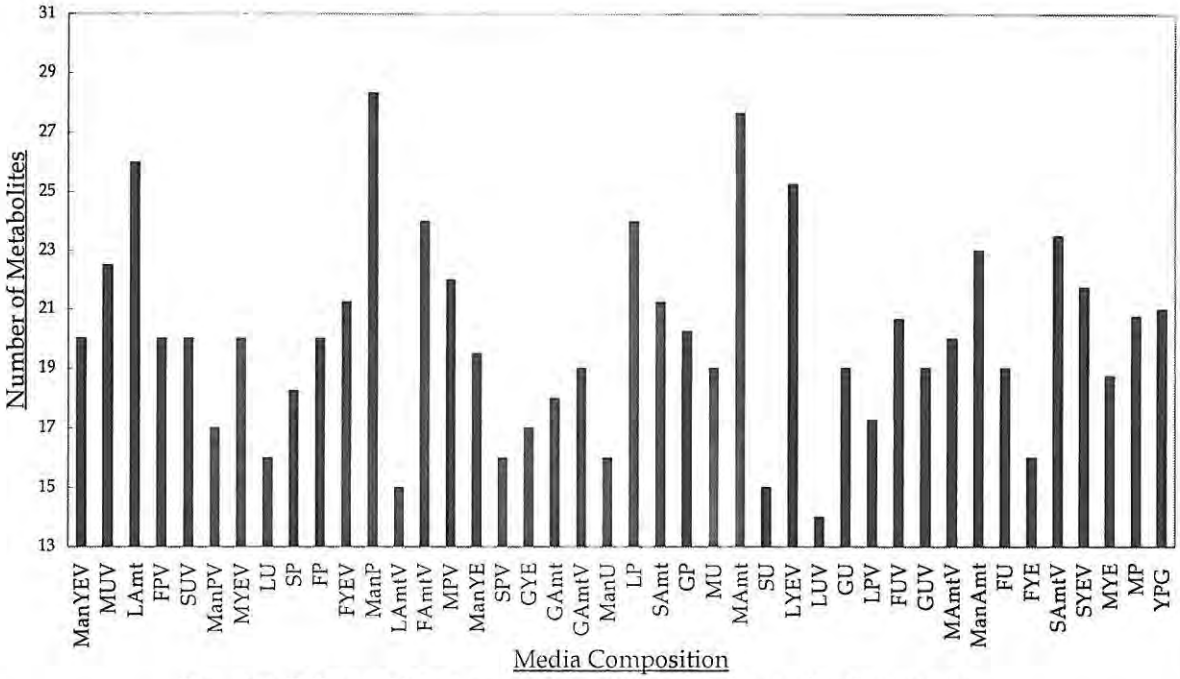


Fig. 4.7: Effect of Media Composition on Metabolic Creativity

G= glucose; L= lactose; M= maltose; F= fructose; Man= mannitol; S= sucrose; P= peptone; Amt= ammonium tartrate; YE= yeast extract; U= urea; V= vitamins.

4.3.3.2.3. Metabolic Creativity

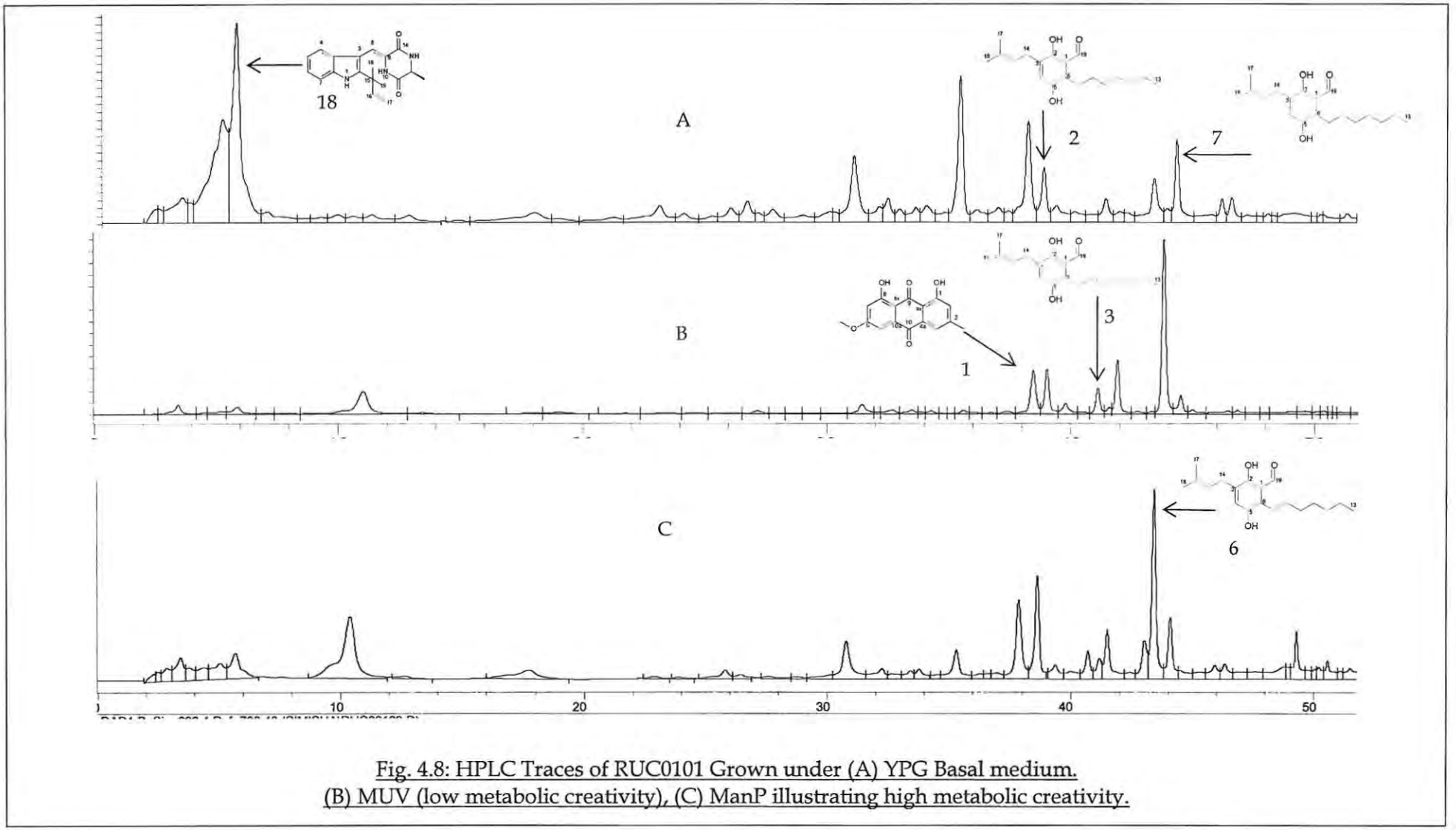


Fig. 4.8: HPLC Traces of RUC0101 Grown under (A) YPG Basal medium.
(B) MUV (low metabolic creativity), (C) ManP illustrating high metabolic creativity.

4.3.3.2.4. Metabolic Productivity

Based on these estimations, SYEV (sucrose, yeast extract, vitamins), FP (fructose, peptone), SP (sucrose, peptone) and FYEV (fructose, yeast extract, vitamins) produced the highest amounts of both compounds. These amounts are better than previously found for flavoglaucin (7) (Gould and Raistrick, 1934). Previous reports with *A. amstelodami* show that flavoglaucin and echinuline are produced in large amounts when grown in Czapek-Dox medium supplemented with sucrose, and that increasing amounts of sucrose (up to 50%) causes an increase in the crude flavoglaucin and echinuline (Allen, 1972). Once again, low yields of both compounds were produced in media with urea and ammonium tartrate.

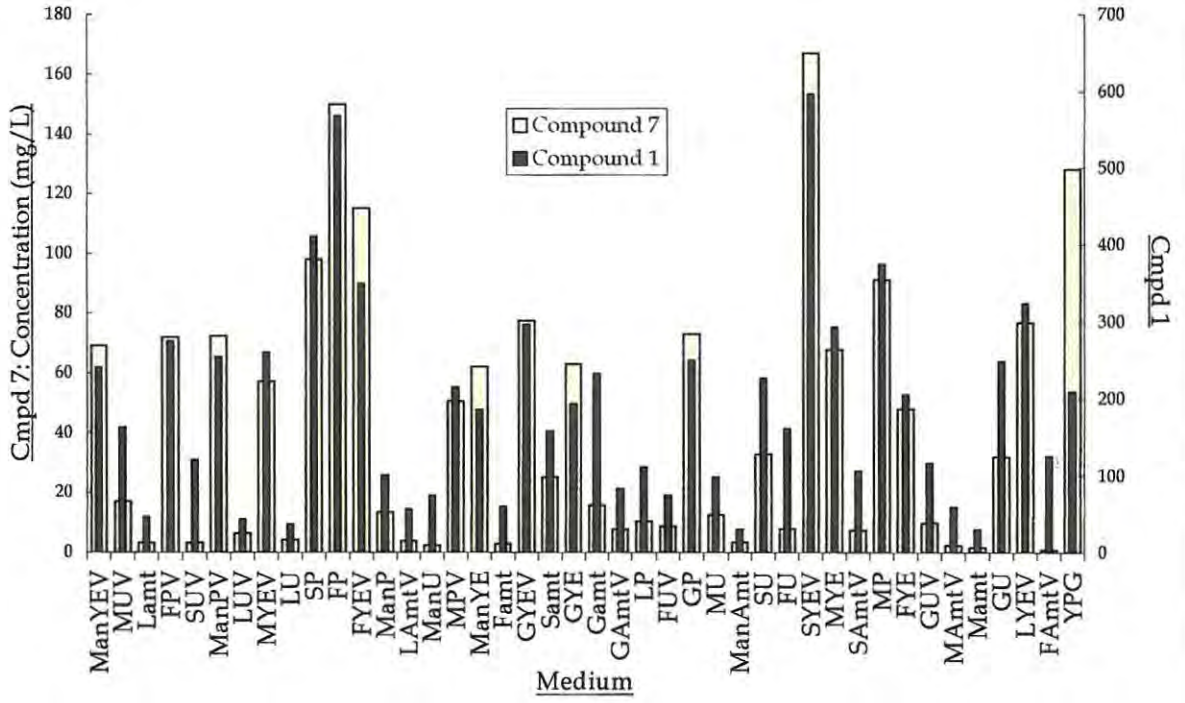


Fig. 4.9: Effect of Media Composition on Metabolite Production.
G= glucose; L= lactose; M= maltose; F= fructose; Man= mannitol; S= sucrose; P= peptone; Amt= ammonium tartrate; YE= yeast extract; U= urea; V= vitamins.

Due to the same medium being found to be optimum for both growth and productivity of physcion and flavoglaucin, it can be assumed that these nutrients cause a general increase in biomass of the fungus, rather than having a direct effect on the biosynthesis of them. Anke *et al.* (1980a) reported that with *A. cristatum*, although yeast extract produced higher mycelial weights, many of the anthraquinones had decreased. This might hold true for RUC0101 because only one anthraquinone, physcion, was isolated. However the HPLC

chromatograms (Fig. 4.8) show that another compound (retention time = 30min) that wasn't isolated exhibited a very similar UV spectrum to that of physcion and thus may be a related compound.

Table 4.5: Summary of Optimisation Experiments

MEDIA	INCUBATION TIME (DAYS)	PRODUCTIVITY (mg/L)		METABOLIC CREATIVITY
		Flavoglaucin (7)	Physcion (1)	
ManYEV	11	76	256	21
ManYEV	11	62	225	19
MUV	11	11	111	23
MUV	11	22	212	21
LAmt	24	4	55	26
LAmt	24	1	36	29
FPV	14	59	228	18
FPV	14	84	319	20
SUV	12	5	188	20
SUV	12	1	52	20
ManPV	25	75	263	17
ManPV	25	68	245	17
LUV	13	6	41	14
LUV	13	6	43	14
MYEV	10	62	231	21
MYEV	10	51	288	19
LU	13	4	40	16
LU	13	3	33	16
SP	11	112	486	17
SP	11	83	335	19
FYEV	11	145	398	19
FYEV	11	84	301	21
ManP	18	3	39	30
ManP	18	22	160	22
MPV	16	54	251	22
MPV	11	46	177	23
ManYE	10	53	147	21
ManYE	10	71	224	18
GYEV	18	73	228	20
GYEV	15	81	363	20
SAmt	13	7	89	24
SAmt	13	42	226	19
GYE	9	38	296	19
GYE	10	87	89	15
GAmt	14	8	127	18
GAmt	20	22	337	17
GAmtV	13	11	112	20
GAmtV	24	4	52	18
LP	13	13	182	22
LP	13	7	38	19
FUV	13	7	71	19
FUV	11	9	76	22

MEDIA	INCUBATION TIME (DAYS)	PRODUCTIVITY (mg/L)		METABOLIC CREATIVITY*
		Flavoglaucin (7)	Physcion (1)	
GP	8	76	236	22
GP	8	69	262	21
MU	11	16	117	25
MU	11	8	77	17
ManAmt	22	5	52	20
ManAmt	13	1	6	26
SU	13	40	285	13
SU	13	25	167	17
FU	13	6	169	20
FU	14	8	153	19
LPV	13	50	132	16
LPV	11	39	118	18
MAmtV	13	2	59	19
MAmtV	14	1	58	22
MAmt	13	1	6	24
MAmt	13	1	52	37
LYEV	13	83	326	30
LYEV	13	70	320	20
GU	13	37	293	18
GU	14	26	203	20
FAmt	12	2	55	20
FAmt	13	2	64	20
GUV	13	8	106	22
GUV	12	10	125	16
SAmtV	23	2	78	23
SAmtV	12	12	134	25
FP	27	141	543	20
FP	19	158	592	22
SYEV	19	141	489	23
SYEV	19	192	703	19
MYE	10	77	269	20
MYE	10	57	315	19
MP	11	114	457	22
MP	13	67	291	21
LAmtV	13	3	56	15
LAmtV	13	5	53	16
FAmtV	14	1	74	25
FAmtV	14	1	80	25
ManU	12	2	21	16
ManU	12	5	25	16
FYE	8	48	205	16
FYE	8	50	200	16

* Metabolic creativity = number of compounds produced that elicited a response of more than 1% of the total area produced by all the compounds in that extract.

4.4. Conclusion

Although the optimisation study involved a considerable amount of time (3 months from culturing to HPLC analyses and interpretation) these results correlate with those found for the *Aspergillus glaucus* group. Additionally, the simultaneous optimisation of (1) incubation time, (2) metabolic creativity and (3) metabolic productivity can be used with confidence.
The HPLC method can be used for metabolic profiling and can be used in conjunction with the NMR - bioassay screening route. However some modifications such as decreasing the acetonitrile: water ratio must be made for better separation of 0-10minutes.

Chapter 5:

Isolation and Characterisation of Secondary Metabolites

5.1. Introduction

5.1.1. Dereplication of Metabolites

It is important to determine early on in the study of an organism if compounds produced have been isolated and characterised previously for bioactivity. This prevents time and money spent on compounds already studied. There are two general methods used for dereplication:

(1) Direct comparison to reference compounds by chromatography (Borders, 1999). Rapid screening requires the availability of a library of reference compounds and methods for the simple identification of active compounds from the crude extract (Verpoorte, 1998). These include thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and hyphenated HPLC techniques such as HPLC-MS (HPLC- mass spectrometry); HPLC-DAD (HPLC-diode array detection) and GC-MS (gas chromatography-mass spectrometry).

(2) Comparison of the chemical properties of pure compounds to those in databases and in literature. There are various databases available, the most comprehensive of which is the Chemical Abstracts Services which can be searched using SciFinder Scholar. In addition, MarinLit, Chapman Hall (>103 000 compounds), NAPRALERT and Beilstein contain information on ^{13}C and ^{1}H nuclear magnetic resonance (NMR), infra-red spectroscopy (IR) and mass spectral data of natural products. Berdy (>23 000), DEREP (>7 000), Antibase (>17 000) and Kitasato (>17 000) databases are usually based on ultraviolet (UV) chromophores, molecular weight (MW) and general biological properties (Borders, 1999). Chapmann and Hall only contains information on compounds that are well characterised. Searches of this database utilise molecular formula (MF), MW, structure and substructures. The Antibase system is one of the more recent commercial databases for antibiotics and other microbial metabolites. The database can be searched by biological properties and UV spectra, MW, MF and ^{13}C NMR signals. NAPRALERT provides information on biological properties and cannot be searched by MF, MW or UV chromophores (Borders, 1999).

Although minute quantities of crude metabolites are needed by the first method, an adequate number of reference compounds are needed. This takes a considerable amount of time to build up, but works well when developed. The problem with the second method is that a lot of time and money is invested by (1) first purifying the "new" metabolites, (2) and

then attaining the various spectra for comparison. For this a few milligrams of the pure compounds are needed.

5.1.2. Extraction and Purification Methods

An important factor in determining the optimal downstream processing steps for microbial metabolites is the physical properties of the molecule (MW, polarity and ionic charge) (Borders, 1999). The microbial metabolites range from extremely water-soluble compounds to very organic solvent-extractable substances that have all types of ionic charges and a range of MW's (Borders, 1999). The most difficult to purify are the water-soluble compounds. Ion exchange methods are generally used for these types of compounds, however some may be suitable to reversed-phase chromatography.

An important consideration is to extract the metabolites in a way that will not interfere with assay results e.g. if enzymes or proteins are to be assayed then special precautions need to be taken. Yarbrough *et al.* (1992) has highlighted the most common methods of metabolite extraction as follows:

1. Filtration of the broth supernatant through a high molecular weight exclusion filter with subsequent freeze-drying
2. Extraction of the broth with an organic solvent
3. Extracting the broth with resins

Depending on the solvent used in the extraction method, different quantities and different types of compounds can be extracted from the organism or the culture broth. Many kinds of extraction schemes have been designed, some highly selective for a certain group of compounds, others very general. In a programme screening for antitumour compounds, scientists at the NCI compared a series of extraction methods and found that with dichloromethane-methanol (1:1), all known antitumour compounds were extracted (Verpoorte, 1998). With all extraction methods, several primary metabolites will be extracted and usually in larger quantities than the secondary metabolites. In non-polar extracts for example, steroids and lipids will be present in large amounts, whereas in polar extracts sugars will be the major component (Verpoorte, 1998). Liquid extraction has been widely used for the extraction of secondary metabolites from fungi. Methanol, ethyl acetate, chloroform and dichloromethane have been used successfully to remove most of the secondary metabolites. The fractions of low and medium polarity can be separated by

normal or reversed-phase column chromatography and then by HPLC to attain the purified compounds.

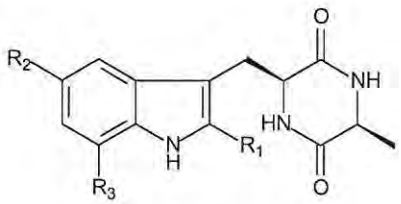
5.1.3. Structure Elucidation

Once the pure compounds have been isolated, the structures are elucidated by a combination of Nuclear Magnetic Resonance, Mass Spectrometry and Infra Red methods. Together these provide a map of the carbon hydrogen framework of the organic molecule. The NMR techniques include ^{1}H, ^{13}C, heteronuclear multiple bond correlation (HMBC), showing protons correlating to neighbouring carbons; heteronuclear multiple quantum coherence (HMQC), showing protons directly attached to carbons; ^{1}H-^{1}H-homonuclear correlation spectroscopy (COSY) which shows proton-proton correlations; and distortionless enhancement by polarization transfer (DEPT), showing methines (CH), methylenes (CH_2) and methyls (CH_3). Ta ken together, these techniques make it possible to determine the complete structures of a compound.

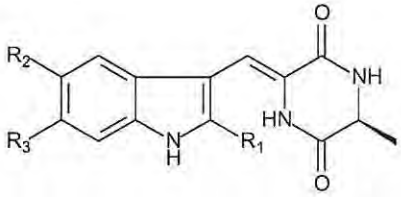
Since the *Aspergillus glaucus* group has been well studied, several compounds have been isolated and identified. Table 5.1 lists the compounds that have been identified.

Table 5.1: Secondary Metabolites Isolated from the *Aspergillus glaucus* Group.

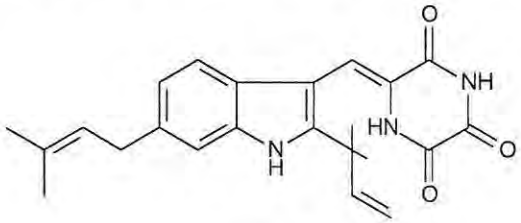
Metabolite:	**Reference:**
ω-hydroxyemodin-5-methylether (HEME) (**5.25**)	Arai *et al.*, 1989
ω-hydroxyrubrocristin (HRC) (**5.24**)	Arai *et al.*, 1989
Aflatoxin B_1 (**5.28**)	Leitao *et al.*, 1989
Asperentin (**5.29**)	Podojil *et al.*, 1978
Asperentin 8-methylether (**5.30**)	Podojil *et al.*, 1978
Asperflavin (**5.27**)	Arai *et al.*, 1989
Aspergin (**5.14**)	Arai *et al.*, 1989
Asperinine A (**5.34**)	Arai *et al.*, 1989
Asperinine B (**5.34**)	Arai *et al.*, 1989
Auroglaucin (**5.17**)	Gould and Raistrick, 1934
Catenarin (**5.20**)	Anke *et al.*, 1980a
Crytoechinuline G (**5.12**)	Gatti, 1978
Echinuline (**5.1**)	Quilico, 1964
Emodin (**5.23**)	Anke *et al.*, 1980a
Erythroglaucin (**5.20**)	Gould and Raistrick, 1934
Flavoglaucin (**5.13**)	Gould and Raistrick, 1934
Gliotoxin (**5.33**)	Cole and Cox, 1981
Isoechinuline A (**5.6**)	Nagasawa *et al.*, 1976
Isoechinuline B (**5.10**)	Nagasawa *et al.*, 1976
Isoechinuline C (**5.11**)	Nagasawa *et al.*, 1976
Isodihydroauroglaucin (**5.16**)	Arai *et al.*, 1989
Isotetrahydoauroglaucin (**5.15**)	Arai *et al.*, 1989
L-alanyl-L-tryptophan anhydride (ATA) (**5.3**)	Arai *et al.*, 1989
L-alanyl-2-(1,1-dimethylallyl)-L-tryptophan anhydride (ADTA) (**5.2**)	Arai *et al.*, 1989
Neoechinuline A (**5.4**)	Barbetta *et al.*, 1969
Neoechinuline C (**5.9**)	Cole and Cox, 1981
Neoechinuline D (**5.5**)	Cole and Cox, 1981
Neoechinuline E	Cole and Cox, 1981
Neoechinuline B (**5.8**)	Cole and Cox, 1981
Ochratoxin A (**5.32**)	Cole and Cox, 1981
Ochratoxin B (**5.33**)	Cole and Cox, 1981
Physcion (**5.18**)	Gould and Raistrick, 1934
Questin (**5.19**)	Frisvad, 1987
Rubrocrsitin (**5.22**)	Anke *et al.*, 1980a
Sterigmatocystine (**5.31**)	Schroeder and Kelton, 1975
Viocristin (**5.26**)	Anke *et al.*, 1980a
Xanthocillin	Frisvad, 1987



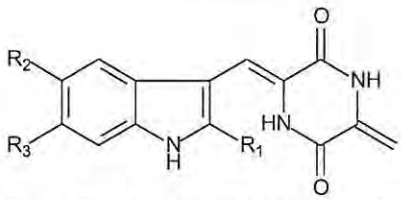
Echinulinine (**5.1**) R_1 = ~$C(CH_3)_2CH{=}CH_2$; $R_2 = R_3$ ~$CH_2CH{=}C(CH_3)_2$
ADTA (**5.2**) R_1 = ~$C(CH_3)_2CH{=}CH_2$; $R_2 = R_3$ =H (Preechinuline)
ATA (**5.3**) $R_1 = R_2 = R_3$ = H



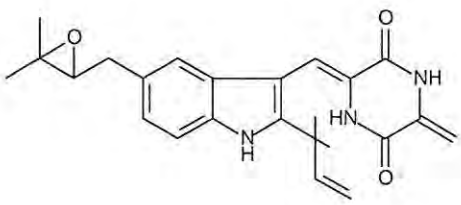
Neoechinuline A (**5.4**) R_1 = $C(CH_3)_2CH_2$; $R_2 = R_3$ = H
Neoechinuline D (**5.5**) R_1 = $C(CH_3)_2CH_2$; R_2 = H; R_3 = ~$CH_2CH{=}C(CH_3)_2$
Isoechinuline A (**5.6**) R_1 = $C(CH_3)_2CH_2$; R_2 = ~$CH_2CH{=}C(CH_3)_2$=H; R_3 = H



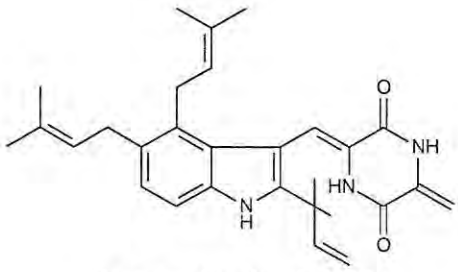
Neoechinuline (**5.7**)



Neoechinuline B (**5.8**) R_1 = ~$C(CH_3)_2CH{=}CH_2$; $R_2 = R_3$ = H
Neoechinuline C (**5.9**) R_1 = ~$C(CH_3)_2CH{=}CH_2$; R_2 = H; R_3 = ~$CH_2CH{=}C(CH_3)_2$
Isoechinuline B (**5.10**) R_1 = ~$C(CH_3)_2CH{=}CH_2$; R_2 = $CH_2CH{=}C(CH_3)_2$=H; R_3 = H

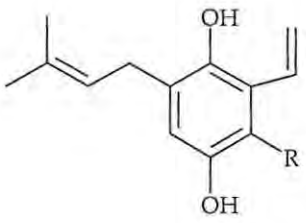


Isoechinuline C (**5.11**)



Cryptoechinuline G (**5.12**)

Fig. 5.1: *A. glaucus* Group Metabolites



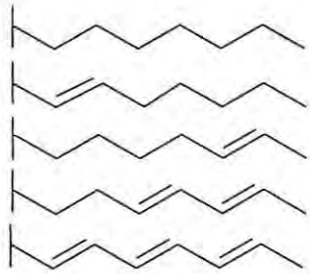
Flavoglaucin (**5.13**)

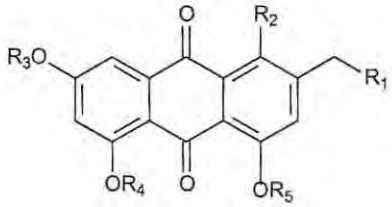
Aspergin (**5.14**)

Isotetrahydroauroglaucin (**5.15**)

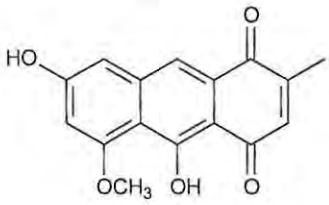
Isodihydroauroglaucin (**5.16**)

Auroglaucin (**5.17**)

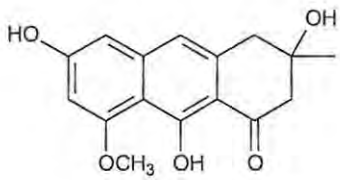




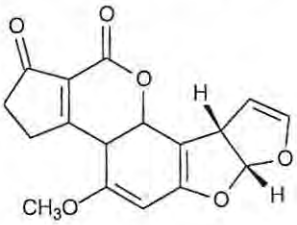
	R_1	R_2	R_3	R_4	R_5
Physcion (**5.18**)	H	H	CH_3	H	H
Questin (**5.19**)	H	H	H	CH_3	H
Erythroglaucin (**5.20**)	H	OH	CH_3	H	H
Catenarin (**5.21**)	H	OH	H	H	H
Rubrocristin (**5.22**)	H	OH	H	CH_3	H
Emodin (**5.23**)	H	H	H	H	H
HRC (**5.24**)	OH	OH	H	CH_3	H
HEME (**5.25**)	OH	H	H	CH_3	H



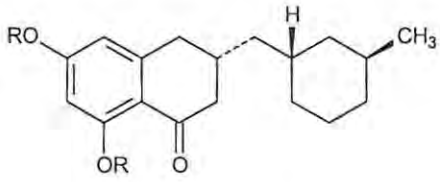
Viocristin (**5.26**)



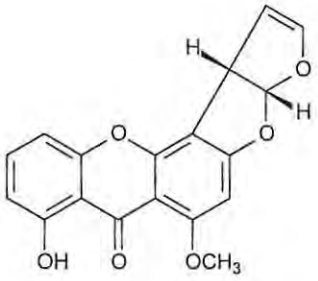
Asperflavin (**5.27**)



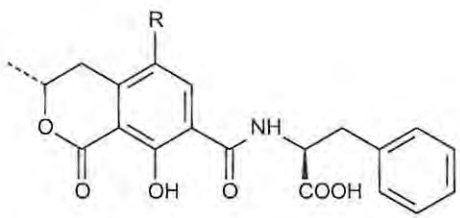
Aflatoxin B_1 (**5.28**)



Asperentin (**5.29**) R = H
Asperentin dimethylether (**5.30**) R = CH_3

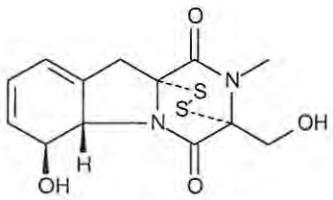


Sterigmatocystin (**5.31**)

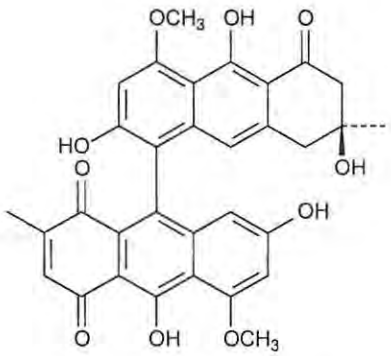


Ochratoxin A (**5.32**) R= Cl
Ochratoxin B (**5.33**) R = H

Fig. 5.1: *A. glaucus* Group Metabolites CONTD.



Gliotoxin (**5.33**)



Aperinine A and B (**5.34**)

Fig. 5.1: *A. glaucus* Group Metabolites CONTD.

The objective of this study was to isolate and characterise the major secondary metabolites from the marine fungal isolate RUC0101 and to use them as standards during our optimisation experiments and bioassays.

5.2. Materials and Methods

5.2.1. General Experimental

Normal phase TLC was performed on Silica Gel $60F^{254}$ plates (Merck) and reverse phase TLC on $RP18F^{254}$ plates. The plates were viewed under UV light (254nm). HPLC separations were performed using a Spectra-Series P100 liquid chromatography pump (Thermo Separations), Spectra-Series UV 100 detector and a rheodyne injector equipped with either an analytical HPLC C_{18} μBondupak reverse phase column (4.6 x 250mm) attached to a guard column (Security Guard) or a preparative HPLC C_{18} reversed-phase column (Phenomenex Luna 10μ; 250 x 10mm) attached to guard column (Security Guard).

Solvents were either of HPLC grade or were distilled before use. Fractions and pure compounds were dried under vacuum using a Buchi Rotavapour RE 120 or with N_2 using a Pierce Reacti Therm Heating Module.

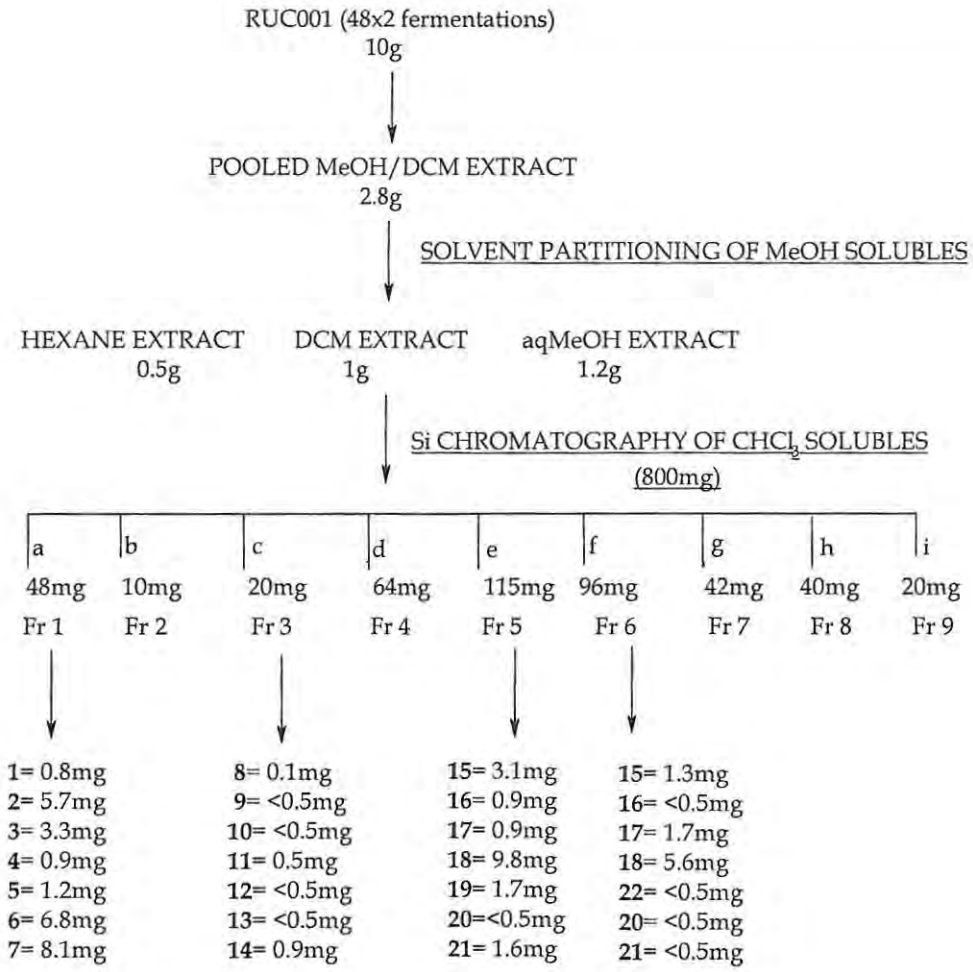
The ^1H (400MHz), ^{13}C (100MHz), DEPT-135, HMQC, HMBC and COSY-90 were recorded on an Avance Bruker 400 MHz spectrometer using standard pulse sequences at 27°C. Either deuterated chloroform ($CDCl_3$) or deuterated DMSO (DMSO-*d*6) was used. Chemical shifts are reported in ppm and referenced to undeuterated solvent resonances ($CHCl_3$ δH 7.25, δC 77.0; DMSO δH 2.50, δC 39.43) and coupling constants are reported in Hz.

Infrared spectra were recorded on a Perkin-Elmer Spectrum 2000 FT-IR spectrometer on NaCl or CsCl disks. ESI-MS spectra were recorded on a Finnigan Mat LCQ mass spectrometer. Optical rotations were measured using a Perken-Elmer 141 Polarimeter.

5.2.2. Isolation of Pure Metabolites

The individual mycelial extracts (Chapter 4) resulting from the optimisation experiments were pooled to give a total of 10g organic material. A portion of this crude extract was fractionated according to Scheme 5.1. Thus, 2.8g of the MeOH solubles were partitioned between hexane (3 x 50mL) and 10% aqueous MeOH (55mL). Water (20mL) was added to the aqueous MeOH fraction which was further partitioned with DCM (3 x 50mL). The hexane (470mg), DCM (970mg) and aqueous MeOH (1.16g) fractions were dried over $NaSO_4$ (anhydrous), filtered and concentrated *in vacuo*. These fractions were analysed by TLC hexane/EtOAc (8:2) and on a RP-C_{18} HPLC column (Waters μBondapak, 4.5 x 250 mm) using CH_3CN/H_2O/TFA (50: 50:0.05) at 1mL/min; 0.05 AUFS, except for the aqueous MeOH fraction which was insoluble in the mobile phase. Since the DCM fraction contained most of the compounds extracted, the pure compounds were purified from this fraction.

800mg of the DCM extract was separated using silica chromatography (Kiesel silica gel 60) (5 x 40cm) using a solvent combination of increasing polarity (Hexane: EtOAc, 10:0; 9:1; 8:2; 7:3; 4:6; 6:4; 8:2; 0:1 then MeOH/DCM 1:9; 1:1; 1:0 and finally MeOH/H_2O 1:1; 0:1). Fractions were combined according to TLC analysis to give 10 fractions. Final purification was achieved by reversed phase HPLC (Scheme 5.1).



Scheme 5.1: Extraction and Isolation Procedure for RUC001.

(a): 90% hexane/EtOAc; (b): 80% hexane/EtOAc; (c) 70%-60% hexane/EtOAc; (d) 40% hexane/EtOAc; (e) 20% hexane/EtOAc; (f) 100%EtOAc; (g) 10% MeOH/$CHCl_3$; (h) 50% MeOH/$CHCl_3$; (i) 100% MeOH+ 50% MeOH/H_2O.
I: CH_3CN/H_2O/TFA (60:40:0.05), 2 AUFS; 3mL/min;
II: CH_3CN/H_2O/TFA (70:30:0.05), 2 AUFS; 1mL/min;
III: 85% MeOH/H_2O, 2 AUFS; 2mL/min; 254nm;
IV: 80% MeOH/H_2O.

5.3. Results and Discussion

5.3.1. Extraction and Isolation

The crude mycelial extract from the 96 fermentations were pooled (2.8g) resulting in a blood-red extract that showed several interesting signals at δ 12, δ 10, δ 5 -7 and δ 2-4 in the ^{1}H NMR spectrum. The crude extract was fractionated according to Scheme 5.1. Solvent partitioning experiments showed that the DCM fraction contained a number of compounds that were present in the crude fraction. Silica chromatography of this fraction (800mg) produced nine further fractions, which were then purified by HPLC. The non-polar fractions (a-f) were yellow becoming red while the polar fractions (g-i) were red-brown in colour. The latter could not be separated due to its high polarity. Although 22 compounds were isolated, only six compounds physcion (compound **1**), isodihydroauroglaucin (compound **2**), isotetrahydroauroglaucin (compound **3**), aspergin (compound **6**), flavoglaucin (compound **7**) and neoechinuline A (compound **18**) were pure enough and isolated in sufficient quantity for their structures to be elucidated. Furthermore, the quantities of pure compounds isolated from the DCM fraction are not representative of the total amount contained in the mycelial crude extracts. A better indication of the quantities produced by both physcion and flavoglaucin can be obtained using the optimisation experiments (Chapter 4).

5.3.2. Structure Elucidation

5.3.2.1. Compound 7

It was evident from the ^{1}H, ^{13}C and mass spectrum of compound 7 that there were 19 carbons, 28 protons and 3 oxygens. The 19 carbon resonances were attributable to 6 x C; 3 x CH; 7 x CH_2 and 3 x CH_3 (Table 5.2). The ^{1}H NMR spectrum (Fig. 5.2) showed typical signs of an isoprenyl group with signals at δ 1.69 (3H, s); δ 1.75 (3H, s); δ 3.28 (2H, d, J = 7Hz) and δ 5.36 (1H, t). A strong ^{1}H NMR signal at δ 10.24 suggested the presence of an aromatic aldehyde, which was confirmed by the IR spectrum containing a band at 1628cm^{-1}. Also present in the ^{1}H NMR spectrum were two exchangeable protons s at δ 11.9 (1H, 2-OH, hydrogen bonded) and δ 4.3 (1H, br, 5-OH) as well as an aromatic proton (δ 6.88, s). This confirmed the presence of the three oxygens two as OH's and one as a carbonyl. The UV maxima at 250nm supported the presence of an aromatic ring. The remaining protons (δ 0.88 - 1.6) were contained in an aliphatic chain. HMBC correlations were used to position the different groups on a pentasubstituted aromatic ring (Fig. 5.4). Key HMBC correlations are

shown in Fig. 5.3. Strong and unambiguous HMBC correlations (Fig. 5.4) from the phenolic OH at δ 11.9 (2-OH) to signals at δ 117.3 (C-1), 155.8 (C-2) and 128.5 (C-3) and from the methylene at δ 2.87 to signals at δ 128.5 (C-6), 117.3 (C-1) and 144.9 (C-5) were particularly useful. The remaining signals could only be accommodated in a heptyl chain. This structure complies with that of flavoglaucin (**5.13**) with all spectroscopic data in agreement with previous results (Podojil *et al.*, 1978; Allen and Barrow, 1977).

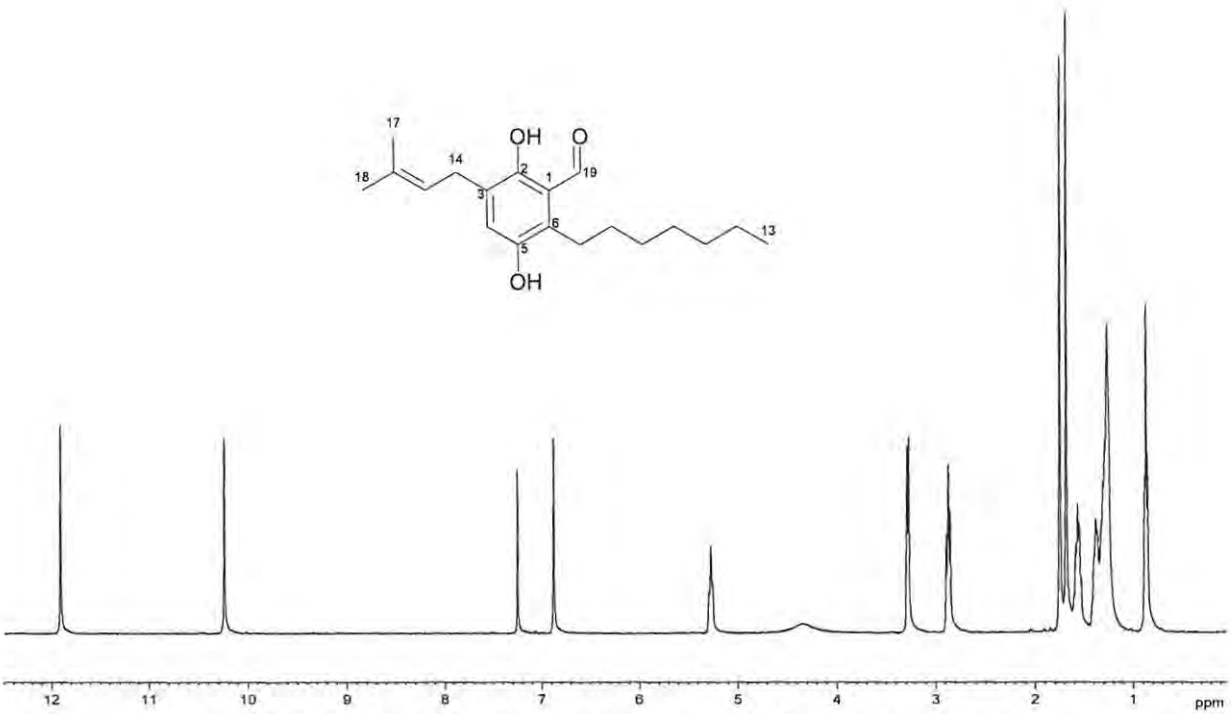


Fig. 5.2: ^{1}H NMR Spectrum of Compound 7 ($CDCl_3$; 400MHz).

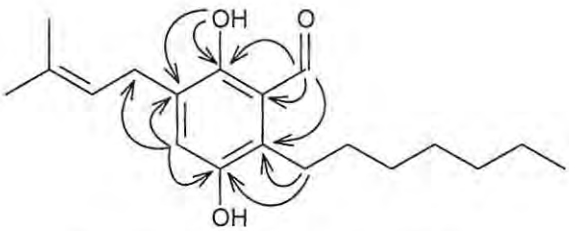


Fig. 5.3: Key HMBC Correlations

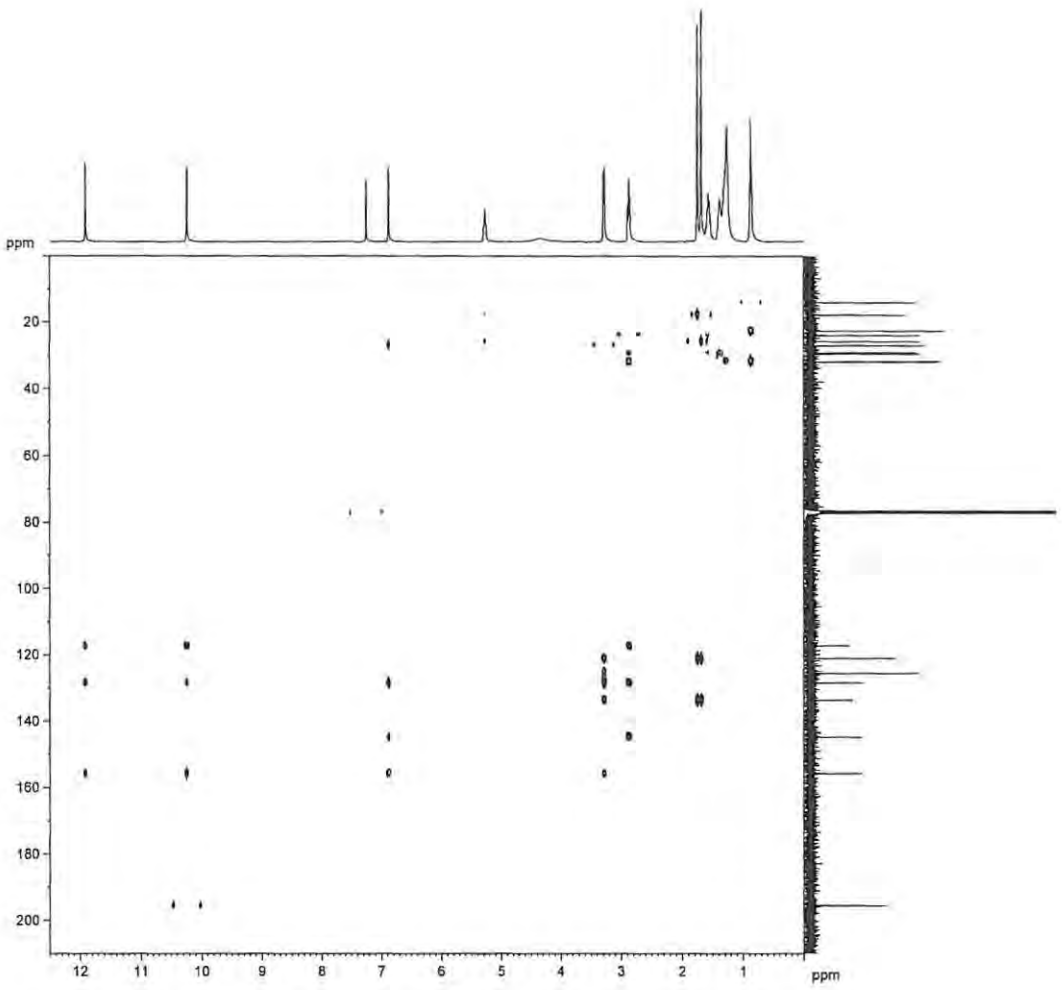


Fig. 5.4: HMBC Spectrum of Compound 7

Flavoglaucin has previously been isolated from several members of the *A. glaucus* group and shown to be cytotoxic (Kawai *et al.*, 1983), possess antioxidant properties (Ishikawa *et al.*, 1984; Tanaka *et al.*, 2001) and antimicrobial properties (Betina *et al.*, 1981). Its structure was originally determined by chemical degradation and synthesis of a reduced intermediate (Raistrick *et al.*, 1937; Cruickshank *et al.*, 1938 and Ashley *et al.*, 1939). Biosynthetic studies have shown that flavoglaucin is a regular polyketide which is modified by isoprenylation (Allen and Barrow, 1977). The putative acyclic biosynthetic precursor of flavoglaucin is shown in Fig. 5.5.

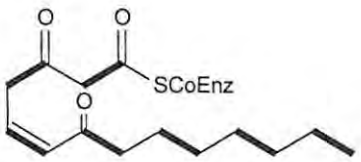


Fig. 5.5: Acyclic Precursor of Flavoglaucin

Table 5.2: Spectral Data for Compound **7** in $CDCl_3$.

Atom no.	δ_C	DEPT	δ_H [mult., *J* (Hz)]	COSY	HMBC
1	117.3	qC			
2	155.8	qC			
3	128.6	qC			
4	125.7	CH	6.88, s	2-OH (lr), H-14 (lr)	C-14; C-3; C-4
5	144.9	qC			
6	128.5	qC			
7	23.9	CH2	2.87, t, 7.6	H-9; H-19 (lr)	C-10; C-9; C-1; C-3; C-4
8	29.1	CH2	1.39, m	H-9	
9	31.9	CH2	1.57, m	H-8; H-7	C-7; C-17; C-3
10	29.6	CH2	1.39, m	H-9	
11	31.8	CH2	1.27, m	H-13	C-13; C-12; C-11
12	22.6	CH2	1.27, m	H-13	
13	14.0	CH3	0.88, t, 6.8	H-11	
14	27.0	CH2	3.28, d, 7.4	H-17 (lr); H-15, H-4	C-15; C-6; C-16
15	121.1	CH	5.26,br t, 7.4	H-14; H-17 (lr)	C-18; C-14
16	133.8	qC			
17	25.8	CH3	1.75, s		C-3; C-4
18	17.8	CH3	1.69, s	H-14; H-15	C-17; C-15; C-16
19	195.5	CH	10.24, s		C-1; C-6; 155.8
2-OH	-	-	11.9, s		
5-OH	-	-	4.3,br s		

5.3.2.2. Compound **6**

A comparison of the ^1H NMR spectra of Compounds **6** and **7** immediately suggested that the two were related. The ^1H NMR spectrum of compound **6** (Fig. 5.6) showed two exchangeable protons at δ 11.7 (1H, 2-OH) and δ 4.96 (1H, br, 5-OH); an aldehyde proton at δ 10.09 (1H, s) and signals which corresponded to a dimethylallyl moiety [δ5.28,t; δ 3.30,d; δ 1.75,s; δ 1.69, s]. The two additional signals at δ 6.46 (1H, d, *J*=16.4Hz) and δ 5.97 (1H, dt, *J* = 6.9Hz) therefore indicated unsaturation in the heptyl side chain. The low resolution mass spectrum of compound **6** showed a molecular ion peak at *m/z* 302 also suggesting that compound **6** is a dehydro derivative of flavoglaucin. An analysis of HMBC data confirmed the structure of the prenylated 2,5-dihydroxybenzaldehyde skeleton and also clarified the position of the double bond in the heptyl side chain. The doublet at δ 6.46, showed HMBC correlations to signals at δ 117 (C-1), 123 (C-6) and 144 (C-5) thus indicating that the double bond is between C-7 and C-8. This structure is consistent with that of aspergin (**5.14**) previously isolated from an unidentified *Aspergillus* sp. (Sokolov *et al.*, 1971).

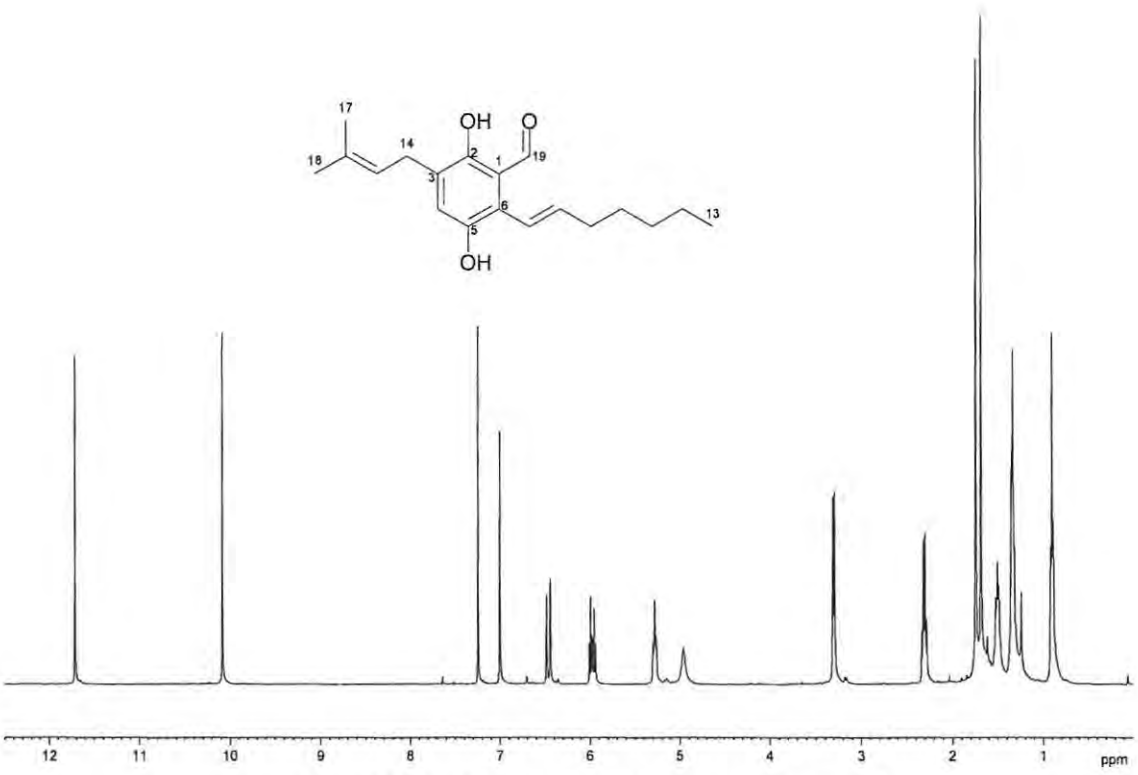


Fig. 5.6: ^{1}H NMR Spectrum of **6** ($CDCl_3$; 400MHz).

Table 5.3: Spectral Data of **6** in $CDCl_3$

Atom	δ_C	DEPT	δ_H [mult., *J* (Hz)]	COSY	HMBC
1	117.3	qC			
2	155.1	qC			
3	130.4	qC			
4	125.1	CH	7.01, s	H-14; 2-OH	C-14; C-3; C-5
5	144.8	qC			
6	123.9	qC			
7	120.1	CH	6.46, d, 16.4	H-14; H-8	C-9; C-1; C-6; C-5
8	142.7	CH	5.97, dt, 16.0, 9	H-14; H-7	C-9
9	33.4	CH2	2.30, m	H-10; H-8;H-7	C-8; C-7
10	28.7	CH2	1.5, m	H-14	
11	31.4	CH2	1.25, m		
12	22.4	CH2	1.35, m	H-13	
13	13.99	CH3	0.92, t, 7	H-12	
14	27.2	CH2	3.3, d, 7.2	H-18; H-15; H-4	C-2
15	121.0	CH	5.28, t, 6.9	H-14; H-18; H-18	C-18; C-17
16	133.9	qC			
17	25.8	CH3	1.75, s		C-15
18	17.8	CH3	1.69, s	H-14; H-15	C-15
19	196.3	CH	10.09, s		C-2; C-1; C-6
2-OH	-	-	11.7, s		C-3; C-2
5-OH	-	-	4.96, br s		

5.3.2.3. Compound **2**

The ^{1}H NMR spectrum of compound **2** (Fig. 5.7) showed signals at δ 11.9 (2-OH), δ 10.22 (1H, s, aldehyde), δ 6.89 (1H, s), δ 5.27 (1H, br t), δ 3.28 (2H, d, J = 7.2), δ 1.75 (3H, s) and δ1.68 (3H, s), which immediately suggested a prenylated 2,5-dihydroxybenzaldehyde derivative. Additional signals at δ 6.01 (2H, br m) and δ 5.59 (2H, br m) indicated the presence of two double bonds in the heptyl side chain. COSY and HMBC correlation data confirmed the structure of the prenylated 2,5-dihydroxybenzaldehyde moiety and assisted in positioning the two double bonds. The methylene at δ 2.97 (2H, t, J=7.2) showed HMBC correlations to signals at δ 117.3 (C-1), δ 145.0 (C-5) and δ 128.9 (C-6) as well as COSY correlations to a methylene at δ 2.33 (2H, br m) thus indicating the presence of a -CH_2CH_2- moiety at C-7 and C-8. The two double bonds can only therefore be accommodated at C-9 and C-11 (Fig. 5.7, Table 5.4). All the spectroscopic data are in agreement with the structure of isodihydroauroglaucin (**5.16**), which had previously been reported from *A. chevalieri* (Hamasaki *et al.*, 1980).

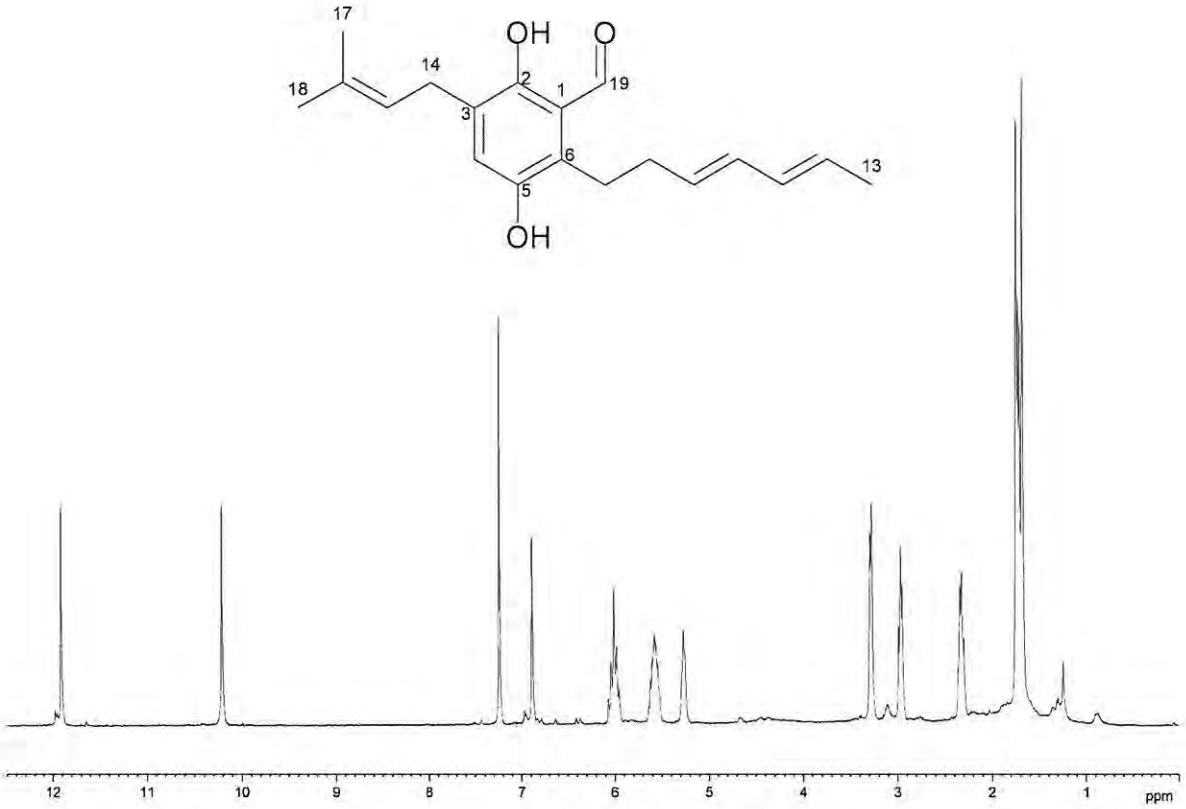


Fig. 5.7: ^{1}H NMR Spectrum of **2** ($CDCl_3$; 400MHz).

Table 5.4: Spectral Data of Compound **2** in $CDCl_3$

Atom	δ_C	DEPT	δ_H [mult., *J*	COSY	HMBC
1	117.3	qC			
2	155.9	qC			
3	127.4	qC	6.89, s	H-14; H-19	
4	125.8	CH	6.89, s	H-14; H-19	C-3; C-5
5	145.0	qC			
6	128.9	qC			
7	24.6	CH2	2.97, t, 7.2	H-8	C-1; C-5; C-8
8	34.2	CH2	2.33, br m	H-13; H-9; H-7; H-11 (lr)	C-9; C-10
9	129.3	CH	5.59, br m	H-8; H-11	C-8
10	132.0	CH	6.01, br m	H-8; H-9, H-11	C-8
11	131.1	CH	6.01, br m	H-8; H-9; H-11	
12	128.3	CH	5.59, br m	H-13; H-8; H-11	C-13
13	17.8	CH3	1.72, m	H-8; H-9; H-11	C-12
14	27.0	CH2	3.28, d, 7.2	H-17; H-7; H-9; H-11 (lr); H-15; H-3, H-4	C-15; C-16; C-3
15	121.1	CH	5.27, br t, 9.8	H-17; H-14	
16	133.9	qC			
17	26.5	CH3	1.75, s	H-8; H-14; H-15; H-9; H-11	C-18; C-16; C-15
18	18.0	CH3	1.68, s	5.29; H-14	C-17
19	195.4	CH	10.22, s		C-2; C-1; C-6
2-OH			11.9, s		C-13; C-2; C-19
5-OH			4.96, br, s		

5.3.2.4. Compound **3**

The final compound isolated in this series was compound **3** (Fig. 5.8). The presence of the prenylated 2,5-dihydroxybenzaldehyde moiety was confirmed by the observation of signals at δ 11.91 (1H, 2-OH), δ 10.23 (1H, s), δ 6.88 (1H, s), δ 5.28 (3H, m), δ 3.28 (2H, d, J = 7.3), δ 1.75 (3H, s) and δ 1.69 (3H, s). In addition, the methyl group at δ 0.92 (cf flavoglaucin) has disappeared and a new peak at δ 1.63 (3H, d, J= 4.5) appeared. It was therefore obvious that this compound contained a double bond at C-12. HMBC data supported this structure (Table 5.5). Isotetrahydroauroglaucin (compound **3**) (**5.15**) has previously been reported from *A. ruber* (Hamasaki *et al.*, 1980).

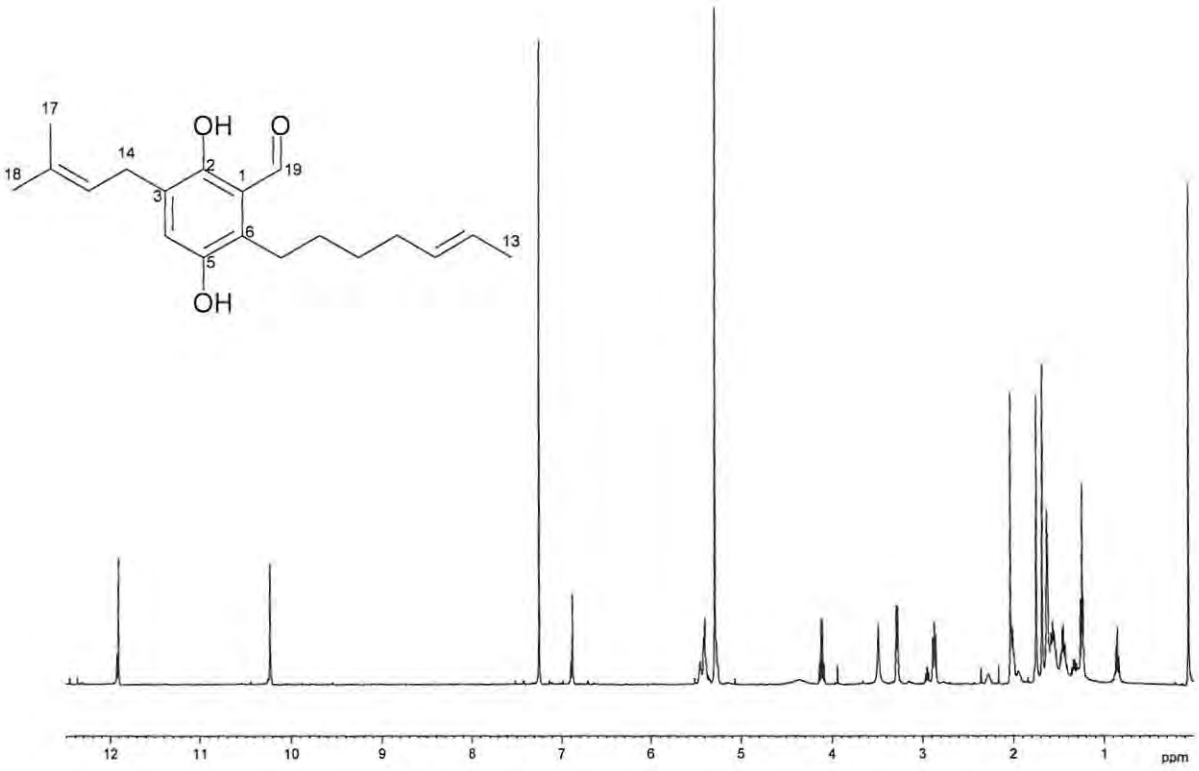


Fig. 5.8: ^{1}H NMR Spectrum of Compound **3** ($CDCl_3$; 400MHz).

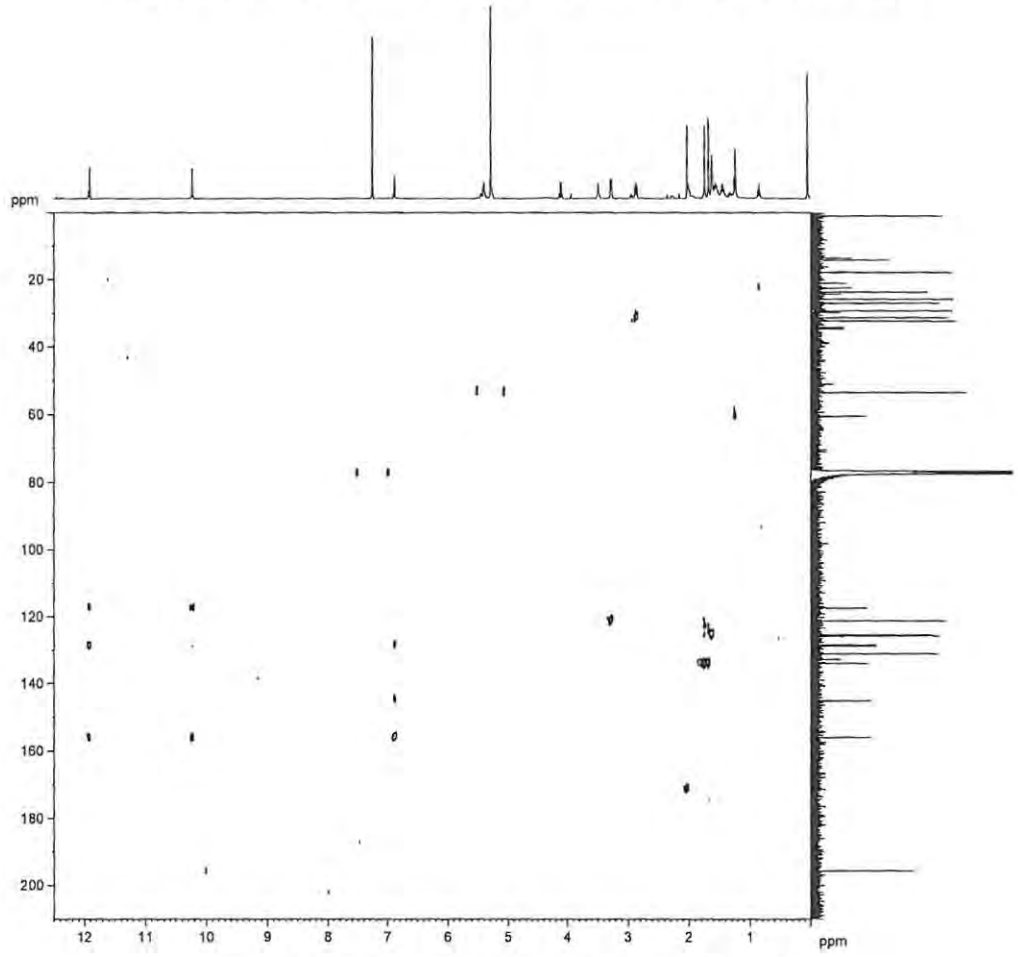


Fig. 5.9: HMBC Spectrum of Compound **3**

Table 5.5: Spectral Data for **3** in $CDCl_3$

Atom	δ_C	DEPT	δ_H [mult., *J* (Hz)]	COSY	HMBC
1	117.3	qC			
2	155.8	qC			
3	128.3	qC			
4	125.9	CH	6.88, s		C-6; C-5
5	144.9	qC			
6	128.3	qC			
7	24.3	CH2	2.87, t, 7.8	H-8	C-8
8	31.3	CH2	1.57, t, 8	H-7	
9	32.3	CH2	1.4, m	H-10	
10	29.7	CH2	2.05, s	H-9	
11	130.9	CH	5.28, m		
12	125.7	CH	5.28, m		C-13
13	17.9	CH3	1.63, d, 4.5		C-4
14	27.0	CH2	3.28, d, 7.3	H-11, H-12	C-15
15	128.7	CH	5.28, m	H-14	
16	133.8	qC			
17	25.8	CH3	1.75, s		C-16
18	17.8	CH3	1.69, s		C-16
19	195.9	CH	10.23, s		C-1; C-3; C-2
1-OH	-	-	4.3, br, s		
2-OH	-	-	11.91, s		C-1; C-2; C-3

5.3.2.5. Compound **18**

The ^1H NMR spectrum of compound **18** was significantly different from the ones previously discussed. The ^{13}C NMR spectrum of compound **18** showed the presence of 19 carbon signals of which 14 were sp^2 hybridized (Fig. 5.12; Table 5.6). The ^1H NMR spectrum (Fig. 5.10) showed signals for seven vinylic methine signals at δ 7.42 (1H, d, *J* = 8 Hz), δ 7.17 (1H, d, *J* = 7.6 Hz), δ 7.08 (1H, t, *J* = 7.4), δ 7.00 (1H, t, *J* = 7.4), δ 6.89 (1H, s), δ6.06 (1H, dd, *J* = 17, 10 Hz) and δ 5.01 (2H, dd, *J* = 17, 10 Hz), three methyl signals (δ1.47, 6H, s and δ 1.37, 3H, d, *J* = 6.8 Hz). ^{13}C NMR resonances at δ 126.0, 135.1, 111.7, 120.0, 119.5 and 118.9 together with a broad exchangeable proton at δ 11.0 in the ^1H NMR spectrum suggested the presence of an indole (Pretsch *et al.*, 2000) which was unsubstituted in the benzene ring. This substructure assignment was supported by an IR absorption band at 3360cm^{-1} and UV maxima at 230 and 290nm. Two amide groups were also deduced from ^{13}C signals at δ 166.6 and 160.0 and IR absorption bands at 1670 and 1630 cm-1. The above information together with a molecular ion peak at m/z 323 in the low resolution mass spectrum suggested a molecular formula of $C_{19}H_{21}N_3O_2$ and 11 degrees of unsaturation for compound **18.** HMBC and COSY-90 data were useful in determining the structure of compound **18.** The indolic proton at δ 11.0

showed HMBC correlations to signals at δ 144.1 (C-2), 135.1 (C-7a) and 103.4 (C-3) indicating that the indole ring was substituted at positions 2 and 3. An isopentenyl substituent at C-2 was deduced from a COSY correlation between the terminal methylene (δ 5.03) and the methine at δ 6.06 and HMBC correlations from the two methyl groups at δ 1.47 to carbon signals at δ 111.7 (C-17) and 144.1 (C-2). A 2,5-diketopiperazine ring was inferred from HMBC correlations from the proton at δ 8.61 NH-13 to carbons at δ 160.0 (C-14) and 50.6 (C-12) and from the NH-10 to carbons at δ 166.6 (C-11) and 103.4 (C-3). HMBC correlations from the vinylic proton at δ 6.89 (H-8) to carbons at 144.1 (C-2) indicated that this substituent was attached to the indole ring at position 3. This structure together with all the spectroscopic data comply with that of Neoechinuline A **(5.4)** (Gatti and Fuganti, 1979; Cole and Cox, 1981).

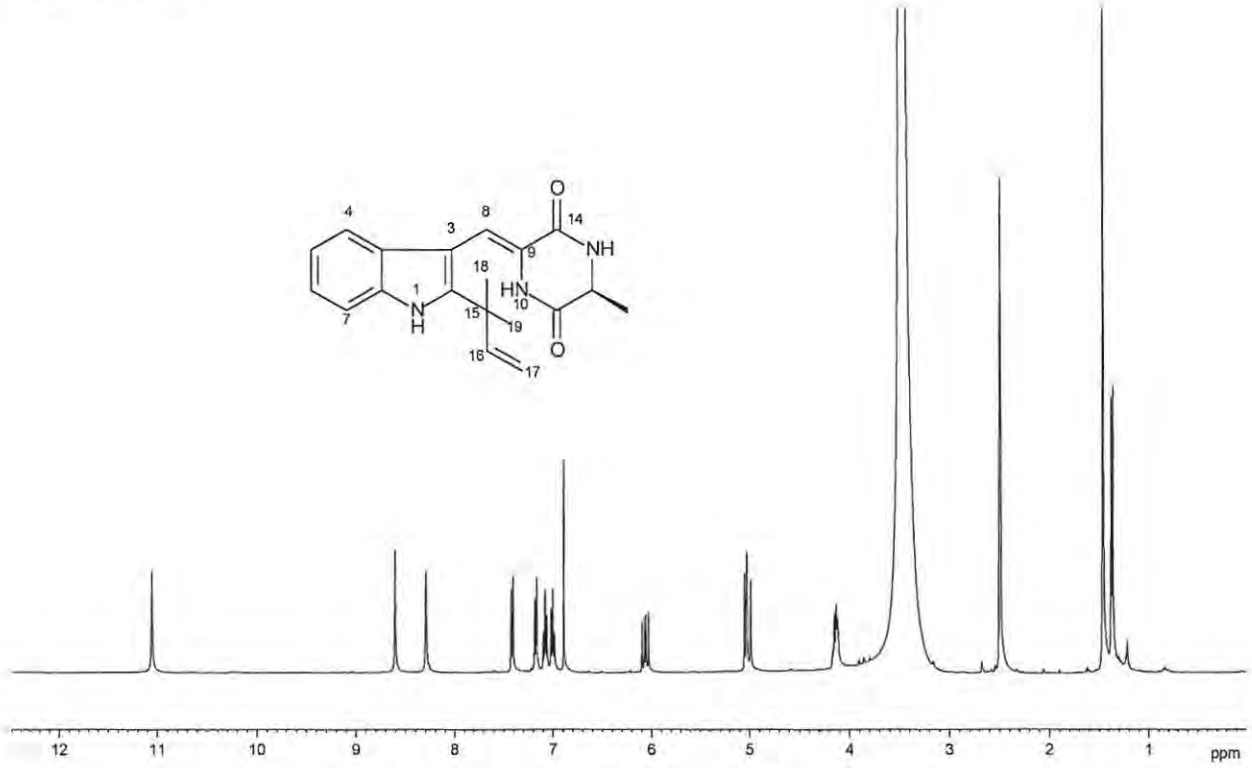


Fig. 5.10: ^{1}H NMR Spectrum of **18** (DMSO)-*d*6; 400MHz)

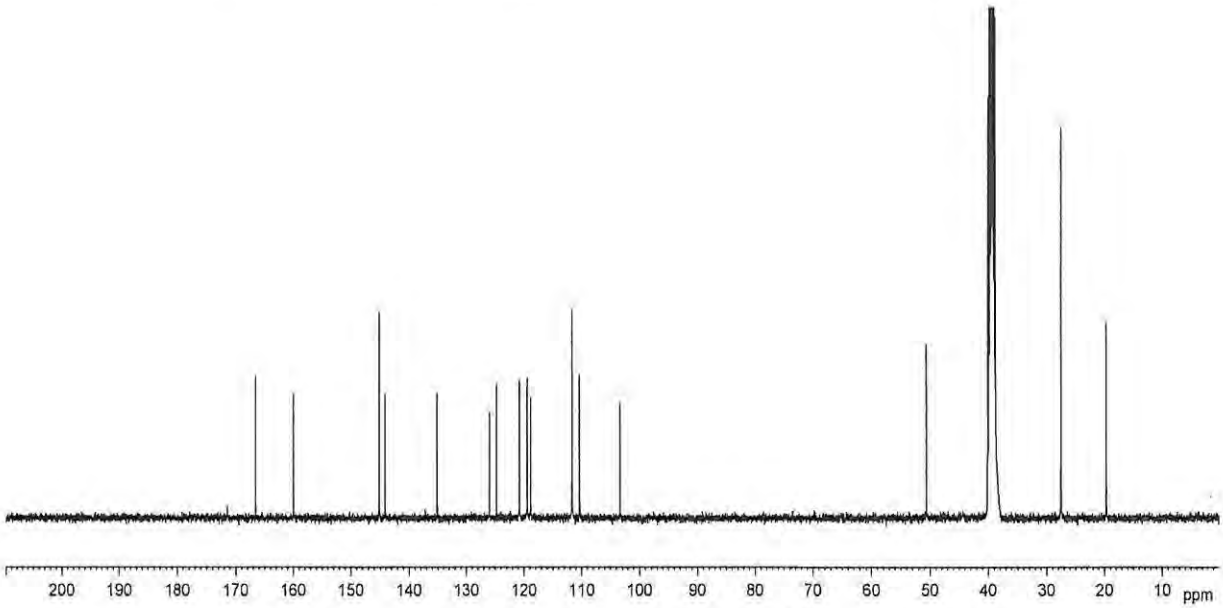


Fig. 5.11: ^{13}C NMR Spectrum of **18** (100MHz; DMSO-d6)

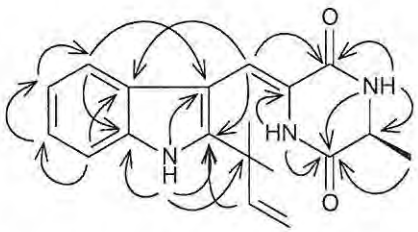


Fig. 5.12: Key HMBC Correlations of Compound **18**

Neoechinuline A is a structurally related dehydrotryptophan derivative of echinuline, an isoprenylated tryptophan derivative that was isolated from *A. amstelodami*. Birch *et al.* (1961) and Birch and Farrar (1963), have clearly established that the biosynthetic precursors of echinuline are tryptophan, alanine and mevalonic acid. It has been further established that L-tryptophan and cyclo-L-alanyl-L-tryptophanyl are *in vivo* precursors of echinuline (Allen, 1972).

Table 5.6: Spectral Data for Compound **18** in DMSO-*d6*

Atom	δ_C	DEPT	δ_H [mult., *J* (Hz)]	COSY	HMBC
1-NH	-	-	11.10, s		C-3a; C-3b; C-7b; C-2
2	144.1	qC			
3	103.4	qC			
4	118.9	CH	7.17, d, 7.6		C-3a; C-6;C-7b
5	119.5	CH	7.00, t, 7.4	H-5	C-7; C-3b
6	120.9	CH	7.08, t, 7.4		C-8; C-4; C-7b
7	111.7	CH	7.42, d, 8		C-4; C-3b
3a	126.0	qC			
7a	135.1	qC			
8	110.4	CH	6.89, s		C-3b; C-2; C-14; C-11
9	124.8	qC			
14	160.0	qC			
13-NH	-	-	8.61, s		C-8; C-9; C-14; C-11
12	50.6	CH	4.15, q, 6.4	H-20	C-14; C-11
11	166.6	qC			
10-NH	-	-	8.20, s		C-9; C-11
20	19.7	CH3	1.37, d, 6.8	H-12	C-11
15	40.2	qC			
18	27.5	CH3	1.47, s		C-7; C-2
19	27.5	CH3	1.47, s		C-7; C-2
16	145.2	CH	6.07, dd, 17, 10	H-17	C-2
17	111.7	CH2	5.03, dd, 17, 10	H-16	C-16

5.3.2.6. Compound **1**

The ^{1}H NMR spectrum of compound **1** (Fig. 5.13) showed two exchangeable proton signals [δ 12.3 (1H, s, 8-OH) and δ 12.1 (1H, s, 1-OH)], four aromatic methine signals [δ 7.63 (1H, s, H-4); δ 7.38 (1H, d, *J* = 2.8 Hz, H-11); δ 7.08 (1H, s, H-2) and δ 6.69 (1H, d, *J* = 2.8 Hz, H-7) and two methyl signals [δ 3.94 (3H, s, 6-OCH_3) and δ 2.45 (3H, s, 3-CH_3)]. Not all the carbons were immediately discernable in the ^{13}C spectrum because of the small amount of material isolated. Here, careful comparison of the ^{13}C spectrum with the HMBC spectrum (Fig. 5.15) was used to identify the remaining quaternary carbons (mainly carbons at ring junctions). Fourteen of the sixteen carbons for compound **1** were sp2 hybrizided and immediately suggested a polycyclic structure. Carbon signals at δ 190.8 (C-9), δ 182.2 (C-10), δ 166.6 (C-6), δ 165.2 (C-1), δ 162.5 (C-8) and δ 22.2 (3-CH_3) in addition to a strong infrared absorption at ν =1619cm^{-1} were typical of a trihydroxyanthraquinone structure of the emodin class. Careful analysis of the HMBC data (Table 5.7) allowed the positioning of the two hydroxyls, the methoxyl and the methyl substituents. A literature search indicated the compound **1** was identical to physcion which had also been previously reported from the genus *Aspergillus* (Anke *et al.*, 1980a; Engstrom *et al.*, 1980), as well as the lichen *Xanthoria* (Manojlovic *et al.*,

2000) and higher plant species (Turner, 1971). Physcion (**5.18**) has been found to exhibit various activities such as antibacterial and anti-fungal activities (Podojil *et al.*, 1978) and act as a sunscreen agent (Wynn-Williams *et al.*, 2002).

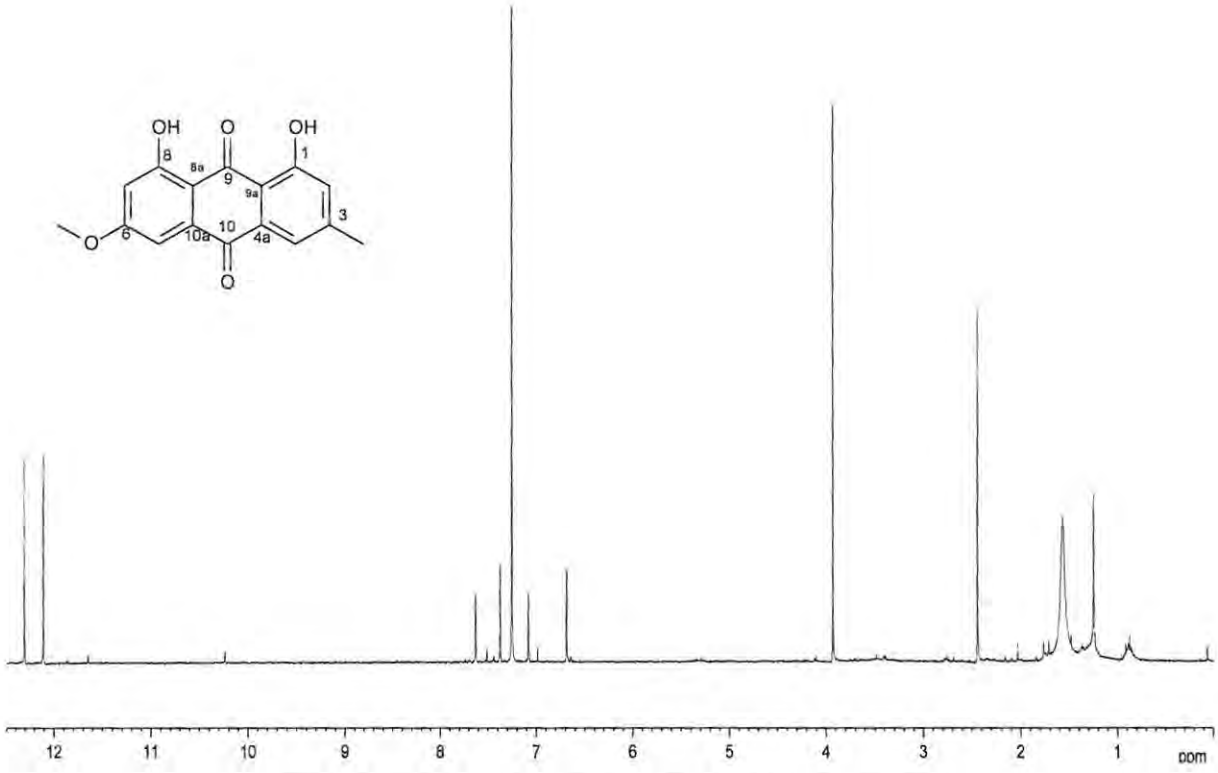


Fig. 5.13: ^{1}H NMR Spectrum of **1** ($CDCl_3$); 400MHz).

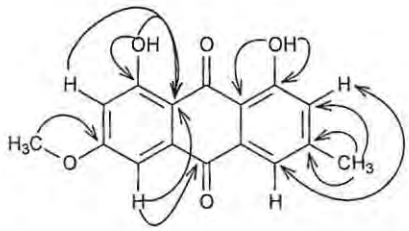


Fig. 5.14: Key HMBC Correlations of Compound **1**

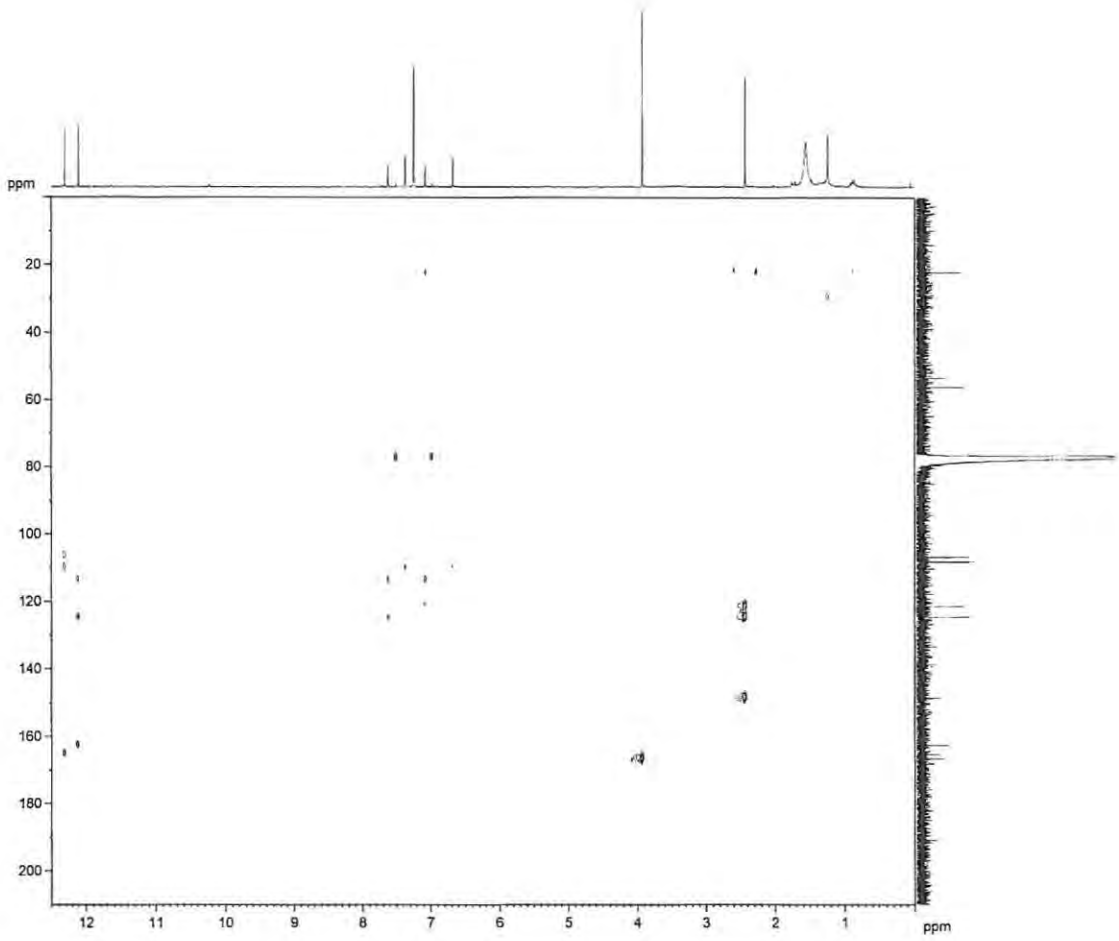


Fig. 5.15: HMBC Spectrum of Compound **1**

Table 5.7: Spectral Data for **1** in $CDCl_3$.

Atom	δ_C	DEPT	δ_H [mult., *J* (Hz)]	COSY	HMBC
1	165.1	qC			
2	124.5	CH	7.08, s	H-5, H-4	
3	148.4	qC			
4	121.3	CH	7.63, s	H-5, H-2	C-2
4a	133.3	qC			
5	22.2	CH_3	2.45, s	H-2	C-3; C-2
6	166.6	qC			
7	106.8	CH	6.69, d, 2.8	H-11	C-8a
8	162.5	qC			
8a	110.3	qC			
9	190.8	qC			
9a	113.7	qC			
10	182.1	qC			
11	108.2	CH	7.38, d, 2.8	H-7	
12	56.1	CH3O	3.94, s		C-6
1-OH	-	-	12.1, s		1C-9a; C-1
8-OH	-	-	12.3, s		C-8a; C-8

5.4. Summary and Conclusion

Six known compounds were isolated from mycelial extracts of the marine derived fungus of the genus *Eurotium*. The structures were determined by interpretation of spectral data and comparison with literature values. The fact that only known metabolites were isolated highlights the problem faced by all natural product chemists involved with the isolation of natural products from microorganisms. The rapid dereplication of known metabolites is therefore essential for natural products research.

It is interesting to note that the metabolites are of polyketide, terpenoid and shikimate origin.

Chapter 6:
Biological Screening

6.1. Introduction

The last stage of a natural products screening programme is to determine whether metabolites isolated exhibit biological activity. There are a number of screens available and with the advent of genomic sequencing, new molecular targets are available for cell-based assays. Determining which screens to use is difficult. However, when pure compounds are available, the biological activity of a compound may be predicted by analysis of its structure. Nevertheless, the bioactivies of many compounds go unrecognised because of the limited number of assay systems being used in the average natural products research group. One of the major limitations is the insolubility of compounds in an assay (Dr Gustafson, 2002). Although techniques such as rocking and sonicating are employed, many of the compounds may precipitate out of solution as the experiment progresses. The other difficulty is that there may be insufficient amounts of the compound for testing. However the shift to use of bioassays in microtitre plates (which require microgram quantities) is now a common practice. Assays also need to be robust to avoid false positives yet sensitive enough to avoid false negatives (which rarely get a second chance).

The metabolites from the *Aspergillus glaucus* group have been shown to possess various biological activities. Anthraquinones in particular possess astringent, purgative, anti-inflammatory and antiviral activity with moderate antitumour and bactericidal effects (Manojlovic *et al.*, 2000). Anthraquinones chrysophanol, physcion (**1**), erythroglaucin, emodin, catenarin, and viocristin have antibacterial and anti-fungal activities (Podojil *et al.*, 1978; Anke *et al.*, 1980b; Agarwal *et al.*, 2000; Babu *et al.*, 2003). In addition, physcion has been suggested to act as possible sun-screen agent (Wynn-Williams *et al.*, 2002; Gauslaa *et al.*, 2003; Solhaug *et al.*, 2003). Emodin has antioxidant activity (Ng *et al.*, 2003) and recently showed cytotoxicity towards human oral squamous cell carcinoma (HSC-2) salivary gland tumor (HSG) cell lines (Shi *et al.*, 2001) and human cervical cancer cells (Srinivas *et al.*, 2003). The auroglaucin analogues also possess antioxidant properties and antibiotic activities (Podojil *e t al.*, 1978a; Ishikawa *et al.*, 1984, Tanaka *et al.*, 2001) and echinuline is active against *Mycobacterium tuberculosis* (Kanokmedhakul *et al.*, 2002). Neoechinuline A (**18**) possesses antioxidant activity (Yagi and Doi, 1999). While these metabolites do exhibit activities that are beneficial to humans, echinuline and erythroglaucin have been shown to be toxic (Wells *et al.*, 1975; Ali *et al.*, 1988; Meuller *et al.*, 1999; Chen *et al.*, 2002). Carcinogens are also a common occurrence e.g.

sterigmatocystin, (Schroeder and Kelton, 1975,) and aflatoxin B_1 (Leitao *et al.*, 1989) ochratoxin and gliotoxin (Turner, 1971).

Since the main focus of our research is the search for antibacterials and anticancer agents, these screens were chosen.

Brine-shrimp Assays (*Artemia salina*, Leach)

The brine-shrimp lethality assay was proposed by Michael (1956) and later developed by Vanhaecke *et al.* (1981) and Sleet and Brendel (1983). Due to most anti-tumour compounds being cytotoxic, the brine-shrimp lethality test is used as a prescreen for potential anti-cancer compounds. The brine-shrimp assay has been shown to give good correlation ($p=0.036$) with the 9KB cytotoxicity assay (Anderson *et al.*, 1991). Brine-shrimp have also been used in other bioassay systems such as testing for mycotoxins (Brown and Wildman, 1968; Harwig and Scott, 1971), toxic marine products (Carballo *et al.*, 2002), presence of heavy metals and cyanobacterial toxins (Carballo *et al.*, 2002) pesticide residues, stream pollutants, anaesthetics, dinoflagellate toxins, morphine-like compounds, determining toxicity of oil dispersants, cocarcinogenicity of phorbol esters and toxicants in marine environments (Meyer *et al.*, 1982). Thus the assay is not specific for the detection of antitumour compounds, however, it is used as a preliminary test assay because (1) it is simple (neither aseptic techniques nor the need for maintaining cultures), (2) is not time-consuming and (3) utilises a large number of easily available organisms required for statistical purposes. Furthermore, the eggs remain viable for several years when stored dry and the hatching is attained within 24 hours (Michael, 1956; Meyer *et al.*, 1982). The method also requires no special equipment or training and a relatively small amount of test sample is needed (Meyer *et al.*, 1982). The use of this system in microtitre plates makes it possible to test large numbers of samples in replicates at different concentrations with only small amounts (0.6mg) of the test sample being needed (Solis *et al.*, 1992).

Using the brine-shrimp cytotoxicity assay, antibiotic assay and crystal violet cytotoxicity assay, the bioactivity of the fractions as well as the pure compounds, flavoglaucin (compound **7**), aspergin (**6**), isodihydroauroglaucin (**2**) neoechinuline A (**18**), physcion (**1**) and isotetrahydroauroglaucin (**3**) were determined. These methods were also evaluated to determine if the bioassays should be included in further screening processes.

6.2. Methods

6.2.1. General Experimental

See chapter 2.

6.2.2. Antibiotic Assays

The crude mycelial extract, solvent partitions and pure compounds were tested in duplicate against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* and marine bacteria *Vibrio alginolyticus, Alteromonas salmonicida* and *A. hydrophila.* 150µL of an overnight culture of the test microorganisms cultivated in nutrient broth were used to inoculate the surfaces of nutrient agar plates (Appendix A). The plates were dried before circular disks (Whatmann No.1; 5mm diameter) containing the crude fractions (200µg) and pure compounds (100µg) were placed on them. Streptomycin sulphate (25µg) (Novo-Nordisk) and Penicillin (25µg) (Penilente-Forte) were used as positive controls for gram-positive and gram-negative bacteria respectively. Disks containing the relevant solvents used for transfer of samples were used as negative controls. All experiments were done in duplicate. Plates containing *E. coli* and *S. aureus* were incubated at 37°C and *P. aeruginosa* and *B. subtilis* at 30°C overnight. Zones of inhibition (in millimeters) indicating anti-bacterial activity were measured.

6.2.3. Cytotoxicity Assays

Brine shrimp were hatched as described in section 2.2.7. 75µL of seawater was added to the wells B-D in the microtitre plate. 100µL of 10mg/mL solution of each extract and pure compound in absolute EtOH was transferred to 650µL of seawater to give a concentration of 1mg/750µL. 150µL of this solution was added to well A. Serial dilutions were made by transferring 75µL from well A to B, and then from B to C, and finally from C to D. 75µL of sea water was added to all wells. 100µL of seawater containing 10 - 20 nauplii were added to each well. Final sample concentrations were therefore 400µg/mL (well A); 200µg/mL (well B); 100µg/mL (well C) and 50µg/mL (well D). The plates were stored in the dark overnight and thereafter the dead nauplii were counted. 100µL MeOH was added to all wells and after 20 minutes the total number of nauplii in all wells was counted. Experiments were done in duplicate with solvent controls.

6.2.4. Anti-cancer Assays

Crystal Violet Cytotoxicity Assay was performed according to Chapter 2.3.8.

6.3. Results and Discussion

6.3.1 Antibiotic Assays

Although metabolites of *E. rubrum* have been reported to exhibit antibiotic activity against a range of bacteria, in our screens, they did not exhibit any activity against any of the marine bacteria, *E. coli*, *S. aureus*, *P. aeruginosa*, and *B. subtilis* however all test pathogens were sensitive to the positive controls at 25µg. In experiments done by Podojil *et al.* (1978), echinuline, physcion (**1**), erythroglaucin and flavoglaucin (**7**) were not effective against *B. subtilis* or *Pseudomonas ovalis* when grown on rich media. Possible reasons for the lack of activity include (a) failure of the test compounds to diffuse into the surrounding aqueous based medium; (b) the compounds may not be active against these specific strains; (c) microbes may be resistant to the compounds when grown in nutrient agar.

Table 6.1: Zones of Inhibition resulting from Antibiotic Assays (mm)

Sample	*E.coli*	*S. aureus*	*P. aeruginosa*	*B. subtilis*	*V. alginolyticus*	*A. salmonicida*	*A. hydrophila*
Crude extract	0	0	0	0	0	0	0
Hexane fraction	0	0	0	0	0	0	0
aq. MeOH fraction	0	0	0	0	0	0	0
DCM fraction	0	0	0	0	0	0	0
Fraction 1	0	0	0	0	0	0	0
Fraction 2	0	0	0	0	0	0	0
Fraction 3	0	0	0	0	0	0	0
Fraction 4	0	0	0	0	0	0	0
Fraction 5	0	0	0	0	0	0	0
Fraction 6	0	0	0	0	0	0	0
Fraction 7	0	0	0	0	0	0	0
Fraction 8	0	0	0	0	0	0	0
Fraction 9	0	0	0	0	0	0	0
Aspergin	0	0	0	0	0	0	0
Flavoglaucin	0	0	0	0	0	0	0
Neoechinuline A	0	0	0	0	0	0	0
Isodihydroauroglaucin	0	0	0	0	0	0	0
Isotetrahydroauroglaucin	0	0	0	0	0	0	0

6.3.2. Brine Shrimp Cytotoxicity Assays

Not all compounds could be tested due to the small amounts isolated. Of the extracts tested, the crude, hexane and DCM partitions, and fractions 1, 2, 5 and 6 showed cytotoxic activity. The aqueous MeOH fraction did not exhibit any activity even at the highest concentration (400µg/mL), while the DCM fraction produced the highest activity (Fig. 6.1). It is also apparent that pure compounds possess stronger activity than when in a crude mixture e.g. compounds isodihydroauroglaucin (**2**) and isotetrahydroauroglaucin (**3**), isolated from fractions 1 and 2, and neoechinuline A (**18**) from fractions 5 and 6 exhibited higher activities.

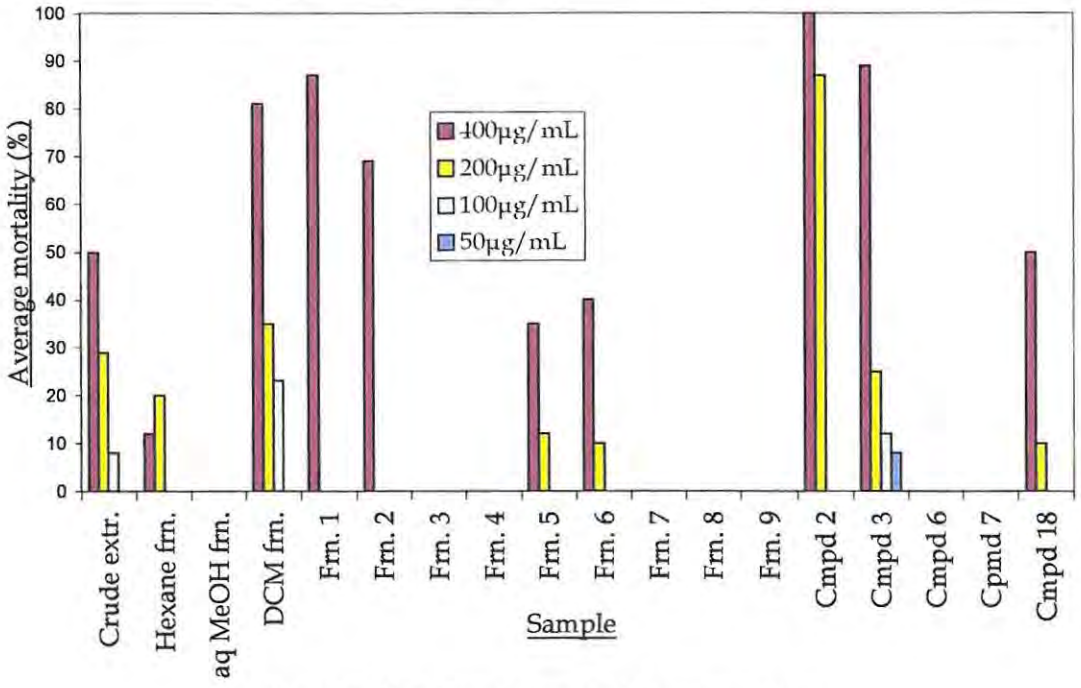


Fig. 6.1: Cytotoxicity against *Artemia salina.*

6.3.3. Anti-cancer Results

The Hexane and DCM partitions, fractions 1 and 6 fractions as well as isodihydroauroglaucin (**2**), isotetrahydroauroglaucin (**3**) and neoechinuline A (**18**) were active against both cervical and oesophageal cells at 10µg/mL. These results correlate well with the brine shrimp results.

6.4. Summary and Conclusion

These results show us that neoechinuline A, isodihydroauroglaucin and isotetrahydroauroglaucin (**3**) show potential for their use as chemotherapeutic agents against oesophageal and cervical cancers. The methods used here are simple enough to be carried out by a natural product chemist, however when working with highly pathogenic microorganisms and facilities do not permit the execution of tests, outsourcing is an option. The combination of general and specific bioassays with chemical screening is thus essential for the screening natural products.

Chapter 7:
Final Conclusion

7.1. Summary of Findings

The use of various isolation media is essential to a natural products programme as it has been shown to result in the isolation of different fungi and thus increase the chances of finding new microorganisms that produce new metabolites for testing.

Culture conditions i.e. static and shaken, produce significantly different metabolites and therefore it is essential to investigate the effects of these culture conditions on an unknown microorganism.

By using a combination of chemical (^{1}H NMR) and biological methods (bioassays) screening RUC0101 was chosen for further study. Using DNA analyses of the ITS1 region, RUC0101 was identified as a close relative of *Eurotium rubrum* (*Aspergillus ruber*).

In order to assess the metabolic creativity of an organism, various fermentation media were employed. Complex nitrogen sources such as yeast extract and peptone had the largest effect on productivity and growth. However, YPG Basal Medium induced one of highest productivities of both physcion and flavoglaucin, and the highest creativy. The HPLC method developed for the analyses of creativity and productivity can also now be used for further metabolic profiling and can be used in conjunction with the NMR-bioassay screening method.

Although 22 compounds were isolated using semi-preparatory HPLC, only six compounds were in sufficient quantity and sufficient purity for structure elucidation using NMR. These six included physcion, neoechinuline A, flavoglaucin, aspergin, isodihydroauroglaucin and isotetrahydroauroglaucin, which are all known metabolites produced by the *A. glaucus* group.

The preliminary biological assessment of the compounds isolated show that neoechinuline A, isodihydorauroglauin and isotetrahydoauroglaucin show potential as chemotherapeutic agents against cervical and oesophageal cancers.

7.2. Recommendations

The purpose of this project was to develop and evaluate methods for the discovery of natural products from marine fungi. This study revealed a number of organisational and technical issues.

It is important early on in the study to identify the isolate. With the advent of DNA analyses using the ITS regions of rDNA, genetic identification is more reliable than taxonomy or chemotaxonomy, however it is expensive and takes time especially when PCR requires optimisation.

The simultaneous optimisation of creativity, productivity and incubation time can be achieved through one experiment. However optimisation is not essential in the early stages of development, especially if a specific metabolite is not being investigated

Extraction of metabolites using solvent extraction has a turnaround time of one day per sample. The purification of metabolites using column chromatography and HPLC may also require time depending on the concentration of compound and its polarity. The bottleneck with regard to the structure elucidation of metabolites is not in the method, but in the analyses. Structure elucidation is essentially experienced-based and requires an extensive background.

The discovery process is undeniably time-consuming. The isolation of fungi, their cultivation, the extraction, isolation and identification of isolates take a great deal of time, especially when working with a large number of samples. The use of automation, robotics and on-line analyses has increased throughput significantly. This is however not feasible in academic institutions. Indeed, some experiments cannot be shortened for fear of lack of accuracy, but advances in biotechnology (fermentation, bioinformatics), molecular biology and chemistry (structure elucidation) have significantly decreased turnaround time. It is thus essential to prioritise extracts. This can be done using simple bioassays in combination with chemical screenings. However once again, collaboration between microbiologists, biochemists and chemists is essential.

Clearly there needs to be a sustained effort to maintain integration in the marine biotechnology /natural products field if it is to be successful. Media optimisation and the physical culture conditions are crucial aspects but are often ignored by natural product

chemists. If the natural products industry is to evolve and to flourish as a means to drug discovery, especially in the face of molecular biology; synthetic and combinatorial chemistry; and bioinformatics, then researchers in this field need to become familiar with all aspects of chemistry and biology.

APPENDICES

APPENDIX A: MEDIA AND REAGENTS

1.1. 1% Agarose Gel

Agarose	1.0g
TBE buffer (1X)	100mL
EtBr (10mg/mL)	3.0µL

1.2. Ethidium bromide (10mg/mL)

EtBr	1.0g
Distilled H_2O	100mL

The solution was stored at 4°C in a dark bottle.

1.3. Gel Loading Buffer

Bromophenol Blue	0.025g
Xylene cyanol	0.025g
Sucrose	4.0g
Distilled H_2O	10mL

1.4. TBE Buffer (10X) (pH=8)

Tris-HCl	108g
Borric acid	55g
EDTA	93g
Distilled H_2O	1000mL

Dilute to 0.5X TBE for use

1.5. YPG isolation agar

Yeast extract	5g/L
Peptone	5g/L
Glucose	10g/L
Agar	17g/L
Seawater	1000mL
Penicillin	75mg/L
Streptomycin	75mg/L

Glucose was autoclaved separately and then and added to the rest of the medium. The antibiotics were added to the sterile medium by filter sterilisation using 0.22µM acetate filter.

1.6. SWA isolation agar

Agar	17g/L
Seawater	1000mL
Penicillin	75mg/L
Streptomycin	75mg/L

The antibiotics were added to the sterile medium by filter sterilisation using 0.22µm acetate filter.

1.7. YPG basal medium

Yeast extract	5g/L
Glucose	10g/L
Peptone	5g/L
Seawater	1000mL

1.8. Universal Buffer

Citric acid	6g
KH_2PO_4	3.9g
Boric acid	1.7g
Diethyl barbuturic acid	5.3g
H_2O	1000mL

Adjusted using NaOH and HCl.

1.9. Vitamin solution (Atlas, 1993)

Thiamine	0.05g
Riboflavin	0.05g
Pyroxidine HCl	0.05g
Calcium pantothenate	0.05g
Nicotinic acid	0.05g
Biotin	0.01g
Folic acid	0.01g
p-amino benzoic acid	0.01g
H_2O	100mL

1.10. Universal buffer - Nitrogen free (Teorell and Stenhagen, 1938)

Na_2HPO_4	7.05g
Citric acid	7g
Boric acid	3.54g
1M NaOH	243mL
H_2O	1000mL

1.11. Nutrient Agar for marine microbes

Nutrient broth	16g/L
Agar	15g/L
Sea water	1000mL

1.12. Nutrient agar for human pathogens

Nutrient broth	16g/L
Agar	15g/L
ddH_2O	1000mL

1.13. Somogyi-Nelson Glucose assays

Alkaline Copper Tartrate Solution:

$CuSO_4.5H_2O$ (2g), Na_2CO_3 (12g), NaK tartrate (8g) and Na_2SO_4 (90g).

Nelson's Aresenomolybdate Reagent:

Dissolve 25g $[(NH_4)_6MO_7O_{24}.4H_2O]$ in 450mL water. Add 21mL concentrated H_2SO_4. Add 3g $Na_2HASO_4.7H_2O$. Make up to 500mL. Make up fresh and store for 24 hours before use.

Standard curve:

Made up 1mg/mL stock solution of glucose

Prepared the following in duplicates:

Tube no.	**1**	**2**	**3**	**4**	**5**
Standard (mL)	0.05	0.1	0.15	0.2	0.25
Water (mL)	5.95	5.9	5.85	5.8	5.75
Cu reagent (mL)	1				
Heat on water bath at 70°C					
Aresenomolybdate (mL)	1				
Leave to stand for 5 minutes					
Read absorbance at 510nm					
Glucose concentration (μg/mL)	6.25	12.5	18.75	25	31.25

Test solution:

Tube	**1**	**2**
Volume of test solution (mL)	0.1mL	
Water (mL)	5.9mL	
Cu reagent (mL)	1mL	
Heat on water bath at 70°C for 20 minutes		
Arsenomolybdate (mL)	1mL	
Leave to stand for 5 minutes		
Read absorbance at 510nm		

1.14. Anticancer Assays

Cell culture

Cells were routinely maintained at 37°C, 5% CO_2. WHCO1 cells (an oesophageal cancer cell line) were cultured in DMEM supplemented with 10% fetal calf serum, 100U/ml penicillin and 100µg/ml streptomycin. ME180 (ATCC #HTB-33) cells (a cervical cancer cell line) were maintained in McCoy's 5A supplemented with 10% fetal calf serum, 100U/ml penicillin, and 100µg/ml streptomycin.

Crystal Violet Assay

Cells were rinsed and lifted with trypsin. They were then neutralised with an equal volume of media and counted using a haemocytometer. An appropriate volume of cell suspension was spun down at 10000rpm for 5 minutes, supernatant removed, and resuspended at 1 000 cells/90µl. Cells were plated in CellStar 96 well plates, 90µl/well (4 wells for each compound), and incubated at 37°C, 5% CO_2 for 24 hours. Compounds were added in 10µl to a final concentration of 1, 10 and 50µg/ml, with solvent (DMSO) at 0.2%. Following 48 hours incubation, observations of cell number and morphology were made, and the plates were then processed for staining. Media was discarded, the plates allowed to drain, and 100µl absolute methanol applied to each well for 10 minutes. Methanol was discarded and replaced with staining solution (1% crystal violet, 50% methanol) for 20 minutes. Plates were rinsed with dH_2O, and 100µl dH_2O added to each well for 1 hour. dH_2O was discarded and replaced with a further 100µl dH_2O. Plates were read at 595nm on an Anthos microtitre plate reader 2001.

APPENDIX B: PRESERVATION OF FUNGI

Preservation methods were carried out as described by Monhaghan *et al.* 1999.

Preservation in dH_2O

Plugs of mycelia grown on YPG agar were cut out and transferred to eppendorfs containing 1mL sterile dH_2O. The vials were stored at 4°C.

1. Preservation with Mineral Oil

The fungus was cultivated on agar slants until a vigorous colony had developed. Thereafter sterile mineral oil was added to cover the entire surface (upright) and stored at 4°C.

2. Preservation on Filter Paper

Pieces of agar containing fruiting bodies were placed on sterile filter paper in a Petri dish, dried in a dessicator, under vacuum and stored at 4°C.

3. Cryopreservation

YPG agar plugs of RUC0101 were added to eppendorfs containing 1mL of 10% glycerol/YPG broth. The eppendorfs were frozen with N_2 and stored at -70°C.

4. Preservation in Liquid Nitrogen

A suspension of spores and mycelia were added to eppendorfs containing 10% glycerol YPG broth. The eppendorfs were frozen and stored at -70°C.

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