ANTIMICROBIAL ACTIVITY OF SOUTH AFRICAN RED ALGAL SECONDARY METABOLITES

BY

DENISE CAMERON

MAY 2001

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN THE FACULTY OF SCIENCES, UNIVERSITY OF THE WESTERN CAPE

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DEDICATION

I dedicate this project to my Lord and Saviour, Jesus Christ, my husband and best friend, P. Saravanakumar, and my parents. Thank you for allowing me to soar like an eagle.

KEYWORDS

ANTIMICROBIAL ACTIVITY OF SOUTH AFRICAN RED ALGAL SECONDARY METABOLITES

Denise Cameron

Antibiotics

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Red algae

Bioactive compounds

Natural products

Bioautography

DECLARATION

I declare that Antimicrobial activity of South African red algal secondary metabolites is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

DENISE CAMERON MAY 2001

SIGNED:.....

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INTRODUCTION

ANTIBIOTICS

Infectious diseases, for example, measles, scarlet fever, malaria, tuberculosis, and cholera have claimed many lives. These infections are caused by pathogenic viruses, bacteria, or fungi that invade the body's tissues and multiply. According to the On-line medical Dictionary (2000), the multiplication may be clinically barely visible or result in local cellular injury because of competitive metabolism, toxins, intracellular replication or antigen antibody response. An infection may remain localized, sub-clinical, and temporary if the body's defensive mechanisms are effective or it could persist, spread, and become an acute, sub-acute, or chronic clinical infection or disease state. A local infection may also become systemic when the microorganisms gain access to the lymphatic or vascular system (On-line Medical Dictionary, 2000). Wound infections are also known as deadly killers.

Antibiotics are medications used to assist the body in defending itself against pathogenic bacteria and fungi. Their effectiveness has enabled many of the progressive developments in modern medicine (Baquero and Blázquez, 1997). These developments are in danger of becoming insignificant with the increase of bacterial resistance against our current supply of antibiotics. Unless antibiotics are modified effectively or new antibiotics are found that can destroy these pathogens, a simple infection could become fatal again. Already, there has been a

recent increase in the incidence of infectious diseases, caused by bacteria and fungi, and the number of deaths brought about by them (Rasoanaivo and Ratsimamanga-Urverg, 1993).

Bacteria may become resistant through spontaneous mutations, the degradation of antibiotics, and the production of enzymes that deactivate the antibiotics or alter the drug target. They can also develop membranes with low permeability to limit the amount of drugs penetrating the cells, or use a multidrug pump efflux system, whereby the antibiotics are pumped out of the cells (Nikaido, 1994). The genes of a pathogen that has developed an effective defense mechanism can be transferred from one pathogen to another and result in an even faster development of resistance (Evans, 1996). For new antibiotics to be effective, they will have to outsmart the pathogens by having mechanisms for which the pathogens have no resistant mechanisms or which override the resistant mechanisms of the pathogens.

One of the main reasons for the development of bacterial resistance is the widespread and indiscriminate use of antibiotics in human and veterinary medicine. The misbelief that antibiotics can be used for almost any ailment, including viral infections such as colds, flu, measles, and herpes, led to large quantities of antibiotics being consumed (Baquero and Blázquez, 1997). In addition, the side effects associated with antibiotics often cause patients to end a recommended antibiotic course prematurely. This allows pathogenic bacteria to remain alive and to develop resistance mechanisms against the antibiotics. Where veterinary medicine is concerned, each year large quantities of antibiotics are added to animal feed to prevent disease and promote animal growth (Pelczar et al., 1993). These sub-therapeutic levels of antibiotics lead to the development of resistant bacteria that could survive in meat if not properly cooked and infect humans. As a result, there is an increase in the number of reports on antibiotic resistant bacteria detected in patients (Baquero and Blázquez, 1997; Carmeli et al.,

1999; Speller, et al. 1997; Travis, 1994). Now antibiotic resistance is described as a "clinical problem and a major public health threat" (Carmeli et al., 1999) which can turn a simple infection into a life threat.

Many surveys have been done to determine the impact of antibiotic resistance on hospitalized patients and the effectiveness of antibiotics. The bacteria of interest in these surveys were mostly Pseudomonas aeruginosa and Staphylococcus aureus, which are known to cause infections in hospitalized patients (Yuan and Williams, 1997; Trautmann et al., 1998; Carmeli et al., 1999).

An example of a survey involving the gram-negative bacterium, Pseudomonas aeruginosa, which showed increased resistance to anti-pseudomonal antibiotics, has been undertaken by Bonfiglio et al. (1998). One thousand and five Pseudomonas aeruginosa isolates were tested against a range of antibiotics namely, Meropenem, Ticarcillin/Clavulanate, Piperacillin, Amikacin, Imipenem, Carbenicillin, Ceftazidime, and Ciprofloxacin. Approximately 50% of the isolates showed resistance. Of the 1005 isolates, 187 were isolated from patients in intensive care units and these showed the most resistance to the antibiotics. The presence of resistant Pseudomonas aeruginosa critically influences the mortality rate in patients. In another study done at a teaching hospital in Boston, Massachusetts, it was found that the mortality rate in patients with resistant Pseudomonas aeruginosa were three times higher than in those with susceptible bacteria. Patients who survived with resistant bacteria stayed longer in hospital and eventually ran a higher hospital charge than patients with susceptible bacteria (Carmeli et al., 1999).

Cystic fibrosis patients are prone to pseudomonal infection and for them bacterial resistance to anti-pseudomonal antibiotics can be fatal. Saiman et al. (1996) from Columbia University tested Pseudomonas aeruginosa isolates that have been obtained from cystic fibrosis patients who were not responding to conventional antibiotics. The isolates were tested against various antibiotic pairs to determine whether the potential synergy of various antibiotics and the effects of an increased concentration of the antibiotic, Tobramycin, would improve the management of cystic fibrosis patients. Of the 1296 isolates tested, 172 were multiple resistant strains. The antibiotics tested included: Ticarcillin, Ticarcillin+Clavulanate, Piperacillin, Ceftazidime, Imipenem, Aztreonam, Tobramycin, Gentamicin, Amikacin and Ciprofloxacin. The 172 multiple resistant strains resisted Ticarcillin, Ticarcillin+Clavulanate, Piperacillin, Imipenem, and Aztreonam. Tobramycin showed the most activity against the 1296 isolates. More than 50% of the multiple resistant isolates were inhibited by combinations of the antibiotics. Where Tobramycin was part of a combination, the pair mostly produced greater inhibition. Pairs with Imipenem did not inhibit the growth of the isolates significantly. Only 21% of the 1296 isolates and 66% of the multiple resistant isolates were resistant to Ciprofloxacin. Fifty-five percent of the 172 multiple resistant strains were resistant to all the antibiotics screened. Most of the strains were resistant to standard concentrations of Tobramycin and Gentamicin.

Another example of resistance is that of the gram-positive bacterium Staphylococcus aureus. Between 1989 and 1995, the rate of resistance of Staphylococcus aureus to Methicillin and other antibiotics was evaluated in approximately 200 clinical laboratories in Wales and England (Speller et al., 1997). The other antibiotics included Benzylpenicillin, Erythromycin, Fusidic acid, Tetracycline, Gentamicin, Ciprofloxacin, Trimethoprim, Clindamycin, and Rifampicin. Isolates of Staphylococcus aureus that were obtained from blood or cerebrospinal fluids from patients over seven years were screened for resistance against the antibiotics. Each year the

same patients were sampled. From 1989 to 1991, 1.5% of the isolates were resistant to Methicillin. The percentage resistance increased to up to 13.2% in 1995. Tetracycline resistance decreased from 9.7% to 5.5%. In general, the isolates tested against Erythromycin, Rifampicin, Ciprofloxacin, Clindamycin and Trimethoprim showed increased resistance. These increases were mainly observed in methicillin-resistant Staphylococcus aureus (MRSA) strains compared to the methicillin-sensitive isolates (MSSA) demonstrating that methicillin-resistant isolates resisted more antibiotics with time.

Drug resistance cannot be discussed without the mention of tuberculosis that has generated alarming statistics globally and which has badly effected the population of South Africa. One of the main difficulties encountered in the treatment of tuberculosis globally is the multi-drug resistance of the pathogen, Mycobacterium tuberculosis (Fourie, 2001). Of the countries with the highest incidences of tuberculosis, South Africa has the second highest rate of co-infection with HIV/AIDS, 2540 per 100 000 total population (MRC Newsletter, 2001). Zimbabwe has the highest rate of co-infection at 4603 per 100 000 total population and more people die of tuberculosis in Zimbabwe and South Africa than anywhere else in the world (MRC, 2001). Currently, the WHO/IUATLD Global Working Group on Tuberculosis Drug Resistance Surveillance is conducting surveys in southern Africa to determine the extent of the multi-drug resistance problem. Survey results for four countries in southern Africa, namely, Botswana, Lesotho, South Africa, and Swaziland have been made available. Overall, it was found that initial resistance to TB-drugs, such as, Isoniazid, Streptomycin, Rifampicin and Ethambutol, in patients being treated for the first time is relatively low, but patients that has previously being treated unsuccessfully, showed acquired resistance that were three times higher than that of first time patients. In addition, patients infected with these multi-drug resistant pathogens require expensive medication, which only a few countries are able to afford. Besides the demand for

better control programmes to ensure that patients complete their treatments, especially for first time patients, there is an equally big demand for new antibiotics to treat the infections in multi-drug resistant patients.

For more than 20 years, no new antibiotics have been introduced. Instead, improvements have been made on existing compounds (Hancock, 1997). Antibiotics are classified as secondary metabolites by the On-line Medical Dictionary, which defines them as "chemicals that are usually unique to one organism or class of organisms, and are not essential to cell survival (On-line Medical Dictionary, 2000). The first antibiotics were isolated from filtered culture broths of bacteria, before the much-celebrated isolation of penicillin from the green Penicillium mould (Sneader, 1986). Since then, many other antibiotics were isolated from soil bacteria and fungi (Sneader, 1986; Burkholder and Sharma, 1969), others were synthesized and derivatives were isolated from non-microbial natural sources, for example lichens, higher plants and animals (Evans, 1996). The antibiotics were isolated, but could not be put to medicinal use, as they did not pass the first test stages in determining their suitability for human and animal consumption. During the last few decades, the search for new antibiotics was extended to marine organisms with the introduction of the self-contained underwater breathing apparatus (SCUBA) (Fenical, 1982).

MARINE ALGAL EXTRACTS AND ISOLATES

Marine algal extracts have yet to be explored to the same extent as the extracts of terrestrial plants. Early work on marine algae involved the establishment of algal taxonomy and nomenclature (South and Whittick, 1987). In addition, marine macroalgal extracts were used by maritime nations as antibiotics, vermifuges, to treat gout, goitre, in fever relief, for hypertension,

wound infections, and many other ailments. Today, interest in seaweeds has extended far beyond earlier uses. This is mainly due to the introduction of the SCUBA and the development of sophisticated instruments and laboratory procedures for algal investigations. Phycologists are using these aids to study algal "cytology, physiology, biochemistry, genetics, molecular biology, ecology, taxonomy and systematics, applied biology (e.g. aquaculture, agriculture, industrial extraction, and uses of phycocolloids), pharmacology, medicine, space biology, planktology, and others" as outlined by South and Whittick (1987).

In general, these developments have enabled natural product chemists to isolate various compounds from marine organisms, of which over 5000 structures have been published (Wright, 1998). In the process, they have shown that the marine secondary metabolites belong to a variety of chemical classes, for example, terpenes, shikimates, polyketides, acetogenins, peptides, alkaloids, and many others. Many of these compounds have been isolated from marine algae. Table 1.1 shows a few examples of seaweeds and the compounds that have been isolated by phycologists. The red algal genus, Laurencia has been studied more than any other marine algal genus (Rovirosa et al., 1999) and more than 250 metabolites (Masuda et al., 1997) have been isolated of which only a few isolates are shown in Table 1.1. The isolates have been reported to be sesquiterpenoids, diterpenoids, triterpenoids, or C_{15} bromoethers (Masuda et al., 1997). Genera such as, Chondria and Digenea have been investigated mainly for the presence of the anthelminthic compounds, such as, kainic acid and domoic acid (see Table 1.1). Plocamium species have been found to produce many polyhalogenated monoterpenes (König et al., 1999). Sterols and amides have been isolated from Gracilaria species (see Table 1.1), but this genus is primarily known for producing pharmaceutical significant polysaccharides. Sterols have also been isolated from the green alga, Codium decorticatum. Bromophenols have been isolated from the red algae, Odonthalia dentata and Rhodomela confervoides.

Some natural product chemists have been interested in finding novel compounds to gain information about the chemistry of marine algae. Graber and Gerwick (1996), in their quest to describe the organic chemistry of algal-derived oxylipins have isolated a novel oxylipin from the red alga, Agardhiella subulata. Glombitza, together with other researchers with an interest in algal phlorotannins, have isolated and characterized many phlorotannins from different species of brown algae, for example, from the brown algae, Sargassum spinuligerum (Glombitza and Keusgen, 1995; Glombitza et al., 1997), Cystophora spp. (Sailler and Glombitza, 1999), and Carpophyllum angustifolium (Glombitza and Schmidt, 1999) (see Table 1.1). In some studies, the novel compounds that were isolated may not be structurally important, but could provide information about the biosynthetic pathways, and help to build up a secondary metabolite profile of the algae (Cannell, 1998), for example, in describing the secondary metabolite chemistry of a Tasmanian Plocamium sp., König et al. (1999a) have isolated a new polyhalogenated monoterpene.

Isolations and characterizations of marine algal compounds have advanced our knowledge of the chemistry of marine algae and have enabled scientists to describe some of the similarities and differences between terrestrial and marine plants. An example of a similarity in the chemistry of marine algae and terrestrial plants can be seen in the isolation of the polysulfide, lenthionine which was isolated from the red seaweed, Chondria californica and the mushroom, Lentinus edodes (Fenical, 1982). Ecological marine studies have shown that the halide-rich seawater environment of marine organisms enables them to produce an even wider variety of compounds than terrestrial plants. Halogens, which are rarely used by terrestrial plants, play significant roles in biosynthesis and allow marine organisms to produce organic compounds featuring bromine, chlorine, and iodine (Fenical, 1982). This was demonstrated when the halogen, bromine was left out from the growth medium of the red seaweed, Delisea pulchra

(Dworjanyn et al., 1999). The alga produced no furanones, which are important secondary metabolites responsible for antifouling, and other ecological functions. A difference in the chemistry of terrestrial plants and marine algae is that in the marine environment the focus of seaweeds is on producing high molecular weight terpenes, especially the diterpenes (C_{20}), while with terrestrial plants the synthesis of monoterpenes (C_{10}) is more prominent. In addition, in the production of terpenes through cyclization mechanisms, seaweeds make use of halogens to initiate cyclization instead of the hydrogen ions used by terrestrial plants (Fenical, 1982).

Others phycologists have been interested in the biological activities of the isolated compounds, for example, monoterpenes that have been isolated from the red alga, Plocamium hamatum were tested for antialgal activity and cytotoxicity (König et al., 1999b). These particular activities might have been selected to establish whether the isolated compound should be subjected to further testing. In this case, the compounds were first isolated and then tested. In other cases, the algal crude extracts were first tested for biological activity and then the bioactive compounds were isolated and tested, for example, when Caccamese et al. (1981) isolated laurencienyne from the antibacterial crude extract of Laurencia obtusa. Overall, many studies of the biological activities of marine algal crude extracts have been done.

ANTIBIOTIC COMPOUNDS IN MARINE ALGAE

This investigation is primarily looking at the antimicrobial activity of red marine algal extracts, therefore some of the biological activities that have been reported for the red algal crude extracts are shown in Table 1.2. Most of these reports are about the antibiotic activity of crude red algal extracts. Other biological activities, such as, immunomodulating (Okai et al., 1996; Liu et al, 1997; Okai et al., 1998), anticoagulant (Sen Sr et al., 1994), and antioxidant activity (Foti et al.,

1994) have also been reported for red algal extracts. In addition, although only the antibiotic activity of red algae was shown in Table 1.2, it must be noted that brown and green algae have shown significant antibiotic activities. In fact, in some cases the extracts of brown and green algae have been even more active than that of red algae. In studies done on seaweeds from the Brazilian northeastern coast (De Campos-Takaki et al., 1988), southern coast of Italy (Caccamese et al, 1985), and the coastline of Japan (Horikawa et al., 1999), the brown algal extracts showed greater antimicrobial activity when compared with the extracts of red and green algae. In another study done on algae collected from the east coast of the Gulf of Aqaba, the red algae showed the highest antibiotic activity (Mahasneh et al., 1995). The green algal extracts have also shown great potential. From 84 different marine algae, the green alga Dunaliella primolecta has been reported to produce the greatest degree of antibiotic activity (Chang et al., 1993).

According to Reichelt and Borowitzka (1984), researchers have been investigating marine algal antibiotic production to determine the ecological role of antibiotics in preventing algal disease or in preventing fouling by bacteria and other marine organisms or in the search for novel antibiotics. Many reports on the antimicrobial activity of marine algae from various places around the world for example, the reports on antimicrobial activity of British marine algae (Hornsey and Hide, 1974, Hornsey and Hide, 1976), Mediterranean macrophytes (Ballesteros et al., 1992), southern Africa macroalgae (Vlachos et al., 1997), and many more have been published. These researchers appear to report their findings in relation to that of other researchers, either geographically or in terms of the ecological importance of the antibiotic compounds.

Other phycologists have investigated the potential of seaweeds as sources of antibiotics exclusively and have made various discoveries. Pratt et al. (1951), in the process of determining the potential of seaweeds as sources of antibiotics, noted that there are seasonal variations in the antibiotic compounds in the seaweeds. Heríquez et al. (1979), while wanting to isolate bioactive compounds, reported that 52% of the petroleum ether extracts that they tested showed antibacterial activity and Caccamese et al. (1980), in their search for antimicrobial compounds, discovered that the algal lipid extracts contained antimicrobial compounds, which varies depending on the life phases of the whole plant. Later, in another investigation, Caccamese et al. (1981) found that antimicrobial activity of the samples were enhanced by fractionation and isolated a novel compound from Laurencia obtusa, namely, laurencienyne. After another investigation, Caccamese et al. (1985) concluded that the brown families Cystoseiraceae and Dictyotaceae and the red family Rhodomelaceae had the greatest antimicrobial activity of all the Mediterranean marine seaweed species that they tested. De Campos-Takaki et al. (1988) contributed to the pool of information of the antimicrobial activity of Brazilian marine algae to continue the search for antibiotic compounds, as did Mahasneh et al. (1995). Reichelt and Borowitzka (1984), after a large scale screening program that looked at a large variety of marine organisms, reported that algae, particularly red algae, were not significant sources of therapeutically relevant antimicrobial compounds. Most of the isolated compounds proved to be too toxic in vivo. However, they mentioned that their sample size was very small in comparison with the large number of algae that remained to be tested before this statement could be qualified.

Overall, from the findings of these studies, algae have shown great potential as sources of antibiotic compounds. Unfortunately, only a few of these marine algal antimicrobial compounds have been isolated and identified, for example, pentabromopyrone and dimethyl gloiosiphone

(see Table 1.2). Where antimicrobials have been isolated, many proved to be highly toxic while only a few showed activities in vivo. In addition, active isolates have been found to be "inactivated by binding with serum proteins" in vivo, failed to be absorbed and transported to the site of the infection or were metabolized into inactive compounds (Reichelt and Borowitzka, 1984). Although these findings are true for a few algal species, it should not be accepted as true for all marine algae, because there are many species still to be investigated. In addition, it would be worthwhile to see if a modification of the isolates before in vivo testing could reduce the toxicity. A simple modification helped Sturino et al. (1997) to stabilize antimicrobials that became inactive due to chromatography. During the characterization procedure, they treated the bioactive fractions with diazomethane.

The syntheses of isolated antimicrobials are imperative. This would make enough material available for studies aimed at modifying the antibiotic compounds to ensure stability, reducing the toxicity, and determining the mechanisms that antibiotics use to inhibit the growth of the bacteria. A novel antibiotic mechanism would qualify further in vivo testing. The possible synergies of novel isolates with other exciting antibiotics should also be explored. Clearly, many investigations will have to be undertaken before an opinion of algal antibiotics can be afforded.

Reports of the synthesis of algal isolates are few. One reason for the scarcity could be that the difficulty of isolation and synthesis itself. Examples of syntheses are the algal metabolite, dimethyl gloiosiphone A that has been isolated from the red alga, Gloiosiphonia verticillaris (Sturino et al., 1997) and a novel polyhalogenated monoterpene that has been isolated from a Plocamium sp. (Whitney et al., 1997). New methods had to be developed for the synthesis of dimethyl gloiosiphone (Sturino et al., 1997) and this is often the case with most syntheses,

making the procedures lengthy, and time consuming. In some cases, the antimicrobial compounds could be lost during the isolation procedures. When laurencienyne has been isolated from the red alga, Laurencia obtusa, it was observed that it was only mildly active and not the compound responsible for the antimicrobial activity of the crude extract (Caccamese et al., 1981). These procedures require more time than simply reporting on the antibiotic activity of a number of algae using existing methods and this could be one of the main reasons why no antibiotic has been developed from marine isolates.

In selecting algae for this investigation, a closer look at South Africa's algal resources was necessary. The seaweeds of the South African coastline are diverse. South African seaweeds have the following divisions: Cyanophyta (with the single class Cyanophyceae – blue-green algae), Rhodophyta (with the single class Rhodophyceae – red algae), Chromophyta (including Phaeophyceae – brown algae, and Xanthophyceae), and Chlorophyta (with the single class Chlorophyceae – green algae) (Silva et al., 1996). Stegenga et al. (1997) estimated the total number of marine algae in South Africa to be nearly 800. Most of the 800 species are red algae, with the result that more red than brown and green seaweeds could be considered potentially harvestable and/or economically important (Critchley et al., 1998). This makes red algae excellent candidates for research. Currently, red seaweeds are harvested for the purpose of export. All harvesting of red algae takes place on the coasts of the Eastern with beach casts of only Gracilaria taken in the Western Cape. The red algal species that are harvested includes, Gracilaria and Gelidium. Red seaweeds, such as Gigartina polycarpa, Sarcothalia stiriata, Aeodes orbitosa, Hypnea spicifera and Gymnogongrus spp., all carrageenophytes, hold possibilities for harvesting and mariculture. Porphyra spp. could be used in abalone food, while Suhria vittata and Carpoblepharis flaccida are potential agarophytes. In spite of the potential of South African seaweeds as carrageenophytes and agarophytes the import of carrageenans, agars,

and alginates are continuing and not much else has been done to optimize the uses of these valuable plants. This is mainly because it is very costly to run processing plants unless a large and predictable supply of raw materials is available.

As a result, the selection of red algae was narrowed down to those with potential economic value and those that are potentially harvestable. Therefore, in this investigation, common red algae, such as Polysiphonia virgata and Gelidium pristoides were selected for antimicrobial screening. Some of these and other red species have been investigated previously and are known to have antimicrobial compounds, for example, Gelidium abbottiorum (Vlachos et al., 1997), Polysiphonia lanosa (Hornsey and Hide, 1976), Polysiphonia virgata (Vlachos et al., 1997), Plocamium rigidum (Vlachos et al., 1997), Plocamium hamatum, (König et al., 1999) and Gracilaria corticata (Sastry and Rao, 1994) (see Table 1.2). These species have not been part of studies with antibiotic research as the rationale, but the reported activities are nonetheless significant. In Table 1.2, other red algae that produced extracts with antibiotic activity are shown.

South African marine algae have not previously been investigated with the rationale of discovering new antibiotics for therapeutic use. In addition, antibiotic compounds have not been isolated from South African marine algae. In the previous screen of southern African marine algae, the rationale appeared to be to report on the antimicrobial activity of the algae to compare it with the findings obtained for similar studies done in other countries (Vlachos et al., 1997). The agar disc diffusion assay was used to determine the antimicrobial activity of the crude extracts. The brown seaweed extracts showed the greatest inhibition, compared with the red and green seaweed extracts. No attempt was made to isolate any of the antimicrobial compounds that have been produced in the 80% ethanol extracts.

In this investigation, the primary objective is to establish whether South African red algae are potential sources of antimicrobial compounds with the rationale of contributing to antibiotic research. Instead of selecting a wide array of microorganisms, only three bacteria and one fungus were chosen, each representing the catogories of pathogens that have become are becoming increasingly resistant to the current supply of antibiotics. Mycobacterium smegmatis, which is closely related to the pathogen responsible for tuberculosis, Mycobacterium tuberculosis, was specifically chosen to screen the extracts for anti-tuberculosis antibiotics as well. Both the agar disc diffusion and the agar overlay bioautography method will be used to assess the inhibitory activity of the algal extracts in a preliminary screen. There are currently no reports available of studies where the agar overlay bioautography method was used to determine the antimicrobial activity of South African marine algae. In addition, the extraction solvents and procedures that have been used in previous studies will be evaluated to optimize the quality of the extracts of secondary metabolites. The secondary objective is to isolate and characterize at least one antimicrobial compound from the algal crude extracts. The extracts or the isolate(s) will not be tested in vivo in this investigation. Thus, the therapeutic relevance of the crude extracts or the isolates will not be determined in this investigation, but the results of this screen should indicate whether South African red algal secondary metabolites hold the potential for further antibiotic research. Should the results show that South African red seaweeds are potential sources of antibiotics, the results of this investigation should lay a foundation for the further isolation, characterization, synthesis, etc. of the antibiotics to materialize the development of algal antibiotics.

In the second chapter, an extraction procedure for red algal secondary metabolites is outlined. This is necessary because different marine natural product chemists make use of many various extraction procedures, using a variety of extraction solvents to obtain crude algal extracts. Some

extraction procedures are very complicated. During the extraction procedure, many secondary metabolites can be destroyed if certain precautionary measures are not followed. Factors, such as heat, vacuum, extraction solvents, etc., all affect the yields of the secondary metabolites (Cronin et al., 1995). These were all taken into account and a simple and short extraction procedure was developed. Two bioassays were evaluated, namely, the agar disc diffusion assay and the agar overlay bioautography method, to see which is more suitable for the screening of red algal secondary metabolites against the microorganisms, Staphylococcus aureus, Pseudomonas aeruginosa, Mycobacterium smegmatis and Candida albicans. In the third chapter, seventeen red algal species are screened for antimicrobial activity, using the extraction procedure developed in Chapter 2. In the fourth chapter, an antimicrobial compound is isolated from the crude extract of the alga, Plocamium corallorhiza and the isolate purified and identified using preparative thin-layer chromatography, NMR-, and GC-Mass spectroscopy.

TABLES

Table 1.1. Seaweeds and examples of compounds isolated from them.

SEAWEEDS	ISOLATED COMPOUNDS	REFERENCES
BROWN SEAWEEDS		
Bifurcaria bifurcata	Diterpenes	Hougaard et al., 1991a
Bifurcaria bifurcata	Diterpenes	Hougaard et al., 1991b
Carpophyllum angustifolium	Trihydroxyphlorethols	Glombitza and Schmidt, 1999
Cystophora retroflexa	Phlorotannins	Sailler and Glombitza, 1999
Cystoseira tamariscifolia	Meroditerpenoid	Bennamara et al., 1999
Notheia anomala	Epoxy lipids	Murray et al., 1991
Sargassum spinuligerum	Fucophlorethols	Glombitza and Keusgen, 1995
Sargassum spinuligerum	Fuhalols, phlorethols	Glombitza et al., 1997
Spatoglossum variabile	Spatozoate, Varinasterol	Atta-ur-Rahman et al., 1999
Sporochnus pedunculatus	A Phenol	Gunasekera et al., 1995
Stoechospermum marginatum	Spatane diterpenoids	De Rosa et al., 1999
GREEN SEAWEEDS		
Codium decorticatum	Sterols, alcohols	Ahmad et al., 1994
Ulva lactuca	Lectin	Sampaio et al., 1998
RED SEAWEEDS		
Agardhiella subulata	Agardhilactone	Graber and Gerwick, 1996
Alsidium helminthocorton	Kainic acid	Calaf et al., 1989
Champia novae-zealandiae	Polysaccharide	Miller et al., 1996
Chondria armata	Domoic acid	Zaman et al., 1997
Digenea simplex	Kainic acid	Karamanos et al., 1994
Digenea simplex	Kainic acid	South and Whittick, 1987
Gracilaria asiatica	Eicosanoids	Sajiki and Kakimi, 1998
Gracilaria coronopifolia	Malyngamides M & N	Kan et al., 1998
Gracilaria coronopifolia	Manauealides	Nagai et al., 1997
Gracilaria edulis	Sterols	Das and Srinivas, 1993
Gracilaria longa	Cholesterol, Chlorophyll a, Lutein	Pollesello et al., 1992
Gracilariopsis lemaneiformis	Floriside, Heterosides, Isethionic acid, amino acids	Broberg et al., 1998
Grateloupia carnosa	Amino acid – Carnosadine	Wakamiya et al., 1984
Hypnea musciformis	Isethionic acid	Holst et al., 1994
Laurencia claviformis	Terpenes, C_{15}-acetogenins	Rovirosa et al., 1999
Laurencia majuscula	Acetogenins and terpenes	Wright et al., 1993
Laurencia nipponica	C_{15}-bromoethers, Terpenes	Masuda et al., 1997
Odonthalia dentata	Bromophenol	Craigie and Gruenig, 1967
Pantoneura plocamioides	Terpenes	Cueto et al., 1998
Plocamium cartilagineum	Poly(β-hydroxybutyrate), Floridoside	Abreu et al., 1997
Plocamium cartilagineum	Terpenes	Cueto et al., 1998
Plocamium cartilagineum	Terpenes	Abreu and Galindro, 1996
Plocamium costatum	Polysaccharide	Miller et al., 1999
Plocamium hamatum	Terpenes	König et al., 1999
Plocamium leptophyllum	Terpenes	Sakata et al., 1991
Plocamium sp.	Terpene	Whitney et al., 1997
Polysiphonia paniculata	Dimethylsulfoniopropionate	Nishiguchi and Goff, 1995
Ptilota filicina	Lectin	Sampaio et al., 1998
Rhodomela confervoides	Bromophenol	Craigie and Gruenig, 1967
Vidalia obtusaloba	Vidalols A and B	Wiemer et al., 1991

Table 1.2. Red seaweeds and the reported biological activities of their extracts

RED SEAWEED	BIOLOGICAL ACTIVITY	ISOLATED COMPOUND	REFERENCE
Asparagopsis taxiformis	Antibacterial		Horikawa et al., 1999
Callophyllis megalocarpa	Antibacterial		Pratt et al., 1951
Falkenbergia rufolanosa	Antibacterial		Ballesteros et al., 1992
Gracilaria corticata	Antibacterial		Sastry and Rao, 1994
Halosaccion glandiforme	Antibacterial		Pratt et al., 1951
Iridophycus flaccidum	Antibacterial		Pratt et al., 1951
Laurencia brongniartii	Antibacterial		Horikawa et al., 1999
Laurencia obtusa	Antibacterial	Laurencienyne	Caccamese et al., 1981
Laurencia obtusa	Antibacterial		Mahasneh et al., 1995
Laurencia okamurae	Antibacterial		Horikawa et al., 1999
Laurencia papillosa	Antibacterial		Mahasneh et al., 1995
Odonthalia corymbifera	Antibacterial		Horikawa et al., 1999
Plocamium hamatum	Antibacterial		König et al., 1999b
Rhodomela teres	Antibacterial		Horikawa et al., 1999
Amphiroa ephedraea	Antimicrobial		Vlachos et al., 1997
Arthrocardia carinata	Antimicrobial		Vlachos et al., 1997
Beckerella pinnatifida	Antimicrobial		Vlachos et al., 1997
Callophylis sp.	Antimicrobial		Heríquez et al., 1979
Cheilosporum sagittatum	Antimicrobial		Vlachos et al., 1997
Chondria coerulescens	Antimicrobial		Caccamese et al., 1985
Chondria dasyphylla	Antimicrobial		Hornsey and Hide, 1974
Chondrus crispus	Antimicrobial		Hornsey and Hide, 1976
Delesseriacean sp.	Antimicrobial	Almazole D	N'Diaye et al., 1996
Delisea fimbriata	Antimicrobial	Fimbrolides	Reichelt & Borowitzka, 1984
Delisea hypnoides	Antimicrobial		Reichelt & Borowitzka, 1984
Gelidium abbottiorum	Antimicrobial		Vlachos et al., 1997
Gloiosiphonia verticillaris	Antimicrobial	Dimethyl gloiosiphone	Sturino et al., 1997
Gracilaria domingensis	Antimicrobial		De Campos-Takaki et al., 1988
Gracilaria sjoestedtii	Antimicrobial		De Campos-Takaki et al., 1988
Grateloupia sp.	Antimicrobial		Heríquez et al., 1979
Haliptylon sp.	Antimicrobial		Heríquez et al., 1979
Hypnea musciformis	Antimicrobial		De Campos-Takaki et al., 1988
Hypnea musciformis	Antimicrobial		Melo et al., 1997
Iridaea membranacea	Antimicrobial		Heríquez et al., 1979
Laurencia complanata	Antimicrobial		Vlachos et al., 1997
Laurencia hybrida	Antimicrobial		Hornsey and Hide, 1974
Laurencia obtusa	Antimicrobial		Caccamese et al., 1980
Laurencia pinnatifida	Antimicrobial		Hornsey and Hide, 1976
Mazzaella capensis	Antimicrobial		Vlachos et al., 1997
Plocamium rigidum	Antimicrobial		Vlachos et al., 1997
Polysiphonia furcellata	Antimicrobial		Caccamese et al., 1985
Polysiphonia lanosa	Antimicrobial		Hornsey and Hide, 1976
Polysiphonia virgata	Antimicrobial		Vlachos et al., 1997
Portierra hornemannii	Antimicrobial		Vlachos et al., 1997
Pterocladia capillacea	Antimicrobial		Khaleafa et al., 1975
Ptilonia australasica	Antimicrobial	Pentabromopyrone	Reichelt and Borowitzka, 1984
Trematocarpus dichotomus	Antimicrobial		Heríquez et al., 1979
Botryocladia botryoides	Antiviral		Caccamese et al., 1980
Callithamnion pikeanum	Antiviral		Kim et al., 1997
Campylaephora hypnaeoides	Antiviral		Ohigashi et al., 1992

Table 1.2 cont.

RED SEAWEED	BIOLOGICAL ACTIVITY	ISOLATED COMPOUND	REFERENCE
Chondrus ocellatus	Antiviral		Hudson et al., 1999
Corallina pilulifera	Antiviral		Kim et al., 1997
Corallina pilulifera	Antiviral		Hudson et al., 1999
Corallina vancouveriensis	Antiviral		Kim et al., 1997
Digenea simplex	Antiviral		Sekine et al., 1995
Gracilaria pacifica	Antiviral		Kim et al., 1997
Grateloupia turuturu	Antiviral		Kim et al., 1997
Grateloupia turuturu	Antiviral		Hudson et al., 1999
Laurencia intermedia	Antiviral		Ohigashi et al., 1992
Laurencia obtusa	Antiviral		Caccamese et al., 1980
Mazzaella cornucopiae	Antiviral		Kim et al., 1997
Nothogenia fastigiata	Antiviral	Xylomannans	Kolender et al., 1997
Nothogenia fastigiata	Antiviral		Witvrouw & De Clercq, 1997
Nothogenia fastigiata	Antiviral		Damonte et al., 1994
Odonthalia floccosa	Antiviral		Kim et al., 1997
Pachymeniopsis elliptica	Antiviral		Kim et al., 1997
Polysiphonia hendryi	Antiviral		Kim et al., 1997
Schizymenia pacifica	Antiviral		Witvrouw & De Clercq, 1997
Symphyocladia latiuscula	Antiviral		Hudson et al., 1999
Symphyocladia marchantioides	Antiviral		Hudson et al., 1999
Symphyocladia spp	Antiviral		Kim et al., 1997
Galaxaura marginata	Cytotoxic	Desmosterols	König et al., 1994
Gelidium amansii	Cytotoxic		Numata et al., 1991
Gracilaria textorii	Cytotoxic		Numata et al., 1991
Laurencia implicata	Cytotoxic	Acetogenins	König et al., 1994
Mazzaella cornucopiae	Cytotoxic		Kim et al., 1997
Peyssonnelia rosa-marina	Cytotoxic		Ballesteros et al., 1992
Scinaia japonica	Cytotoxic		Numata et al., 1991
Portieria hornemannii	Antitumor	Halomon, monoterpenes	Fuller et al., 1994
Hypnea musciformis	Diuretic		Solimabi et al., 1980
Chondria armata	Hypotensive		Solimabi et al., 1980
Plocamium telfairiae	Insecticidal	Telfairine, Aplysiaterpenoid a	Watanabe et al., 1990
Peyssonnelia rosa-marina	Antimitotic		Ballesteros et al., 1992
Gelidiella acerosa	Antifertility		Solimabi et al., 1980
Plocamium costatum	Antifouling		König et al., 1999a
Plocamium leptophyllum	Antifouling		Sakata et al., 1991
Chondria atropurpurea	Anthelminthic	Chondriamides A, B, C	Davyt et al., 1998
Chondria atropurpurea	Anthelminthic	3-indoleacrylic acid	Davyt et al., 1998
Digenea simplex	Anthelminthic	Kainic acid	Evans, 1986
Chondria atropurpurea	Anthelminthic	Chondriamides A, B, C	Davyt et al., 1998
Plocamium hamatum	Anti-algal		König et al., 1999b
Carpoblepharis flaccida	Anti-inflammatory		Stirk et al., 1996
Carpoblepharis flaccida	Anti-inflammatory		Stirk et al., 1996
Corallina elongata	Anti-inflammatory		Bustos et al., 1992
Corallina elongata	Anti-inflammatory		Bustos et al., 1992
Galaxaura oblongata	Anti-inflammatory		Bustos et al., 1992
Galaxaura oblongata	Anti-inflammatory		Payá et al., 1990
Hypnea musciformis	Anti-inflammatory		Payá et al., 1990
Laurencia obtusa	Anti-inflammatory		Bustos et al., 1992
Sarcothalia stiriata	Anti-inflammatory		Stirk et al., 1996
Vidalia obtusaloba	Anti-inflammatory		Wiemer et al., 1991

Chapter 2

A PILOT STUDY TO DETERMINE AN EXTRACTION PROCEDURE FOR DETECTING ANTIMICROBIAL ACTIVITY IN RED ALGAL SECONDARY METABOLITES

ABSTRACT

Various procedures were used to extract secondary metabolites from red algae. Each extraction procedure was evaluated based on the ease with which it was carried out, the amount of sample that was wasted, and the time it took to obtain a dried crude sample. The resultant extracts were tested against bacteria Staphylococcus aureus, Pseudomonas aeruginosa, Mycobacterium smegmatis, and the yeast Candida albicans in the agar disc diffusion assay and the agar overlay bioautography method. In the first extraction procedure, dried and wet algae were boiled in 80% ethanol. In the second procedure, the algae were either freeze-dried or homogenized and extracted using methanol, dichloromethane, and water successively. In the third procedure, wet algae were homogenized in a solution of dichloromethane (DCM) and methanol (MeOH), centrifuged, and dried in a Speedvac. Extraction procedure four was the same as extraction procedure three, except that the samples were dried using a rotary evaporator. In extraction procedure five, the wet algae were homogenized in DCM:MeOH (2:1), filtered, and the solvents evaporated using a rotary evaporator. In extraction procedure six, the algae were extracted with dichloromethane, methanol, and water successively. Extraction procedure five was found to be the most suitable, because it was simple to perform, the resultant extract was stable, and the process was less time consuming. Most of the extracts showed no antimicrobial activity in the agar disc diffusion assay, but when tested in the agar overlay bioautography method, showed significant antimicrobial activity. These results suggested that the agar disc diffusion assay was

not suitable for this investigation and that the bioautography method was more sensitive for assessing the antibiotic activity of the less polar crude algal extracts.

INTRODUCTION

Secondary metabolites from marine organisms have been studied at length during the last decade and more than 5000 structures have been published (Wright, 1998). Marine natural product chemists use of various extraction, purification, and isolation procedures. Wright looked at 115 reports of marine natural products that were published in 1995 and found that 77% of the researchers extracted fresh or frozen materials while the other 23% extracted lyophilized or air-dried material. The most common extraction solvents were alcohols followed by mixtures of alcohol and less polar solvents. In 28% of the work, no solvent partitioning was used. In the case of column chromatography for isolating bioactive compounds or specific compounds, 10% did not use column chromatography, while the majority of chemists used silica-gel stationary phases (Wright, 1998). The method of extraction has to be considered carefully as it seriously influences the results (Rios et al., 1988). Bearing in mind that there is no single correct way of extracting a natural product (Cannell, 1998), recommendations from previous studies were considered when an extraction procedure for this was determined, especially the recommendations of Cronin et al., (1995).

In determining a suitable extraction procedure for testing the antimicrobial activity of South African algae, Vlachos recommended the extraction of air-dried and milled seaweeds with 80% ethanol by boiling the alcohol broth for 4 hours on a water bath (Vlachos, 1996). Ethanol was recommended because it was less volatile compared to the other solvents tested and it did not evaporate as rapidly during boiling even though the methanol extracts produced the best

antimicrobial activity (Vlachos, 1996). Cronin, however, found that when methanol was used alone in extractions; it was not as effective as when it was used in conjunction with dichloromethane (Cronin et al. 1995). When samples of brown seaweeds were extracted with methanol alone, artifacts formed during the extraction. Combining dichloromethane (DCM) and methanol (MeOH) in a 2:1 ratio, produced a mixture with a higher percentage of lipophilic secondary metabolites because this mixture contains a low-polarity solvent, DCM, and a high-polarity, water-miscible solvent, MeOH, that is able to penetrate cell membranes and extract a wide variety of lipids (Cronin et al., 1995). Boiling the samples in the 80% ethanol could result in the loss of heat-labile antimicrobial compounds (Vlachos et al., 1996).

Other factors that are known to alter the yields of secondary metabolites include biochemical activity, light, vacuum, the extraction solvent, the drying procedure and the duration of the extraction (Cronin et al., 1995). Freeze-drying and rotary evaporation should therefore be used with caution, as extreme vacuum could reduce the amount of secondary metabolites. Cronin recommended that samples should be extracted and analyzed as soon as possible as decomposition could occur during storage. Immersing the algae in DCM:MeOH (2:1), before storage, assists in preventing the loss of some of the secondary metabolites. A mixture (2:1) of DCM:MeOH also minimized the formation of artifacts (Cronin et al., 1995).

In this investigation, various extraction procedures were tried using 80% ethanol, methanol, dichloromethane, and water. Dried and wet algae were extracted and extracts were obtained either through centrifuging, using a pressure filter or a Buchner funnel. The extracts were dried using a Speedvac at 43°C, rotary evaporator, or a freeze-dryer. The different solvents and equipment were observed for the ease of the procedure, the yields of the crude extracts and the time it took to obtain the sample masses of the crude extracts.

The antimicrobial activities of algal extracts have been mostly determined by using diffusion methods where the extracts were brought in contact with inoculated agar, incubated and inhibition zones around the reservoirs measured (Pratt et al., 1951; Hornsey and Hide, 1974; Khaleafa et al., 1975; Hornsey and Hide, 1976; Heríquez et al., 1979; Solimabi et al., 1980; Caccamese et al., 1980; Caccamese et al., 1981; Reichelt and Borowitzka, 1984; Caccamese et al., 1985; De Campos-Takaki et al., 1988; Munro et al., 1989; Tariq, 1991; Ballesteros et al., 1992; König et al., 1994; Sastry and Rao, 1994; Mahasneh et al., 1995; N'Diaye et al., 1996; Robles-Centeno et al., 1996; Vlachos et al., 1996; Melo et al., 1997; Crasta et al., 1997; Vlachos et al., 1997; König et al., 1999; Horikawa et al., 1999). Different types of reservoirs have been used, namely, filter paper discs (disc diffusion method) and wells made in the agar medium (hole-plate method) (Vanden Bergh and Vlietinck, 1991). Alternatively, dilution methods have also been used where a series of dilutions of the extracts were mixed with inoculated mediums, the mixture incubated and the growth of the microorganisms determined by using visual or turbidimetric comparisons with controls and the results recorded as minimum inhibition concentration values (MIC-values) (Vanden Berghe and Vlietinck, 1991).

The bioautography method is highly recommended for the identification of bioactive compounds (Wright, 1998), especially for the detection of compounds with antibiotic activity (Betina, 1973). When screening terrestrial plants for antifungal compounds, bioautography was considered more suitable than the agar disc diffusion assay since it combined bioactivity and the location of active compounds (Hostettmann and Marston, 1994). Simple bioautography, for the detection of antifungal substances, was previously done by using Whatman no.3MM paper (Homans and Fuchs, 1970). This method was time consuming, so the Whatman paper was replaced by DC-Alufolie Kieselgel F_{254} thin-layer chromatography (tlc) plates (Merck), which enabled rapid plate development, but the actual assay methodology required acquired skill and

the plates could not be recycled (Homans and Fuchs, 1970). Homans and Fuchs also found that it was easier and more effective to spray the chromatograms with a spore suspension of the test fungus instead of pressing the tlc-plate on agar seeded with a suitable microorganism or seeding solid agar, which has previously been molten and poured onto the tlc-plate (Homans and Fuchs, 1970). The method held a few complications, for example, Candida albicans could not be sprayed directly onto the developed thin-layer chromatography (tlc) plate as was done in direct bioautography. Instead, to optimize the conditions for growth, the fungus was introduced into the agar medium, spread over the developed tlc-plate before solidification and after incubation, sprayed with methylthiazoyltetrazolium chloride (MTT) reagent to make the zones of inhibition more visible (Nostro et al., 2000; Rahalison et al., 1991). When spreading the inoculated agar medium it should be done in such a way as to keep the layer as thin as possible to assist the diffusion of active compounds during incubation (Rahalison et al., 1991). Rahalison et al. (1991) also found that glass-backed thin-layer chromatography (TLC) plates were more suitable than aluminium-backed TLC-plates. In this investigation the agar disc diffusion assay, previously used by Salie et al., (1996) for the screening of terrestrial plant extracts for antimicrobial activity, together with the agar overlay bioautography method, using glass-backed thin-layer chromatography plates (Gibbons and Gray, 1998), were the assays selected to determine the antimicrobial activity of red algal crude extracts.

The microorganisms used in the assays of this study included the gram-positive bacterium Staphylococcus aureus, the gram-negative bacterium, Pseudomonas aeruginosa, the bacterium Mycobacterium smegmatis and fungus, Candida albicans. These pathogens were selected firstly, because of the demand for new antibiotics to treat the infections caused by them and secondly, because they were readily available as a result of an antimicrobial screen that have been done previously on terrestrial plants at the University of the Western Cape (Salie et al.,

1996). Mycobacterium smegmatis is closely related to the pathogen Mycobacterium tuberculosis, that is the causative organism of tuberculosis (Sneader, 1986). This is especially significant since South Africa, and more specifically the Western, Eastern, and Northern Cape, have been reported to be the areas with the highest rates of tuberculosis incidences (MRC, 2001). Staphylococcus aureus and P. aeruginosa are the pathogens responsible for causing infections in hospitalized patients (Yuan and Williams, 1997; Trautmann et al., 1998; Carmeli et al., 1999). Staphylococcus aureus causes localized cellulitis or abscess, bacteraemia, endocarditis, mediastinitis, osteomyelitis, septic arthritis and pneumonia (Barg, 1998). Pseudomonas aeruginosa causes localized and systemic infections, with systemic infections holding the potential of developing septic shock, multiorgan failure, and death, mainly in intensive care units (Trautmann et al., 1998). Candida albicans causes candidiasis (On-line Medical Dictionary, 2000).

MATERIALS AND METHODS

The red algae used in this investigation were collected during March to December 1999, from the rocky shores and subtidal zone of Holbaaipunt, Kommetjie and Kalk Bay. Voucher specimens were deposited in the Herbarium of the Botany Department at the University of the Western Cape (UWC). In the laboratory, sand, herbivores, and epiphytes were removed from the algae using distilled water, after which the algae were blotted with paper towel.

EXTRACTION PROCEDURE 1

Fresh and dried seaweeds were extracted with 80% ethanol. For the wet extraction, a mass of 40 g of a freshly chopped seaweed sample was homogenized in 80% ethanol using a Kinematica homogenizer. The mixture was filtered using a Buchner funnel with Advantec no.2 filter paper.

The filtrate was placed in test tubes and dried in an evaporator under vacuum at 43°C. For the dried extraction, half of the seaweeds were placed in an oven at 40°C. The dry specimens were milled as recommended by Vlachos et al. (1996). The milled samples were treated in three different ways:

1. 20 mL of ethanol (80%) was added to 10 g of sample and the mixture boiled on a water bath for 4 hours. It was filtered using a pressure filter and evaporated under vacuum at 43°C.
2. 20 mL of ethanol (80%) was added to 10 g of sample and the mixture was stirred for 24 hours, at room temperature. It was filtered using a pressure filter and evaporated under vacuum at 43°C.
3. 20 mL of ethanol (80%) was added to 10 g of sample and the mixture was stirred at 4°C for 24 hours. It was filtered using a pressure filter and evaporated under vacuum at 43°C.

EXTRACTION PROCEDURE 2

The seaweeds were either frozen in preparation for freeze-drying or homogenized in methanol while fresh. The homogenized samples were shaken at 4°C overnight after which they were centrifuged at 4000 rpm for 20 minutes. The resultant supernatants were removed and concentrated under vacuum in a rotary evaporator at ~50°C. The smaller volumes were transferred to test tubes using methanol and evaporated under vacuum at 43°C. Another volume of methanol was added to the plant material and the extraction procedure repeated. This was done thrice, after which the procedure was repeated with dichloromethane and water. The freeze-dried samples were ground and extracted with methanol, dichloromethane, and water.

EXTRACTION PROCEDURE 3

Of each seaweed, 5 g was weighed out and the alga dipped in a DCM:MeOH (2:1) solution before freezing as recommended by Cronin et al. (1995). The dipped samples were placed in small plastic bags and stored at approximately -80°C overnight. The next morning the samples were allowed to thaw and then homogenized in DCM:MeOH (2:1). For every 1 g of wet sample, 6 mL of the mixed solvent was used. The mixture was centrifuged at 4000 rpm for 20 minutes. The supernatant was decanted and evaporated under vacuum using a rotary evaporator, until a small volume of extract remained. This was transferred to small test tubes to be evaporated under vacuum in a Speedvac at 43°C. Many of the samples were lost in the Speedvac when the vacuum was applied. The remaining samples were placed in an oven at 40°C. After 2 days, most of the samples were still wet. The samples were then transferred to weighed round bottom flasks and the solvent evaporated under vacuum using a rotary evaporator at approximately 50°C and placed in a desiccator to ensure complete drying. The dried flasks were reweighed to determine the yields and placed in a desiccator.

EXTRACTION PROCEDURE 4

40 mL of DCM:MeOH (2:1) was added to 10 g of each sample and the sample left in the freezer at –20° C. Two days later, the samples were homogenized and centrifuged at 2500 rpm for 15 minutes, after which the supernatant was removed from the tubes using needles and syringes. The homogenization and centrifuging were repeated thrice, each time with fresh solvents. To ensure that most of the metabolites were extracted, the samples were shaken for 15 minutes with 40 mL 80% MeOH and 100% MeOH respectively, thrice with each solvent. The solvents were all evaporated under vacuum using a rotary evaporator at 40°C and the dried samples placed in

a desiccator under vacuum, overnight, to ensure total dryness. To redissolve the sample, the first attempt was made by adding DCM:MeOH (1:1) solution to the dried extract. The sample did not dissolve completely. No matter how much of this solution was added, the sample did not dissolve completely. Even when water was added the sample did not dissolve. Different combinations of dichloromethane, methanol and water were tried, namely, DCM:MeOH (80%) (1:1) and DCM:MeOH (90%) (1:1), but it did not work. Acetone was also unsuccessful. Adding the solvents individually for DCM:MeOH (80%) (1:1), first DCM, followed by methanol and finally water, worked very well. The extracts were filtered using Whatman nylon syringe filters, with pore sizes of 0.45 μm. Of each sample, 100 μl were spotted onto 20 x 5 cm silica gel F_{254} Merck plates with 0.25 mm thickness and developed in the mobile phase hexane:chloroform:methanol (7:2:1). The plates were vacuum packed in plastic and stored at –20°C. The rest of the 10 mg/mL sample solution was stored at –20°C.

EXTRACTION PROCEDURE 5

40 mL of DCM:MeOH (2:1) was added to 10 g of a chopped seaweed sample. The samples were stored at –20°C. After two days, the samples were homogenized, centrifuged at 1500 rpm for 15 minutes, and filtered into weighed round bottom flasks using Whatman no.541 filter paper instead of using syringes, as in procedure no. 4. This was repeated until the supernatant lost most of its green color. Lastly, the samples were extracted with 100% MeOH and 80% MeOH. All the samples were evaporated under vacuum using a rotary evaporator at approximately 40°C and placed in a desiccator under vacuum. The dried samples were redissolved with DCM:MeOH (80%) [1:1], by adding the solvents individually and made up to volume to prepare solutions of 10 mg/mL concentration. Of each sample, 100 μl were spotted onto 20 x 5 cm silica gel F_{254} Merck plates with 0.25 mm thickness and developed in the mobile

phase hexane:chloroform:methanol (7:2:1). The plates were vacuum packed in plastic and stored at –20°C. The rest of the 10 mg/mL sample solution was stored at –20°C.

EXTRACTION PROCEDURE 6

The seaweeds were extracted with three solvents to see which solvent would give activity in the agar disc diffusion assay. Each sample was divided in three and each mass was extracted extensively with DCM, MeOH, or water. The DCM extraction was performed six times, until the green color of the extract faded sufficiently. The DCM samples were made up to 100 mg/mL solutions in DCM. The DCM solubles were separated from the initial water solubles using a separating funnel. The methanol extraction was repeated seven times until the extracts green color disappeared. The water extract was frozen at –20°C in preparation for freeze-drying. The DCM-samples were evaporated under vacuum using a rotary evaporator at approximately 40°C and the methanol extracts at 50°C.

THE AGAR DISC DIFFUSION ASSAY

Staphylococcus aureus and Pseudomonas aeruginosa were maintained on Difco nutrient agar, Mycobacterium smegmatis on Difco Mycobacteria 7H11 agar supplemented with Bacto Middlebrook OADC enrichment and Bacto glycerol and Candida albicans on Sabourand dextrose agar. Sterile 6 mm discs were impregnated with 50 µl of the 10 mg/mL sample solution. For each sample, this was done in triplicate. The discs were placed onto the agar plates that were inoculated with a microbial culture. Control discs were impregnated with 50 µl of the dissolution solvent, DCM:MeOH(80%) (1:1). The antibiotic controls that were used in the assay were Ciprofloxacin (40 µg/disc) and Amphotericin B (25 µg/disc). Ciprofloxacin was used as a

control for S. aureus, P. aeruginosa, and M. smegmatis. Amphotericin B was used as a control for C. albicans. The agar plates with the discs and the microorganisms were incubated at 37°C for 24 hours for the bacteria, S. aureus and P. aeruginosa and the fungus C. albicans. The agar plates with the bacterium M. smegmatis were incubated for 48 hours. After the incubation period, the inhibition zones were measured in millimeters as the distance between the discs and the growth free zones around the discs. When no inhibition zones were observed the first time around the assay was repeated with increased sample volumes. The assay was repeated with all the sample extracts and the extracts obtained with extraction procedure no. 6., but this time; the amounts impregnated on the sterile discs were increased. Where the sample volumes allowed it, 50 µl was spotted up to four times. Instead of impregnating the 6 mm disc with 50 µl of the 10 mg/mL crude extract, each disc was impregnated with 200 µl of the 10 mg/mL extract. After spotting each 50 µl, the discs were allowed to dry in the incubator, before the next application. The methanol extracts of 25 mg/mL were spotted 50 µl x 4 times and the DCM extracts, 100 mg/mL solutions, were spotted 50 µl x 2 times.

AGAR OVERLAY BIOAUTOGRAPHY

A mobile phase that would allow good separation of the compounds of the sample solutions had to be determined. From the literature, some suggestions for mobile phases were followed, namely, 10% MeOH-$CHCl_3$ (Graber and Gerwick, 1996) hexane:chloroform:methanol (7:2:1) (Rovirosa et al., 1999). Additionally, hexane:chloroform:methanol:acetic acid (70:20:10:5) and ethyl acetate:formic acid:acetic acid:water (100:11:11:27) were also tried. Two of the crude extracts were spotted onto glass-backed, Kieselgel 60 F_{254}, 20 x 5 cm, Merck Art 5715 thin-layer chromatography (TLC) plates with a thickness of 0.25 mm and allowed to develop in the abovementioned mobile phases. The best separation on the TLC-plates was obtained with

hexane:chloroform:methanol (7:2:1). To ensure that the best separation for the solvent combination was obtained, different ratios of hexane:chloroform:methanol were tested: 7:2:1, 6:3:1, 6:2:2, 7:1:2, 5:4:1, 5:3:2, 5:2:3, 5:1:4, 8:1:1. The best separation was still obtained with the mobile phase hexane:chloroform:methanol (7:2:1). To determine which sample volume produced the best results 20 µl, 40 µl, 80 µl and 100 µl sample volume was spotted onto a TLC-plate and allowed to develop in hexane:chloroform:methanol (7:2:1). The 100 µl sample volume produced the best separation.

Glass-backed 20 x 5 cm Kieselgel 60 F_{254} TLC-plates, with a thickness of 0.25 mm, were eluted with methanol to remove contaminants, dried with a hairdryer and stored in a desiccator before the crude extracts were applied. Of the 10 mg/mL solutions, 180 µl were spotted in 2 cm bands using a Linomat applicator. The chromatograms were allowed to develop using the mobile phase, hexane:chloroform:methanol (7:2:1). After development, the plates were dried using a hairdryer, left in a fume cupboard for a few minutes, and stored in a freezer at approximately -20°C.

The developed TLC-plates were removed from the freezer and maintained at 37°C in an incubator. The agar mixtures were allowed to cool until they could be held in the hand comfortably, c. 37°C, and the temperature maintained by keeping the solutions in an oven. The microorganisms were added to the agar mixtures, and the solutions were mixed to form suspensions. 10 mL of the suspension was pipetted with a heated pipette, onto the TLC-plates lengthwise, over the 20 x 5 cm TLC-plates, ensuring that the chromatograms were covered. After solidification of the agar layers, the TLC-plates were placed into plastic trays with a beaker of water to maintain a humid atmosphere that prevented the TLC-plates from drying out. The trays were covered with plastic bags and left in the incubator at 37°C. Staphylococcus

aureus, P. aeruginosa, and C. albicans were incubated for 24 hours, while M. smegmatis was incubated for 48 hours. After the incubation period, the plates were sprayed with a 0.1% aqueous solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT-reagent). The mitochondrial enzymes of live organisms reduce MTT-reagent, which is a pale yellow substance, to a dark blue formazan product. The sprayed plates were left in the incubator for 1 hour at 37°C after which the inhibition zones were recorded. The R_f–values of the zones were calculated as follows: R_f-value = Inhibition zone distance from origin (midpoint) in (mm)/Solvent front distance from origin in (mm).

RESULTS

In extraction procedure 1, the resultant mixtures formed with the wet seaweed samples were difficult to filter. A pressure filter took care of this difficulty, but much of the sample was lost in the process. The viscosity of the samples varied and so did the ease of filtration. Another option to get a clear extract was to centrifuge the samples. The effective time for centrifuging depended on the viscosity of the solution and took long for some of the samples, as did the evaporation process. Some of the samples took days to dry. Drying the seaweeds at 40°C to prevent the loss of volatile compounds while producing dried seaweeds was a time-consuming process. Another problem encountered was that the ethanol of the boiled samples evaporated and had to be refilled to keep to the extraction time of 4 hours. Drying the filtrates also took days. In extraction procedure 2, the water extract was very difficult to filter. Centrifuging and pressure filtration was equally difficult. The extracts took days to dry in the freeze-dryer and in the oven at 43°C after which it was difficult to remove the dried samples from the test tubes. In extraction procedure 3, the samples that were placed in the Speedvac for drying were repeatedly lost under vacuum. The extracts took days to dry in the oven at 43°C. In extraction procedures 4 and 5, the

extracts were easily obtained. In extraction procedure 6, the water extracts took up to a week to dry in the freeze dryer.

In the agar disc diffusion assay, no inhibition zones were formed around the sample and solvent control discs of the extracts obtained through extraction procedure 4 and 5, where sample volumes of 50 µl were applied to the sterile discs. The assay was repeated with increased sample volumes, in which case, the DCM:MeOH (2:1) extracts of two of the algae inhibited the growth of Staphylococcus aureus (Table 2.1). With one of the algae, only two of the three discs produced inhibition zones against Staphylococcus aureus. In the agar overlay bioautography method, the antimicrobial activity of the extracts that inhibited the growth of Staphylococcus aureus in the agar disc diffusion assay was confirmed. Additionally, they also inhibited the growth of the bacterium, Mycobacterium smegmatis (Table 2.2). When the same algae were extracted with dichloromethane, methanol, and water, successively in extraction procedure 6, the dichloromethane extract alone showed antimicrobial activity in the agar disc diffusion assay and the agar overlay bioautography method. The dichloromethane extract of Carpoblepharis flaccida inhibited the growth of Candida albicans in the diffusion method (Table 2.1) and of Staphylococcus aureus and Mycobacterium smegmatis in the bioautography method (Table 2.2). In the bioautography method, four zones of inhibition against Staphylococcus aureus and three inhibition zones against Mycobacterium smegmatis indicated the presence of up to four antimicrobial compounds (Table 2.2). Other algal extracts that showed no inhibition in the agar disc diffusion assay, namely, the extracts of Champia compressa, Hypnea spicifera, Nothogenia erinacea and Polysiphonia virgata showed inhibition against Staphylococcus aureus in the bioautography method (Table 2.2).

DISCUSSION

In extraction procedures 1 to 3, the long drying procedures could result in the loss of secondary metabolites. Cronin et al. (1995) noted that the drying of algae before extraction increased the chance of loss of secondary metabolites. This is one of the reasons why most marine natural product chemists prefer to work with fresh material (Wright, 1998). In extraction procedure 4, the filtration time through centrifuging and using micro-syringes, lengthened the extraction procedure that could also lead to changes in the yields of the secondary metabolites (Cronin et al., 1995). Extraction procedure 5 was an improvement on extraction procedure 4 by simplifying the filtration method. This extraction procedure was easier and faster than the other procedures, minimizing the loss of secondary metabolites. In extraction procedure 6, the procedure time was again lengthened by freeze-drying where the samples were exposed to vacuum for days. This could also result in the loss of secondary metabolites (Cronin et al., 1995).

The poor results obtained with the agar disc diffusion method could be because of the incompatibility of the test solvent with the agar medium. In extraction procedure 4, the difficulty experienced to redissolve the dried algal extract showed that the samples were more lipophylic in nature. Thus, a non-polar solvent formed the medium for the test solutions. Agar is a polar mixture and a polar test solution would have assisted with the diffusion of the antimicrobial compounds through the agar (Vanden Berghe and Vlietinck, 1991). The fact that the dichloromethane:methanol extracts showed no inhibition in the agar disc diffusion assay, but significant inhibition in the agar overlay bioautography method, suggests that the antimicrobial substances remained on the sterile discs, and could not impact the growth of the microorganisms. With an increase in the sample mass on the disc, the concentration of the

antimicrobial compounds was increased and so the extent to which the growth of the microorganisms could be inhibited.

After the completion of this investigation, it became known that diffusion methods would have been more suitable for these non-polar extracts. Rios et al. (1998) noted that disc diffusion were in fact unsuitable for testing non-polar extracts (Rios et al., 1988). In recommending methods for testing the extracts of terrestrial plants, Vanden Berghe and Vlietinck (1991) noted that the filter paper disc method is for assaying water-soluble antibiotics, while the dilution methods, where the minimum inhibition concentration values (MIC-values) are determined are more suitable for non-polar extracts. In these methods, "non-polar extracts, essential oils, suspensions of solids or emulsions and antimicrobial substances, which do not diffuse through agar media can be tested directly by incorporating them in the agar media as if they were aqueous solutions" (Vanden Berghe and Vlietinck, 1991). Since the polarity of the solvents used for preparing the test solution is so significant, it would be advisable either to use polar solvents for redissolving the dried seaweed extracts or to use the dilution method. Solvents such as methanol, acetone, solutions of methanol and water, and mixtures of dimethyl sulfoxide (DMSO), methanol and water, did not produce a homogenous solution when attempts were made to redissolve the dried seaweed extracts. Therefore, the agar dilution method would have to be used in the place of the agar disc diffusion assay when extracting algae with dichloromethane:methanol (2:1) as the final extract requires a less polar solvent such as, dichloromethane:methanol (80%) [1:1) (Vanden Berghe and Vlietinck, 1991).

The agar overlay bioautography method produced excellent results. Two problems that are often encountered in the screening of crude plants extracts are, firstly, that the active compounds could be present in very small concentrations or secondly, that the compounds may exert

antagonistic effects in the assay (Rasoanaivo and Ratsimamanga-Urverg, 1993). In the bioautography, the separation of the compounds, brought about by the mobile phase hexane:chloroform:methanol (7:2:1), increased exposure of the antibiotic compounds, which inhibited the growth of microorganisms. The difference between the results obtained in the agar disc diffusion assay and those obtained in the bioautography method showed that the bioautography method is therefore more sensitive for screening non-polar seaweed extracts. Most of the extracts that showed inhibition zones, inhibited Staphylococcus aureus making it the most susceptible microorganism of this investigation, followed by Mycobacterium smegmatis. The inhibition zones often numbered up to two and more, suggesting that the crude extract contained more than one antibiotic compound.

In this investigation, extraction procedure 5 was the most suitable for obtaining algal secondary metabolites, verifying the recommendations made by Cronin et al. (1995). The solvent dichloromethane:methanol (2:1) extracted the relevant antimicrobials. Betina (1973) has described thin-layer chromatography as "an indispensable tool in the study of antibiotics", and its use in this investigation clearly demonstrated it. From these results, the agar overlay bioautography method is as strongly recommended for studies investigating antibiotics in marine algal extracts as it were for studies investigating the antibiotics in terrestrial plants. Overall, the results obtained in this investigation were encouraging and showed that red algal secondary extracts are potential sources of antibiotics.

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TABLES

Table 2.1. Results of agar disc diffusion assay with increased sample volumes (zone sizes in mm).

Date	No.	Seaweeds	S	M	P	C
Jul-99	P97.1	Aeodes orbitosa	0	0	0	0
Jul-99	P97.2	Carpoblepharis flaccida	0	0	0	0
Jul-99	P97.3	Champia compressa	0	0	0	0
Jul-99	P97.4	Gelidium pristoides	0	0	0	0
Jul-99	P97.5	Gigartina polycarpa	0	0	0	0
Jul-99	P97.6	Hypnea spicifera	0	0	0	0
Jul-99	P97.7	Nothogenia erinacea	0	0	0	0
Jul-99	P97.8	Polysiphonia virgata	0	0	0	0
Jul-99	P97.9	Porphyra capensis	0	0	0	0
Jul-99	P97.10	Sarcothalia stiriata*	0	0	0	0
Jul-99	P97.11	Sarcothalia stiriata**	0	0	0	0
Jul-99	P97.12	Suhria vittata	0	0	0	0
Aug-99	P981	Aeodes orbitosa	0	0	0	0
Aug-99	P982	Carpoblepharis flaccida	0	0	0	0
Aug-99	P983	Gelidium pristoides	0	0	0	0
Aug-99	P984	Gigartina polycarpa	0	0	0	0
Aug-99	P985	Pachymenia carnosa	0	0	0	0
Aug-99	P986	Polysiphonia virgata	0	0	0	0
Aug-99	P987	Sarcothalia stiriata	0	0	0	0
Aug-99	P988	Suhria vittata	26	0	0	0
Aug-99	P989	Chondria capensis	25	0	0	0
Aug-99	K981	Aeodes orbitosa	0	0	0	0
Aug-99	K982	Aristothamnion collabens	0	0	0	0
Aug-99	K983	Botryocarpa prolifera	0	0	0	0
Aug-99	K984	Carpoblepharis flaccida	0	0	0	0
Aug-99	K975	Epymenia obtusa	0	0	0	0
Aug-99	K986	Gelidium capense	0	0	0	0
Aug-99	K987	Gigartina polycarpa	0	0	0	0
Aug-99	K988	Gelidium pristoides	0	0	0	0
Aug-99	K989	Gigartina bracteata	0	0	0	0
Aug-99	K9810	Gigartina scutellata	0	0	0	0

*Sarcothalia stiriata – gametophyte
**Sarcothalia stiriata – tetrasporophyte
P = Pringle Bay; K = Kommetjie; S = Saldanha Bay; S = Staphylococcus aureus; M = Mycobacterium smegmatis; P = Pseudomonas aeruginosa; C = Candida albicans;

Table 2.1 cont. Results of agar disc diffusion assay with increased sample volumes (zone sizes in mm).

Date	No.	Seaweeds	S	M	P	C
Aug-99	K9811	Neuroglossum binderianum	0	0	0	0
Aug-99	K9812	Nothogenia erinacea	0	0	0	0
Aug-99	K9813	Pachymenia carnosa	0	0	0	0
Aug-99	K9814	Polysiphonia virgata	0	0	0	0
Aug-99	K9815	Porphyra capensis	0	0	0	0
Aug-99	K9816	Sarcothalia stiriata	0	0	0	0
Aug-99	K9817	Suhria vittata	0	0	0	0
Aug-99	K9818	Gymnogongrus dilatatus	0	0	0	0
Aug-99	K9819	Trematocarpus flabellatus	0	0	0	0
Aug-99	S9820	Gracilaria gracilis	0	0	0	0
Oct-99	d109.1	Carpoblepharis flaccida	0	0	0	12
Oct-99	m109.1	Carpoblepharis flaccida	0	0	0	0
Oct-99	h109.1	Carpoblepharis flaccida	0	0	0	0
Oct-99	d109.2	Polysiphonia virgata	0	0	0	0
Oct-99	m109.2	Polysiphonia virgata	0	0	0	0
Oct-99	h109.2	Polysiphonia virgata	0	0	0	0
Oct-99	d109.3	Suhria vittata	0	0	0	0
Oct-99	m109.3	Suhria vittata	0	0	0	0
Oct-99	h109.3	Suhria vittata	0	0	0	0

*Sarcothalia stiriata – gametophyte
**Sarcothalia stiriata – tetrasporophyte
P=Pringle Bay; K=Kommetjie; S=Saldanha Bay; S=Staphylococcus aureus; M=Mycobacterium smegmatis; P=Pseudomonas aeruginosa; C=Candida albicans;
d=dichloromethane extract; m=methanol extract; h=water extract;

Table 2.2. R_f-values of the active agents in the samples that were assayed in the agar overlay bioautography method. (-) indicates no inhibition zone visible after the plates were sprayed with MTT-reagent. Each R_f–value refers to an inhibition zone.

Date	No.	Seaweeds	S	M	P	C
Jul-99	P97.1	Aeodes orbitosa	-	-	-	-
Jul-99	P97.2	Carpoblepharis flaccida	-	-	-	-
Jul-99	P97.3	Champia compressa	0.7	-	-	-
Jul-99	P97.4	Gelidium pristoides	-	-	-	-
Jul-99	P97.5	Gigartina polycarpa	-	-	-	-
Jul-99	P97.6	Hypnea spicifera	0.2; 0.7			
Jul-99	P97.7	Nothogenia erinacea	0.04;0.7	-	-	-
Jul-99	P97.8	Polysiphonia virgata	0.7			
Jul-99	P97.9	Porphyra capensis	-	-	-	-
Jul-99	P97.10	Sarcothalia stiriata*	-	-	-	-
Jul-99	P97.11	Sarcothalia stiriata**	-	-	-	-
Jul-99	P97.12	Suhria vittata	-	-	-	-
Aug-99	P981	Aeodes orbitosa	-	-	-	-
Aug-99	P982	Carpoblepharis flaccida	-	-	-	-
Aug-99	P983	Gelidium pristoides	-	-	-	-
Aug-99	P984	Gigartina polycarpa	-	-	-	-
Aug-99	P985	Pachymenia carnosa	-	-	-	-
Aug-99	P986	Polysiphonia virgata	-	-	-	-
Aug-99	P987	Sarcothalia stiriata	-	-	-	-
Aug-99	P988	Suhria vittata	0.3	0.01;0.3;0.1	-	-
Aug-99	P989	Chondria capensis	0.1;0.2;0.4	0.1;0.3	-	-
Aug-99	K981	Aeodes orbitosa	-	-	-	-
Aug-99	K982	Aristothamnion collabens	0.2	0.2;0.4	-	-
Aug-99	K983	Botryocarpa prolifera	0.8	-	-	-

*Sarcothalia stiriata – gametophyte
**Sarcothalia stiriata – tetrasporophyte
P=Pringle Bay; K=Kommetjie; S=Staphylococcus aureus; M=Mycobacterium smegmatis; P=Pseudomonas aeruginosa; C=Candida albicans

Table 2.2 cont. R_f-values of the active agents in the samples that were assayed in the agar overlay bioautography method. (-) indicates no inhibition zone visible after the plates were sprayed with MTT-reagent.

Date	No.	Seaweeds	S	M	P	C
Aug-99	K984	Carpoblepharis flaccida	0.04	-	-	-
Aug-99	K985	Epymenia obtusa	0.01;0.1	-	-	-
Aug-99	K986	Gelidium capense	0	-	-	-
Aug-99	K987	Gigartina polycarpa	0.4	-	-	-
Aug-99	K988	Gelidium pristoides	0.02;0.1	-	-	-
Aug-99	K989	Gigartina bracteata	0.2;0.7	-	0.03	-
Aug-99	K9810	Gigartina scutellata	0.8	-	0.03	-
Aug-99	K9811	Neuroglossum binderianum	0.3	0.3	0.1	-
Aug-99	K9812	Nothogenia erinacea	0.6;0.7;0.8	0.2	-	-
Aug-99	K9813	Pachymenia carnosa	0.8	-	-	-
Aug-99	K9814	Polysiphonia virgata	0.00;0.03	-	-	-
Aug-99	K9815	Porphyra capensis	0.8	-	-	-
Aug-99	K9816	Sarcothalia stiriata	0.8	-	-	-
Aug-99	K9817	Suhria vittata	-	0.2	-	-
Aug-99	K9818	Gymnogongrus dilatatus	0.9	-	-	-
Aug-99	K9819	Trematocarpus flabellatus	0.2;0.7	-	-	-
Aug-99	S9820	Gracilaria gracilis	0.8	-	-	-
Oct-99	$P109.1_{DCM}$	Carpoblepharis flaccida	0.4;0.7;0.6;0.8	0.2;0.4;0.5	-	-
Oct-99	$P109.1_{MeOH}$	Carpoblepharis flaccida	-	-	-	-
Oct-99	$P109.1_{H2O}$	Carpoblepharis flaccida	-	-	-	-
Oct-99	$P109.2_{DCM}$	Polysiphonia virgata	-	-	-	-
Oct-99	$P109.2_{MeOH}$	Polysiphonia virgata	-	-	-	-
Oct-99	$P109.2_{H2O}$	Polysiphonia virgata	-	-	-	-
Oct-99	$P109.3_{DCM}$	Suhria vittata	-	-	-	-
Oct-99	$P109.3_{MeOH}$	Suhria vittata	-	-	-	-
Oct-99	$P109.3_{H2O}$	Suhria vittata	-	-	-	-
Oct-99	K101.1	Polysiphonia virgata	0.01;0.2	-	-	-
Oct-99	K101.2	Carpoblepharis flaccida	0.3	-	-	-
Oct-99	K101.3	Aeodes orbitosa	-	-	-	-
Oct-99	K101.4	Suhria vittata	-	-	-	-

*Sarcothalia stiriata – gametophyte
**Sarcothalia stiriata – tetrasporophyte
P=Pringle Bay; K=Kommetjie; S=Staphylococcus aureus; M=Mycobacterium smegmatis; P=Pseudomonas aeruginosa; C=Candida albicans

Chapter 3

ANTIMICROBIAL ACTIVITY OF SOUTH AFRICAN RED ALGAL SECONDARY METABOLITES

ABSTRACT

Seventeen red algal species were screened for antimicrobial activity against Staphylococcus aureus, Pseudomonas aeruginosa, Mycobacterium smegmatis and Candida albicans, using agar overlay bioautography. The crude extracts of nine algae showed antimicrobial activity. Extracts of these nine algae were fractionated, after which the antimicrobial activity was enhanced in some of the fractions. Most of the antimicrobial activity of both the crude extracts and the fractions was against S. aureus and M. smegmatis. The extracts of Plocamium corallorhiza and Polysiphonia virgata produced the greatest inhibition zones.

INTRODUCTION

Numerous red algal extracts have been screened by various researchers for biological activities and many have been found to possess significant antimicrobial activity (Pratt et al., 1951; Hornsey and Hide, 1974; Khaleafa et al., 1975; Hornsey and Hide, 1976; Heríquez et al., 1979; Solimabi et al., 1980; Caccamese et al., 1980; Caccamese et al., 1981; Reichelt and Borowitzka, 1984; Caccamese et al., 1985; De Campos-Takaki et al., 1988; Munro et al., 1989; Tariq, 1991; Ballesteros et al., 1992; König et al., 1994; Sastry and Rao, 1994; Mahasneh et al., 1995; N'Diaye et al., 1996; Robles-Centeno et al., 1996; Vlachos et al., 1996; Melo et al., 1997;

Crasta et al., 1997; Vlachos et al., 1997; König et al., 1999; Horikawa et al., 1999). From these reports, the algae that are commonly reported to have antimicrobial activity belong to the genera Hypnea, Gracilaria, Laurencia, Chondria, and Plocamium. Representatives of these genera are commonly found along the coast of South Africa, but little has been done to investigate the extent of their biological activities. Vlachos screened a few southern African seaweeds for their antimicrobial activity using an agar disc diffusion assay. The red alga, Polysiphonia virgata, has been reported to have the largest inhibition zones against the microorganisms tested (Vlachos et al., 1997). The 80% ethanol extracts of common red algae, such as, Porphyra sp., Plocamium corallorhiza and Aeodes orbitosa were reported to have no significant antimicrobial activity.

The extraction procedure used by Vlachos, included dried algal material and allowed for the destruction of heat-labile compounds (Vlachos et al., 1997). To minimize the loss of secondary metabolites, an extraction procedure that was developed in a pilot investigation was used in this investigation.

Agar diffusion assays are the most common screening procedures used by marine researchers, but a pilot investigation demonstrated that this assay was not suitable for testing the dichloromethane:methanol (2:1) extract because of the lipophilic nature of the compounds extracted. In this investigation, the bioassay that is known to be ideal for bioassay screening and bioassay-guided fractionation, bioautography, was used (Rasoanaivo and Ratsimamanga-Urverg, 1993). Additionally, the bioactive fractions were also tested in the agar disc diffusion method, because at the time of testing the assay was prepared and it was another opportunity to observe the differences in the sensitivity of the two assays when testing a dichloromethane:methanol (2:1) algal extract. The dichloromethane:methanol (2:1) red algal extracts were screened for antimicrobial activity against Staphylococcus aureus, Mycobacterium

smegmatis, Pseudomonas aeruginosa and Candida albicans. Staphylococcus aureus and P. aeruginosa are bacteria responsible for causing infections in hospitalized patients (Yuan and Williams, 1997; Trautmann et al., 1998). Mycobacterium smegmatis is closely related to the bacterium M. tuberculosis that causes tuberculosis (Sneader, 1986). Candida albicans causes candidiasis (On-line Medical Dictionary, 2000).

MATERIALS AND METHODS

Seventeen common red seaweed species were collected during January and February 2000 from the rocky shores and subtidal zone of Kommetjie (Table 3.1). Voucher specimens were deposited in the Herbarium of the Botany Department at the University of the Western Cape (UWC).

EXTRACTION

The samples were put into plastic bags and cooled on ice. In the laboratory, samples were washed with distilled water to remove surface salts, sand, and epiphytes. The seaweeds were dabbed with paper towel to remove excess water, then cut into small pieces. Samples were weighed and volumes of dichloromethane (DCM):methanol (MeOH) (2:1) solution was added (Table 3.2). The samples were homogenized using a Kinematica homogenizer, shaken for 36 hours and filtered using a Buchner funnel with Whatman no. 541 filter paper. The seaweeds were extracted for a second time with the DCM:MeOH (2:1) solution. The samples that could not be processed on a particular day were kept in the freezer at –20°C. The filtrates were evaporated under vacuum in a rotatory evaporator at 50°C and the yields determined. The dried extracts were stored in a freezer at approximately -20°C.

In preparation for the agar overlay bioautography method, 20x 20 cm Silica gel 60F thin layer chromatography (TLC) plates were allowed to run with methanol to clean the plates of impurities. Two mg of each sample was applied to the 20 x 20 cm TLC-plate in triplicate using a 100 µl micropipette. This was done on four different plates to test extracts against the four microorganisms. The plates were allowed to develop in the mobile phase hexane:chloroform:methanol (7:2:1). After development, the plates were dried using a hairdryer and left in a fume cupboard for a few minutes, after which they were wrapped in foil and placed in a freezer at –20°C, until the time of the assay.

BIOAUTOGRAPHY METHOD

The microorganisms used for testing were supplied by the Microbiology Department of the University of the Western Cape. Staphylococcus aureus and Pseudomonas aeruginosa were maintained on Difco nutrient agar, Mycobacterium smegmatis on Difco Mycobacteria 7H11 agar supplemented with Bacto Middlebrook OADC-enrichment and Bacto glycerol and Candida albicans on Sabourand dextrose agar. The developed TLC-plates were removed from the freezer and maintained at 37°C in an incubator. The agar mixtures were allowed to cool until they could be held in the hand comfortably (c.37°C). The agar mixtures and the microorganisms were mixed thoroughly. The plates were removed from the incubator as soon as the agar mixtures were prepared. 40 mL of the agar mixtures were poured over the chromatograms on each 20 x 20 cm TLC-plate, ensuring the chromatograms were completely covered. After solidification of the agar layers, the TLC-plates were placed into plastic trays with a beaker of water to maintain a humid atmosphere to prevent the TLC-plates from drying. The trays were covered with plastic bags and left in the incubator at 37°C. Staphylococcus aureus, P. aeruginosa, and C. albicans were incubated for 24 hours, while M. smegmatis was incubated for

48 hours. After the incubation period, the plates were sprayed with a 0.1% aqueous solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT-reagent). The mitochondrial enzymes of live organisms reduce the MTT-reagent, which is a pale yellow substance, to a dark blue formazan product (Begue and Kline, 1972). The sprayed plates were left in the incubator for 1 hour at 37°C, after which the inhibition zones were recorded.

FRACTIONATION

Only the extracts that inhibited the growth of the microorganisms were fractionated. A 10 mg/mL solution, of which the actual volumes are shown in Table 3.2, was evaporated until dryness under vacuum, using a rotary evaporator at approximately 50°C. The sample was redissolved with dichloromethane and adsorbed onto silica gel 100, then evaporated until dryness. The adsorption was complete when the dried silica mixture was free flowing. Silica gel 60 was packed into a glass column until it was approximately three quarters full. The sample plug was introduced onto the silica gel 60, and cotton wool was placed on top of the sample plug. The mobile phase, hexane:chloroform:methanol (7:2:1) was introduced with a covered funnel, allowed to run through the column, and 25 mL fractions were collected in reagent bottles. For each sample, the position of the active bands was noted (Table 3.3).

The sample was allowed to run through the column until the active band/s passed through. The bands of the Polysiphonia virgata extract did not move through the column as expected. After 75 fractions were collected, the estimated active bands did not elute from the column. After fraction no. 75 was collected, 100% methanol was passed through the column and the resultant fraction collected. Fractions 41 to 46, 47 to 56, 57 to 65, and 66 to 75 were combined and spotted onto the tlc-plates. The fractions were stored at –20°C. Of each fraction, 200 µl were

spotted onto glass-backed 20 x 20 cm Kieselgel F_{254}, 0.25 mm thick TLC-plates, using a Linomat, with 6 fractions per plate. The plates were developed in the mobile phase, hexane:chloroform:methanol (7:2:1). After development, the TLC-plates were dried and stored at –20°C. These samples were assayed against S. aureus and M. smegmatis only since unclear inhibition zones were obtained with C. albicans and P. aeruginosa previously.

BIOASSAY OF THE COMBINED FRACTIONS

The fractions that showed inhibition zones were combined and 200 µl of the resultant solutions were spotted onto 20 x 20 cm square and 0.25 mm thick silica gel F_{254} plates and developed with the mobile phase, hexane:chloroform:methanol (7:2:1). The plates were assayed against S. aureus and M. smegmatis using bioautography. The crude extracts were also assayed against the test organisms using disc diffusion by impregnating 200 µl of each extract onto the 6 mm discs. This was done to see whether the fractions would inhibit the growth of the microorganisms.

AGAR DISC DIFFUSION METHOD

Sterile 6 mm discs were impregnated with 200 µl of the 10 mg/mL sample solution in triplicate. After having dried in the incubator at 37°C, discs were placed onto the agar plates that had been inoculated with microbial cultures. Control discs were impregnated with 50 µl of the dissolution solvent, DCM:MeOH (80%) (1:1). Antibiotic controls included Ciprofloxacin, 40 µg/disc, and Amphotericin B, 25 µg/disc. Ciprofloxacin was used as a control for S. aureus, P. aeruginosa, and M. smegmatis. Amphotericin B was used as a control for C. albicans. The agar plates with the discs and the microorganisms were incubated at 37°C for 24 hours for S. aureus, P. aeruginosa, and C. albicans and 48 hours for M. smegmatis. After the incubation period, the

inhibition zones were measured in millimeters as the distance between the discs and the growth free zones around the discs.

RESULTS

Crude extracts of nine of the seventeen species screened showed inhibition in the bioautography method against one or two of the microorganisms tested (Table 3.4). Most of the antimicrobial activity of the algal crude extracts and their fractions was against S. aureus, followed closely by M. smegmatis. Plocamium corallorhiza was the only alga that inhibited the growth of C. albicans.

Pseudomonas aeruginosa did not show any inhibition zones and when the plates were sprayed with MTT-reagent after incubation, the yellow formazan product of the reagent was not reduced to blue by the mitochondrial enzymes. This was also observed with some of the Candida albicans plates.

The zones obtained when the more purified fractions of the crude extracts were assayed, where much larger than zones observed for the crude extracts (Figures 1 & 2). In some cases, the fractions inhibited microorganisms that were not inhibited by the crude extracts, for example, the fractions of the crude extract of Polysiphonia virgata. The crude extracts inhibited the growth of S. aureus, while the fractions inhibited the growth of S. aureus and M. smegmatis (Table 3.7 & 3.8). Other fractions that followed a similar pattern were those of Neuroglossum binderianum and Plocamium corallorhiza (Table 3.7 & 3.8). Overall, the algal extracts of P. virgata and P. corallorhiza had the most active fractions. When the fractions that showed inhibition in the bioautography method were combined, the antimicrobial activity for some

extracts differed from those of the fractions (Figure 3). Some of the microorganisms that were inhibited by the fractions were not inhibited by the combined fractions (Table 3.5). In addition, the combined fractions of P. virgata and P. corallorhiza each produced two inhibition zones, showing the presence of two antimicrobial compounds. When the combined fractions of the eight fractionated extracts were tested in the agar disc diffusion assay, only the extracts of P. virgata and P. corallorhiza showed antimicrobial activity (Table 3.6).

DISCUSSION

This study had four species in common with an antimicrobial screen that was done previously on southern African marine algae, namely, Plocamium corallorhiza, Polysiphonia virgata, Gelidium pristoides, and Porphyra capensis (Vlachos et al., 1997). The ethanol extract of Plocamium rigidum then showed the broadest spectrum of antimicrobial activity and the extract of Polysiphonia virgata (then Carradoriella virgata) produced the largest inhibition zones. However, the extracts of Plocamium corallorhiza, Gelidium pristoides, and Porphyra capensis did not show significant antimicrobial activity in that study (Vlachos et al., 1997). In this investigation, the results obtained for the extracts of P. virgata were consistent with those obtained by Vlachos (Vlachos et al., 1997). Other species within the genus Polysiphonia have also been reported to have antimicrobial activity (Hornsey and Hide, 1976; Vlachos et al., 1997). In addition, the extracts of G. pristoides and P. corallorhiza showed significant antimicrobial activity. The dichloromethane:methanol (2:1) extract of P. capensis cannot be reported as being without antimicrobial compounds until the fractions of the crude extract are tested. In investigations done previously, the genera Gigartina and Gelidium also produced extracts with significant antimicrobial activity (Munro et al., 1989; Vlachos et al., 1997).

Of all the algae that were tested, Plocamium corallorhiza was selected for further analyses because of its broad spectrum of antimicrobial activity. Further analyses should include the isolation and identification of the bioactive compounds. The remaining seven algae that showed antimicrobial activity and those that were not fractionated are equally important candidates for further antimicrobial screening. Priority should be given to those that are considered to have economic potential, namely, Carpoblepharis flaccida, Gelidium pristoides, Gigartina polycarpa, Porphyra capensis, Sarcothalia stiriata, and Suhria vittata (Critchley et al., 1998). These algae can be easily harvested in large quantities, which are one of the requirements for antibiotic research with the focus of isolating, identifying, and synthesizing bioactive compounds. The isolated compounds should then be tested against the multi-drug resistant strains of Staphylococcus aureus and Mycobacterium tuberculosis.

Other common red algae that were not included in this antimicrobial screen, such as, Hypnea spicifera, Aeodes spicifera and Gymnogongrus spp. should be screened for antimicrobial compounds, because they have also been reported to hold possibilities for harvesting and mariculture (Critchley et al., 1998). In addition, other species of the genus Hypnea were reported to have compounds with significant biological activity (De Campos-Takaki et al., 1988; Melo et al., 1997). A few researchers have studied species of the genus Laurencia (Hornsey and Hide, 1976; Henriques et al., 1979; Caccamese et al., 1980; König et al., 1994; Horikawa et al., 1999) and Gracilaria (Sastry and Rao, 1994; Vlachos et al., 1996; Crasta et al., 1997), either for their biological activities or to determine the chemical composition of their secondary metabolites. Chondria spp., Nothogenia sp. and Grateloupia sp. are algae with compounds possessing anthelminthic activity (Zaman et al., 1997; Davyt et al., 1998), antiviral activity (Kolender et al., 1997), and antimicrobial activity (Heriquez et al., 1979), respectively. These algae are also available along our coast and should be included in future screens for bioactive compounds.

This investigation once again demonstrated the potential of marine red algal extracts as sources of antimicrobial compounds. Most of the extracts strongly inhibited the growth of the microorganisms Staphylococcus aureus and Mycobacterium smegmatis, which were more sensitive to the red algal antimicrobials than Pseudomonas aeruginosa and Candida albicans. This qualifies further investigation into red algal extracts as sources of anti-tuberculosis and anti-staphylococcal antibiotics, particularly against various resistant strains of M. tuberculosis and S. aureus.

The differences in the results obtained for the crude extracts and the fractions suggest that a screen for antimicrobial activity of algal secondary metabolites must include the fractionation of the crude extracts. These differences were noted previously in an investigation of the antimicrobial activity of Indian seaweeds, when the crude extracts were active only against gram-positive bacteria, while the fractions inhibited the growth of both gram-negative and gram-positive bacteria (Sastry and Rao, 1994). It was then suggested that interfering compounds could be masking the bioactive compounds. Thus, this investigation can only report on the antimicrobial activity of eight of the seventeen algae tested, because only eight extracts were fractionated.

Generally, agar overlay bioautography was an easy assay to perform in our laboratory. The problem with the plates that were overlayed with Pseudomonas aeruginosa, showing no sign of live microorganisms after being sprayed with MTT-reagent, needs to be solved. It is possible that the gram-negative bacterium did not grow on the tlc-plates or that the MTT-reagent used was not suitable for this assay. In future, when extracts are tested against P. aeruginosa in bioautography, the optimum laboratory procedure will have to be determined. The agar disc diffusion assay results were poor when compared to that of the bioautography

method. This is mainly due to the non-polar nature of the extracts and its incompatibility with the polar agar medium. The agar disc diffusion assay must be replaced by a dilution method that is more suitable for non-polar extracts (Vanden Berghe and Vlietinck, 1991).

This investigation should serve as an introduction to the complete isolation of marine algal antimicrobial compounds, the characterization of the isolates, and the synthesis of the antibiotic compounds. The results indicate which species are more likely to produce secondary metabolites with antibiotic compounds. The compounds, when isolated and characterized, must be tested in vivo to establish the therapeutic value of the antimicrobials. Should in vivo testing proof the antimicrobials to be high in toxicity, as have been reported for red algae previously by Reichelt and Borowitzka (1984), the mechanisms of the antibiotics should be established, modified and the compounds re-tested. In addition, it would be worthwhile to determine the effectiveness of synergies of marine algal antimicrobials with exciting antibiotics after the toxicity has been reduced.

One limiting factor for further work is funding. Multinational companies, normally at the cutting edge of antibiotic research, have stopped to invest in the discovery of novel antibiotics because of the high cost involved (Hancock, 1997). Hancock (1997) also notes that researchers at universities and research institutions don't regard the crisis of antibiotic resistance with enough urgency and that national granting bodies do not invest in antibiotic research significantly. The result is that there are only a few researchers worldwide able to show progress in antibiotic research, particularly those working with marine algal extracts. Although many reports on the antimicrobial activity of marine algae have been published (Horikawa et al., 1999), only a few researchers have attempted to isolate new algal antibiotics. Those that have succeeded in the isolation and characterization of seaweed antibiotics have not all moved on to

the synthesis of the isolates. This could be because isolation and syntheses are more complicated and time consuming than to determine the antibiotic activity of crude algal extracts. According to Cannell (1998), "the degree of purity achieved in a natural product extraction, and the amount of work required to achieve this, is very approximately exponential ". One of the difficulties with isolation is that the isolate might not be the main compound responsible for the antibiotic activity of the crude extract. When Caccamese et al. (1981) isolated laurencienyne from the crude extract of Laurencia obtusa it was found that it was only mildly active and not the main antibiotic in the crude extract. However, after a successful isolation and characterization, more of the compound will be needed to optimize its application. Syntheses are imperative to enable more investigations and provide better evaluation of the effectiveness of marine red algal antibiotics.

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TABLES

Table 3.1 List of 17 seaweeds collected at Kommetjie in January and February 2000. The nomenclature is according to Silva (Silva et al., 1996).

Herbarium no.	Seaweed Species
6574	Aristothamnion collabens (Rudolphi) Papenfuss
6575	Botryocarpa prolifera Greville
6576	Botryoglossum platycarpum (Turner) Kützing
6577	Carpoblepharis flaccida (Lamouroux) Kützing
6578	Epymenia obtusa (Greville) Kützing
6579	Gelidium pristoides (Turner) Kützing
6580	Gigartina bracteata (S. Gmelin) Setchell & Gardner
6581	Gigartina polycarpa (Kützing) Setchell & Gardner
6582	Neuroglossum binderianum (Kützing)
6583	Nothogenia erinacea (Turner) Parkinson
6584	Pachymenia carnosa (J. Agardh) J. Agardh
6585	Pachymenia cornea (Kützing) Chang
6586	Plocamium corallorhiza (Turner) J. Hooker & Harvey
6594	Polysiphonia virgata (Agardh) Sprengel
6588	Porphyra capensis Kützing
6589	Sarcothalia stiriata (Turner) Leister
6590	Suhria vittata (Linnaeus) Endlicher

Table 3.2. The masses of the algae used in the extraction and the initial solvent volumes used.

Species	Mass (g)	Extraction Vol. (mL)	*Fractionation Vol. (mL)
Aristothamnion collabens	65	700	158.4
Botryocarpa prolifera	300	900	nf
Botryoglossum platycarpum	150	1000	551.4
Carpoblepharis flaccida	150	1000	504.4
Epymenia obtusa	100	1000	260.4
Gelidium pristoides	150	1500	389.4
Gigartina bracteata	300	900	nf
Gigartina polycarpa	300	900	398.4
Neuroglossum binderianum	300	900	850.4
Nothogenia erinacea	150	1500	nf
Pachymenia carnosa	300	900	nf
Pachymenia cornea	300	900	nf
Plocamium corallorhiza	210	900	966.4
Polysiphonia virgata	150	1500	449.4
Porphyra capensis	150	1000	nf
Sarcothalia stiriata	150	1000	nf
Suhria vittata	150	1000	nf

* Volume indicates the volume of a 10 mg/mL solution of crude extract used for sample preparation in open-column fractionation
nf = not fractionated

Table 3.3. Positions of the active bands as obtained for the crude extracts in the agar overlay bioautography method results.

SPECIES	POSITION OF THE ACTIVE BANDS
Gelidium pristoides	Just before the second yellow/orange band
Epymenia obtusa	Just before the second yellow/orange band
Botryoglossum platycarpum	Before the second yellow band
Polysiphonia virgata	At the origin and the second orange and beyond
Aristothamnion collabens	Before the second yellow and after the second green
Carpoblepharis flaccida	Before the yellow band
Plocamium corallorhiza	Before the first green and before the last yellow band
Neuroglossum binderianum	Before the last yellow band
Gigartina polycarpa	Before the yellow band

Table 3.4. Results of the overlay bioautography method for the crude extracts. Only the positive inhibition results are shown.

SPECIES	R_f-values of bioactive zones
Staphylococcus aureus	
Gelidium pristoides	0.26
Epymenia obtusa	0.22
Polysiphonia virgata	0.00
Aristothamnion collabens	0.25
Carpoblepharis flaccida	0.22
Gigartina polycarpa	0.23
Candida albicans	
Plocamium corallorhiza	0.74
Mycobacterium smegmatis	
Gelidium pristoides	0.27
Epymenia obtusa	0.25
Botryoglossum platycarpum	0.27
Aristothamnion collabens	0.30
Carpoblepharis flaccida	0.19
Plocamium corallorhiza	0.72
	0.35
Neuroglossum binderianum	0.20

Table 3.5. Results of the combined fractions obtained in the agar overlay bioautography method.

Species collected	S. aureus	M. smegmatis
Gelidium pristoides	0.20	no zone
Epymenia obtusa	0.20	no zone
Polysiphonia virgata	0; 0.13	no zone
Aristothamnion collabens	0.21	0.23
Carpoblepharis flaccida	0.20	no zone
Plocamium corallorhiza	0.28; 0.54	0.29; 0.55
Neuroglossum binderianum	0.22	no zone
Gigartina polycarpa	0.22	no zone

Table 3.6. Inhibition zones recorded in millimeters in the agar disc diffusion assay. S=Staphylococcus aureus; M=Mycobacterium smegmatis; C=Candida albicans; P=Pseudomonas aeruginosa

Species collected	S	M	C	P
Gelidium pristoides	0	0	0	0
Epymenia obtusa	0	0	0	0
Polysiphonia virgata	1.7	0	0	0
Aristothamnion collabens	0	0	0	0
Carpoblepharis flaccida	0	0	0	0
Plocamium corallorhiza	1.0	4.0	1.0	0
Neuroglossum binderianum	0	0	0	0
Gigartina polycarpa	0	0	0	0

Table 3.7. The R_f-values of the inhibition zones of the fractions of Polysiphonia virgata, Plocamium corallorhiza and Neuroglossum binderianum against Staphylococcus aureus.

Fraction	Polysiphonia virgata (6594)	Plocamium corallorhiza (6586)	Neuroglossum binderianum (6582)
1	no zone	no zone	no zone
2	no zone	no zone	no zone
3	no zone	no zone	no zone
4	no zone	no zone	no zone
5	no zone	no zone	no zone
6	no zone	0.05, 0.15, 0.22, 0.51, 0.78	no zone
7	no zone	0.02, 0.71	no zone
8	no zone	0.72	no zone
9	no zone	0.72	no zone
10	no zone	0.71	no zone
11	no zone	0.68	no zone
12	0.28	0.68	no zone
13	0.29	0.66	no zone
14	0.31	no zone	no zone
15	0.32	no zone	no zone
16	0.28	no zone	0.00
17	0.23	0.45	0.31
18	0.13	0.48	0.14, 0.50
19	0.11	0.42	0.08
20	0.13	0.28	0.09
21	0.17	0.37	0.03
22	0.23	0.29	
23	0.22	0.32	
24	0.22	0.28	
25	0.23	0.27	
26	0.23	0.23	
27	0.22	no zone	
28	0.16	0.03	
29	0.16	0.03	
30	0.16	0.02	
31	0.17	0.03	
32	0.18	0.04	
33	0.17	0.03	
34	0.16	0.04	
35	no zone	0.06	
36	no zone	no zone	
37	no zone		
38	no zone		
39	no zone		
40	0.13, 0.19		
41-46	0.16		
47-56	0.14		
57-65	0.12		
66-75	0.13		
MeOH	no zone		

Table 3.8. The R_f-values of the inhibition zones of the fractions of Polysiphonia virgata, Plocamium corallorhiza, and Neuroglossum binderianum against Mycobacterium smegmatis.

Fraction	Polysiphonia virgata (6594)	Plocamium corallorhiza (6586)	Neuroglossum binderianum (6582)
1	no zone	no zone	no zone
2	no zone	no zone	no zone
3	no zone	no zone	no zone
4	no zone	no zone	no zone
5	no zone	no zone	no zone
6	no zone	0.01, 0.1, 0.57, 0.80	no zone
7	no zone	0.77	no zone
8	no zone	0.75	no zone
9	no zone	0.73	no zone
10	no zone	0.73	no zone
11	0.30	0.70	no zone
12	0.26	0.69	no zone
13	0.28	0.69	no zone
14	0.28	0.43	no zone
15	0.34	0.41, 0.60, 0.69	no zone
16	0.29	0.47, 0.59	no zone
17	0.19	0.52	0.21
17	0.24	0.48	0.13
18	0.15	0.45	0.05
18	0.22	0.37	0.06
19	0.15	0.37	no zone
20	0.17	0.41	
21	0.17	0.33	
22	0.19	0.27	
23	0.18	0.29	
24	0.17	0.22	
25	0.16	no zone	
26	no zone	0.12	
27	no zone	0.09	
28-33	no zone	0.09	
34-40	no zone	0.08	
41-46	no zone	0.04	
47-56	no zone	0.04	
57-65	no zone	0.05	
66-75	no zone	0.07	
MeOH	no zone	no zone	

FIGURES

FIGURE CAPTIONS

Figure 1. The inhibition zones obtained with the crude extracts of Gelidium pristoides (three white zones on left) and Epymenia obtusa (three white zones on right) against Staphylococcus aureus. Each extract was spotted in triplicate.

Figure 2. The inhibition zones of the fractions of the crude extract of Epymenia obtusa obtained against Staphylococcus aureus indicating the presence of antimicrobial compounds in the extract.

Figure 3. The developed chromatograms of the combined fractions that have been covered with agar, inoculated with Staphylococcus aureus and the inhibition zones obtained, indicating the presence of antimicrobial compounds in the sample after fractionation.

Origin

Origin

Origin

Chapter 4

THE PURIFICATION, ISOLATION, AND PARTIAL IDENTIFICATION OF THE ANTIMICROBIAL COMPOUNDS OF THE ALGA, PLOCAMIUM CORALLORHIZA

ABSTRACT

In an investigation done on the antimicrobial activity of red algal secondary metabolites, the dichloromethane:methanol (2:1) extract of Plocamium corallorhiza and its fractions produced the widest spectrum of antimicrobial activity. The microorganisms that were inhibited by the algal extract included the bacteria, Staphylococcus aureus and Mycobacterium smegmatis and the fungus, Candida albicans. In this investigation, the two antimicrobial active fractions of the algal extract of P. corallorhiza were purified and isolated, using preparative thin-layer chromatography. After purification, the total number of bioactive fractions numbered five of which only one could be partially characterized using GC-MS and NMR spectroscopy due to small sample sizes. The spectroscopic analysis suggested that the isolated antimicrobial compounds were a chlorohexene and long chain fatty acid.

INTRODUCTION

In the search for new compounds with significant biological applications, a few marine natural product chemists investigated species of the genus Plocamium (König et al., 1994; Abreu and Galindro, 1996; Abreu et al., 1997; Whitney et al., 1997; Cueto et al., 1998; Miller, 1999; König et al., 1999a; König et al., 1999b). In the process, many compounds were isolated and their biological activities reported. Most of the compounds that were isolated were terpenes. An example of isolated terpenes is the isolation of the monoterpenes, Telfairine and

Aplysiaterpenoid A, from Plocamium telfairiae. Both terpenes showed insecticidal activity against the German cockroach, Blatella germanica and the mosquito larvae, Anopheles gambiae (Watanabe et al., 1990). Another example is that of the monoterpenes isolated from the alga, Plocamium hamatum, which had strong anti-algal activity against the alga Chlorella fusca and proved anti-tubercular toward Mycobacterium tuberculosis. In that study, two of the monoterpenes that were isolated were moderately cytotoxic (König et al., 1999b). Other studies produced isolates with significant antifouling (Sakata et al., 1991; König et al., 1999a) and antimicrobial activity (Vlachos et al., 1997).

In Chapter 3, the antimicrobial activity of an extract of Plocamium corallorhiza was reported. In this investigation, the antimicrobial compounds in the algal extract of P. corallorhiza were isolated and purified using preparative thin-layer chromatography. The resultant compounds were partially identified using GC-MS and NMR spectroscopy.

MATERIALS AND METHODS

A sample of Plocamium corallorhiza was previously extracted with dichloromethane:methanol (2:1) and fractionated using column chromatography. The fractions that showed antimicrobial activity in agar overlay bioautography against Staphylococcus aureus, Mycobacterium smegmatis and Candida albicans were combined and dried. In preparation for purification, the resultant sample was made up to a 10 mg/mL solution using dichloromethane:methanol (2:1).

PURIFICATION

Preparative thin-layer chromatography

The amount of 105 mL of the 10 mg/mL solution, approximately 20 mL per plate, was spotted onto preparative TLC-plates (1 mm, 20 x 20 cm, silica gel F_{254} plates) using a 1 mL pipette. After saturating the developing tanks, the plates were developed in the mobile phase hexane:chloroform:methanol (7:2:1), then viewed at wavelengths 254 nm and 366 nm. Bioactive zones were calculated from the R_f-values 0.3 and 0.5, with zone sizes of 50 mm and 32 mm respectively. At wavelength 254 nm and 366 nm, bands correlating with the R_f -values of 0.3 and 0.5 were visible. In preparation of the desorption of the bioactives from the silica which was done three times, the silica of each marked band of each fraction was scraped off the plate with a spatula, ground using a mortar and pestle, and transferred to centrifuge tubes. Dichloromethane was added and the mixture shaken for 30 minutes, centrifuged at 1000 rpm and the clear solution decanted into a syringe fitted with a 0.45 µm micro-filter. The filtrate was placed in a weighed round bottom flask and the solvent evaporated under vacuum in a rotary evaporator at 40°-50°C. A more polar solvent, DCM:MeOH (2:1), was added to the silica instead of DCM alone to ensure complete desorption. This was done until the UV-spectrophotometer reading at 366 nm and 254 nm showed a reading of zero absorbance. The solution was evaporated under vacuum using a rotary evaporator at 40° to 50°C. The mass was recorded and a 10 mg/mL solution in DCM was prepared. The resultant solution was spotted onto HPTLC-plates, and developed in two different solvent systems to obtain the best separation of the sample compounds. The two solvent systems were hexane:chloroform:methanol (5:3:1) and hexane:chloroform:methanol (5:4:1). Hexane:chloroform:methanol (5:4:1) showed the best separation. The Linomat was used to spot

200 µl of the two fractions in 2 cm bands onto 0.25 mm thick, 20 x 20 cm, silica gel 60 TLC-plates. The plates were developed in the mobile phase, hexane:chloroform:methanol (5:4:1). After the solvent front reached ~1 cm from the end of the plate, the plates were removed from the developing tanks and the solvents evaporated using a hairdryer. Further evaporation was allowed by leaving the plates in a fume cupboard for a few minutes. The plates were wrapped in foil and stored at –20°C.

BIOAUTOGRAPHY METHOD

The inhibition of Staphylococcus aureus and Mycobacterium smegmatis was to be tested. Of the sample solutions, 200 µl was spotted in 2 cm bands using a Linomat. The fraction with $R_f = 0.3$ showed two active zones against S. aureus (Table 4.1). This fraction was the one that appeared green when removed initially from the silica. The fraction with the $R_f = 0.5$ showed a large zone of inhibition. The solutions were spotted onto 0.25 mm Silica gel 60 F_{254} plates and developed in the mobile phase: hexane:chloroform:methanol (5:4:1). These plates were assayed in the bioautography method against Staphylococcus aureus and Mycobacterium smegmatis.

IDENTIFICATION

Five separate isolations were affected to obtain the active fractions by preparative thin-layer chromatography. The masses obtained were very small, ± 1-2 mg of an opaque white solid material. All five samples were dissolved in deuteriochloroform and analyzed using proton nuclear magnetic resonance spectra run on a 200 MHz Gemini 2000 spectrometer. All five samples had the same basic spectrum and the samples which appeared to have the best defined signals was chosen for the purposes of discussion and investigation by Gas Chromatography-

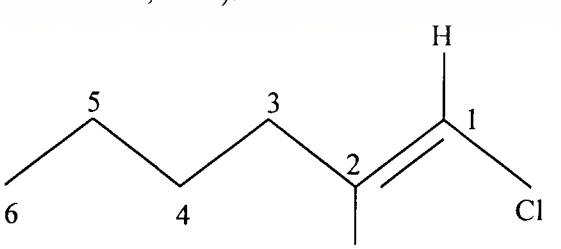
Mass spectroscopy (GC-MS) analysis. Thus, an attempt is presented to rationalize features obtained from the GC-MS with the signals observed in the proton spectrum. The results presented are at the best very preliminary, are not conclusive, and merely illustrate some structural features of the isolates.

RESULTS

The inhibition zones obtained for the fractions are reported in Table 4.1. The purer the fractions became, the more zones of inhibition were observed, bringing the final number of bioactive fractions to five. The active compounds could not be completely characterised because of the small sample sizes of the fractions. The partial identification of the antimicrobial compounds indicated that the bioactives are likely to be long chain fatty acids. The GC-MS of the active fraction showed three major peaks and each are discussed separately according to the elution times (see Figure 1):

FRACTION 1: Elution time = 32.5 minutes

Two prominent peaks at m/z 118 and 120 in the expected isotopic ratio of 3:1 correspond to (E)-1-chlorohexene (1) (Silverstein et al., 1991).



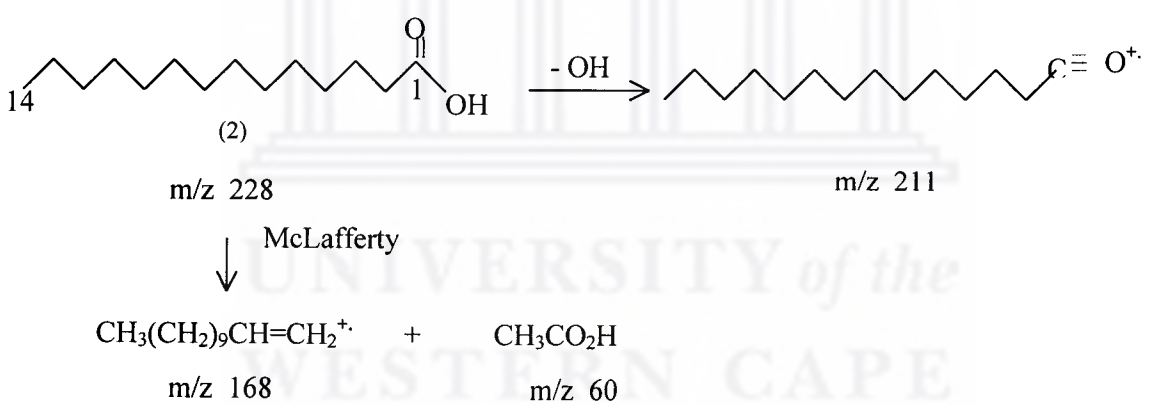
(1)

The proton 1-H appeared at 6.57 ppm as a trans-coupled doublet with J 15.6 Hz showing the expected coupling to 2-H which appeared as a doublet of doublets at 6.33 ppm with trans

coupling of 15.6 Hz to 1-H and vicinal coupling to 3-H of 8.5 Hz (see Figure 2). The anticipated doublet of a triplet for 3-H in the region of 2.0 – 2.2 ppm could not be clearly identified. A broadly defined triplet at 0.85 ppm could be assigned for the terminal methyl group at 6-H while evidence for aliphatic protons was apparent in a broad signal at 1.60 ppm. It is suspected that this molecule is a fragment from a larger compound due to the long retention time not expected for a haloalkene.

FRACTION 2: Elution time = 41.5 minutes

The molecular ion was evident at m/z 228 and from the NIST Search Library of fragmentation patterns the most likely compound to fit the data was given as the long chain fatty acid, tetradecanoic acid (2). The fragmentation pattern is given below. All the peaks are clear.

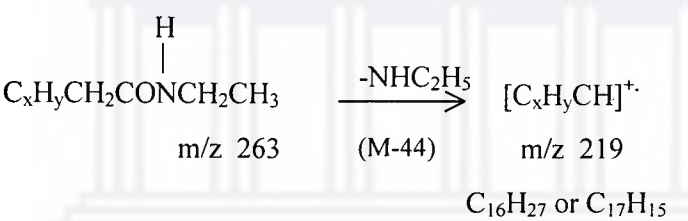


There is also the typical long chain alkyl fragmentation with peaks at m/z values of 29, 43, 57, 71, 85, etc. A second major fragmentation pattern is apparent which arises from an initial loss of a formyl radical HCO followed by hydrogen transfer to give the $[C_{12}H_{24}CH_2OH]^{+\cdot}$ ion at m/z 199 since a new series of M-14 peaks is evident at m/z 199, 185, 171, 157, 143, 129, and 115. The proton nuclear magnetic resonance spectrum shows a D_2O exchangeable peak at 10.12,

typical for a carboxylic acid which together with an ill defined triplet at 0.85, ppm as well as broad peaks between 1.20-1.40 and 1.50-1.80 ppm are in keeping with the fatty acid structure. The expected triplet for the α-protons to the carboxylic acid group at 2.3-2.4 ppm was masked by a strong sharpish signal.

FRACTION 3: Elution time = 43.0 minutes

The fragmentation pattern with the odd molecular ion at m/z 263 did not provide a good match with the NIST library and thus the assignment is even more tentative than fractions 1 and 2. An odd molecular ion and greater elution time could suggest that this is a long chain fatty acid amide. The first fragment peak is at m/z 219, which suggests a loss of an aminoethyl fragment.



No further discovery could be made or deduced without further purification.

DISCUSSION

The increase in the number of bioactive zones from two to five against the bacteria could suggest that a number of antimicrobial compounds are present in the algal extract. A similar trend was observed by Tovar and Ballantine (2000), when one antimicrobial zone on a thin-layer chromatography plate was found to house many antimicrobial compounds when analyzed using HPLC. Plocamium species are known to yield many polyhalogenated monoterpenes (König et al., 1999a). Therefore, when the antimicrobial compounds were isolated, there was an expectation that polyhalogenated monoterpenes would be isolated. Indeed, the chlorinated alkene, E-1-chlorohexene was isolated and identified. In addition, it was shown that another antimicrobial compound, which could only be partially identified, was a long chain fatty acid. The presence of fatty acids in algal extracts has been reported previously (Pohl and Zurheide, 1982). Pohl and Zurheide (1982) noted that the fatty acid content of marine algae are subject to environmental conditions which is a question that could be investigated in the case of P. corallorhiza. It is probable for the antimicrobial compounds to be fatty acids as the antimicrobial activity of a few fatty acids has been reported previously (Ohta et al., 1993; Benyagouls et al., 1996). Benyagouls et al., 1997 reported on two fatty acids with antibiotic activity as metabolites produced by the biocontrol fungus Sporothrix flocculosa. However, none of these fatty acids is used as novel antibiotics. The findings of this investigation must however be verified in a large-scale follow-up study, because the partially characterized compounds should be tested again to make sure that a E-1-chlorohexene and long chain fatty acid are in fact the compounds responsible for the antimicrobial activity of the algal extract. This is especially important since the original two bioactive zones of the fractions of P. corallorhiza resulted in five bioactive zones. A follow-up study must therefore also clarify whether the five bioactive zones were the result of one compound or five different compounds, each significantly antibiotic.

It is evident that another study aimed at completely identifying the antimicrobial compounds of Plocamium corallorhiza is necessary. Future purification should be attempted on using high-performance liquid chromatography to minimize the loss of sample during this step. When the antimicrobial compounds have been isolated and characterized successfully, the extent of the bioactivity of the isolates could then be investigated as well, for example, the inhibition of the antimicrobial of resistant bacterial strains, the cytotoxicity of the compounds and many more. Synthesis of the bioactives could then be pursued since it showed significant activity against the bacteria Staphylococcus aureus and Mycobacterium smegmatis. The synthesized antibiotic compounds should then be subjected to further antibiotic research, including in vivo testing, to establish their usefulness in the development of novel anti-tuberculosis and anti-staphylococcal antibiotics.

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TABLES

Table 4.1. Data of the preparative thin-layer chromatography purification procedure, of an extract of Plocamium corallorhiza.

First preparative tlc data. Sample volume spotted onto plate = 200µl					
Sample no.	Fraction Rf	Mass (g)	Volume (mL)		
202.5	0.3	173.1mg	17.31		
	0.5	113.1mg	11.31		
S. aureus					
No.	Fraction Rf	Front (mm)	Zone distance (mm)	Zone size (mm)	Rf-value
202.5	0.3	172	90	19	0.5
		172	118	26	0.7
	0.5	172	120	12	0.7
M. smegmatis					
No.	Fraction Rf	Front (mm)	Zone distance (mm)	Zone size (mm)	Rf-value
202.5	0.3	157 mm	105	50	0.7
	0.5	159 mm	115	32	0.7
Second preparative tlc data. Sample volume spotted onto plate = 50ul					
S. aureus					
Fraction no.	Fraction Rf	Front (mm)	Zone distance (mm)	Zone size (mm)	Rf-value
202.5/03/05	0.5	170	60	17	0.4
202.5/03/07	0.7	171	70	12	0.4
		171	85	6	0.5
202.5/05/07	0.7	170	95.5	8	0.6
M. smegmatis					
Fraction no.	Fraction Rf	Front (mm)	Zone distance (mm)	Zone size (mm)	Rf-value
202.5/03/05	0.5	160	64	14	0.4
202.5/03/07	0.7	160	70	11	0.4
		160	85	16	0.5
202.5/05/07	0.7	160	70	10	0.4
		160	95	17	0.6
Third preparative tlc data. Sample volume spotted onto plate = 50µl					
S. aureus					
No.	Fraction Rf	Front (mm)	Zone distance (mm)	Zone size (mm)	Rf-value
202.5/03/05/04	0.4	177	42	15	0.2
202.5/03/07/04	0.4	177	49	10	0.3
202.5/03/07/05	0.5	177	64	9	0.4

S. aureus = Staphylococcus aureus M. smegmatis = Mycobacterium smegmatis

Table 4.1 cont.

No.	Fraction Rf	Front (mm)	Zone distance (mm)	Zone size (mm)	Rf-value
202.5/05/07/04	0.4	177	23	2	0.1
		177	68	10	0.4
202.5/05/07/06	0.6	177	22.5	1	0.1
		177	84	2	0.5
M. smegmatis					
No.	Fraction Rf	Front (mm)	Zone distance (mm)	Zone size (mm)	Rf-value
202.5/03/05/04	0.4	182	42	5	0.2
202.5/03/07/04	0.4	182			
202.5/03/07/05	0.5	182	61	14	0.3
202.5/05/07/04	0.4	182	70	18	0.4
202.5/05/07/06	0.6	182	85	12	0.5
Candida albicans					
No.	Fraction Rf	Front (mm)	Zone distance (mm)	Zone size (mm)	Rf-value
202.5/03/05/04	0.4	166	42	10	0.3
202.5/03/07/04	0.4	166	53	10	0.3
202.5/03/07/05	0.5	166	66	10	0.4
202.5/05/07/04	0.4	166	79	13	0.5
202.5/05/07/06	0.6	166	87	12	0.5

S. aureus = Staphylococcus aureus
M. smegmatis = Mycobacterium smegmatis

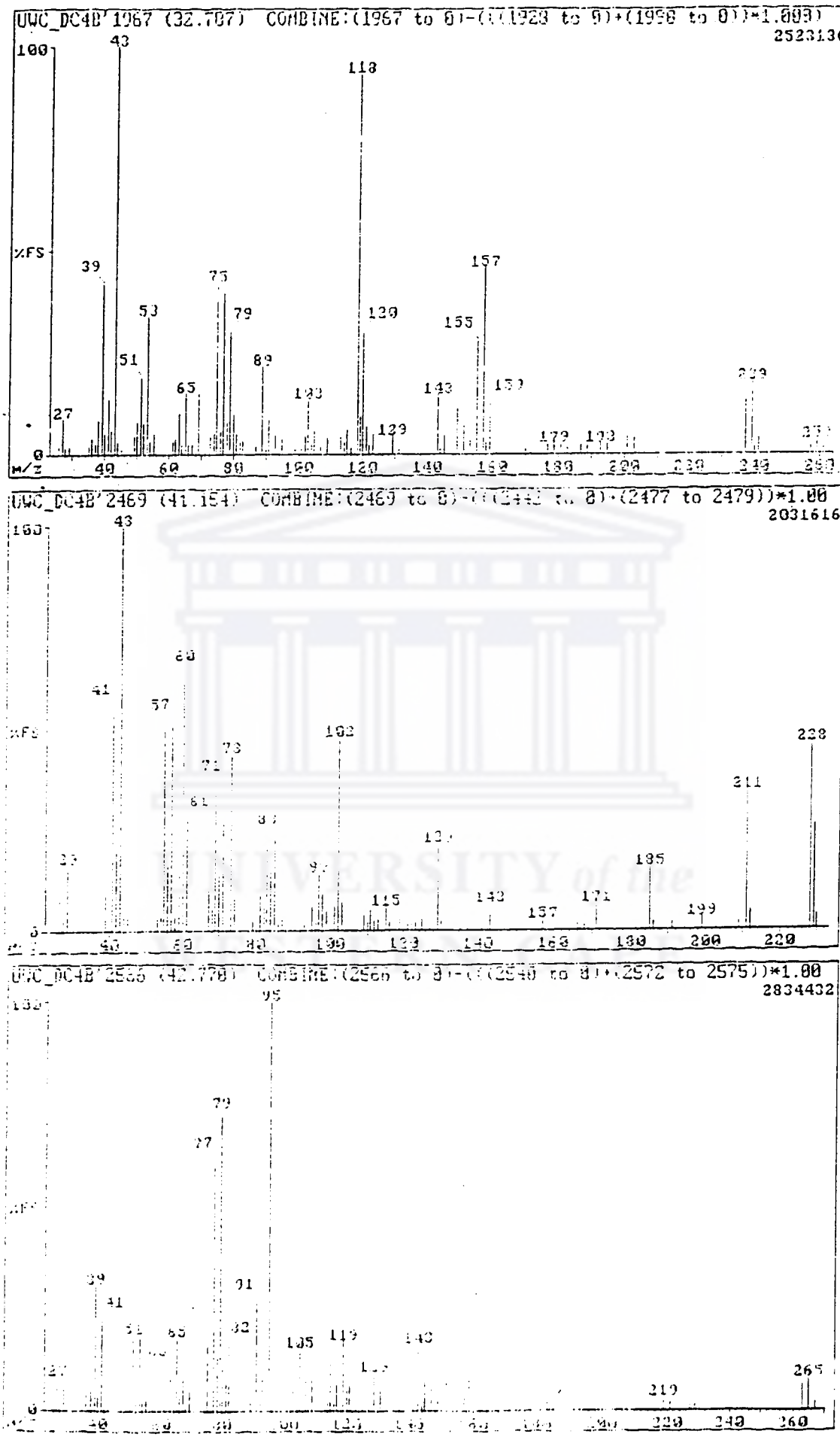
FIGURES

FIGURE CAPTIONS

Figure 1. GC-MS spectrums of the three major peaks of the active fraction according to elution times.

Figure 2. 1H NMR spectrum of the active fraction.

Figure 1



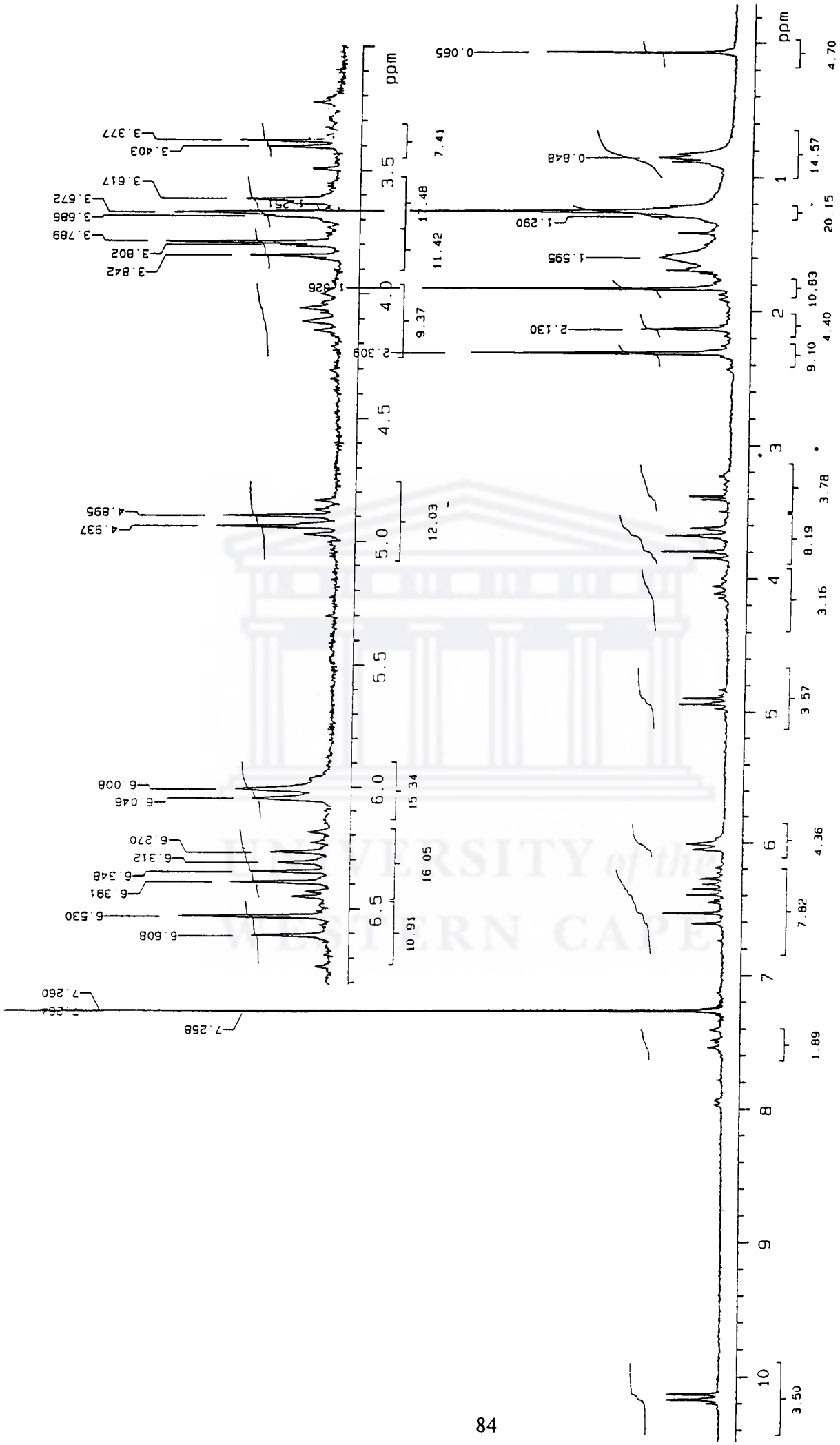


Figure 2

GENERAL DISCUSSION

The results of this investigation showed that South African red algal secondary metabolites should have a strong position in the development of novel antimicrobials, which are much needed in our battle against antibiotic resistant bacteria. Most of the extracts inhibited Staphylococcus aureus, which is responsible for causing infections in hospitalized patients (Yuan and Williams, 1997, Horikawa et al., 1999) and is becoming increasingly resistant to our current supply of antibiotics. In addition, the inhibition of Mycobacterium smegmatis, which is closely related to the pathogen Mycobacterium tuberculosis, cannot be taken lightly; especially in the light of the tuberculosis crisis in which Africa finds itself. Studies investigating the antimicrobial activities of the red algal crude extracts and the fractions on resistant strains of these microorganisms should follow this investigation.

The overall positive results make large-scale investigations aimed at isolating and characterizing the antibiotic compounds from the red algae that have been screened in this investigation imperative. In addition, this must be followed by syntheses and establishing the mechanisms that are used by the algal antibiotics. A novel mechanism is more likely to be effective against bacteria that have developed spontaneous mutations or enzymes that deactivate the existing antibiotics or change the target of the drugs. According to Tovar and Ballentine (2000), red algal extracts produce many different antimicrobial compounds in their natural environment and in laboratory culture. This increases the likelihood of finding antimicrobials in the extracts of the eight algal species that were not fractionated, and similarly, the number of investigations that could result out of the findings of this investigation.

Algae with economic value, for example, Gelidium pristoides and those with possible economic value, for example, Gigartina polycarpa, should be further investigated (Critchley et al., 1998). This should include the characterization of their antimicrobial compounds, synthesis and testing the antibiotic compounds for other biological activities, such as, cytotoxicity, antiviral, and anticoagulant activity. Algae with significant bioactive compounds could then be considered for mariculture particularly for developing algal antibiotics.

Besides establishing that fact that South African red algae are potential sources of antibiotics, this investigation has developed an effective extraction procedure, efficient in capturing the secondary metabolites responsible for the antibiotic activities. Dichloromethane:methanol (2:1) preserved the relevant secondary metabolites as has been suggested by Cronin et al. (1995). The different results reported for the agar disc diffusion assay and bioautography demonstrated the importance of a suitable bioassay that is sensitive to the varying polarities of bioactive compounds and highlighted the effectiveness of bioautography, which was highly recommended by Wright (1998). The agar disc diffusion assay proved to be unsuitable and as recommended by Vanden Berghe and Vlietinck (1991), agar dilution methods should rather be considered.

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APPENDIX 1

JOURNAL OF APPLIED PHYCOLOGY

Instructions for authors

General

Journal of Applied Phycology publishes original articles relevant to practical uses of algae, including fundamental research, development of techniques and commercial applications. The scope includes algal biotechnology and genetic engineering, tissue culture, culture collections, commercially useful micro-algae and their products, marine culture, algal inoculation and soil fertility, pollution and fouling, monitoring, toxicity tests, toxic compounds and antibiotics. Each part of the journal also includes a section of camera-ready copy for brief notes and information on new products, patents and company news.

There is no page charge, provided that the number and size of tables and figures is reasonable. In order to avoid long tables, species lists and other protocols, authors may deposit such material with any official repository and indicate this in the text. The National Technical Information Service of the U.S.A. is an example, but authors may select an institution of their choice, with the only restriction that data must be available for free consultation.

All papers should be written in English. Five categories of contributions are published:

- Research paper: 5--15 printed pages including tables, figures and references to the literature.
- Short research note or comment on recently published papers: 2--4 printed pages including an abstract and key words.
- Review paper: 10--25 printed pages. Papers which provide overviews for a broad readership are especially welcome. Prospective authors should consult the editor before submitting a review. Authors are responsible for obtaining copyright clearance.
- Gene Bulletin: 1--(usually)2 pages. Data on sequencing insufficient for full-length paper. (For examples, see 8: 563--564 and 8: 565--567.)
- Brief notes for the camera-ready appendix to each number of the journal on topics of general interest such as new products, patents and company news.

Editorial policy

Papers should be submitted initially as hard copy (threefold) to the editor or assistant editors. A version on disk is helpful at a later stage and the editor will give advice about this.

Manuscripts will first be checked for language, presentation and style. Substandard manuscripts will be returned to authors without review. Such manuscripts may be resubmitted after all necessary adjustments have been made.

Papers which conform to journal scope and style are sent to outside referees.

Preparing the manuscript

Manuscripts should conform to standard rules of English grammar and style. British or American spelling may be used, but must be consistent throughout the article.

Conciseness improves greatly the readability of a paper. Ambiguous statements, vague expressions and long and pointless series of adjectives should be avoided. Do not include long lists of references to make a single scientific point. Authors are also warned against a sloppy use of scientific expressions. Examples are such terms as physicochemistry where physical and chemical properties (of water) are meant, the non-interchangeable use of variables (or variates, or environmental factors) and parameters etc. Authors are warned against the erroneous use of period and comma in numerical values. Remember that in English ten thousand is written 10,000 while ten, exact to three decimals, is written 10.000. Do not include a comma in four-digit numbers.

Three copies of the manuscript should be submitted. They should be free of handwritten corrections. They should be double spaced throughout, typed on only one side of the paper, with 3 cm left-hand margin. Do NOT adjust vertically the right-hand margin. Tables and illustrations should also be submitted in triplicate, but there is a need for only one high-quality original.

White paper of good quality and standard size (21 x 29 cm) should be used. Word-processed manuscripts should be printed in letter quality or near-letter quality mode.

The contents of the manuscript should be well organized. Page one should show the title of the contribution, name(s) of the author(s), address(es) or affiliation(s) and up to ten key words. An appropriate phone, fax and email number (in parentheses) may follow the corresponding author's address. The abstract should appear on page two. The body of the text should begin on page three. It should be free of footnotes and divided into sections and subsections.

A typical organization might be:

- Introduction
- Materials and methods
- Results
- Discussion
- Acknowledgements
- References
- Tables
- Figure captions.

The Introduction of a research paper should usually end with a clear statement of aims (and not a repeat abstract). Results and Discussion must be separated, unless there is a scientific reason to amalgamate them. Approximate locations for tables and figures should be indicated in the left margin of the text.

Words to be published in italics may be presented in italics.

Tables

Tables should not duplicate figures or *vice versa*. They should be numbered consecutively in Arabic numerals, and bear a descriptive legend on top. Tables should be presented on separate sheets of white paper. Authors should plan tables to fit either one or two columns widths of the journal.

Vertical lines are not to be used and horizontal ones should be kept to a minimum.

Figures

All figures should be numbered in Arabic numerals, either on top or on the back and identified by the author's name. The top of the figure should also be indicated. Figure captions should be grouped on a separate sheet(s) of paper, after the tables.
Do not type captions on the figures themselves.

Photographs should be original, glossy prints, and not cuttings from books or papers.

Colour photographs will only be accepted if the author agrees to pay for the extra cost (about USD 700.00).

Figures will mostly be printed to fit one column width of the journal, so it is important to consider how well the figure will reduce. Particular features to consider are to use thick lines, large symbols and text and sufficient space between text and lines to permit satisfactory reduction. (90% of authors fail to follow these simple guidelines and the figures have to be returned for re-drawing.)

As far as possible, avoid the use of shading. If it is essential for clarity, use types of shading that will reduce satisfactorily.

Do not surround figures with unnecessary boxes or add upper and right-hand lines to graphs, unless these lines are needed to provide additional scales.

While some computer graphics packages are excellent, several widely used ones are inadequate to produce journal-quality figures. If you do not have access to a good software package, you will need to add additional lettering and delete nonsense such as 0.00 for 0 on the axes.

Quantities, units, symbols and their abbreviations

Standard international units (S.I. system) are in general the only ones acceptable, but L is used for liter and other units are used for time besides 's'.

Combined expressions in text, tables and figures should be presented using negative exponents. Examples are given in the table below. (It is recommended that L be used for litre.)

Chemical symbols. Equivalents (or milliequivalents) and moles (or millimoles) are both acceptable, if properly defined. Compounds: more and more abbreviations are being introduced for chemical compounds. Some, like DO, EDTA, HEPES are widely known, but it is advisable to give a full statement of the meaning when first used in the text. Such usage is compulsory for less familiar acronyms. Excessive use of acronyms and other abbreviations is strongly discouraged.

Biological nomenclature. Authors are urged to comply with the rules governing biological nomenclature, as expressed in the International Code of Botanical Nomenclature, the International Code of Nomenclature of Bacteria and the International Code of Zoological Nomenclature.
Authors are urged to check the correct spelling of all scientific names appearing in their texts. When a species name is used for the first time in an article it should be stated in full. The authority for a species used for research purposes should be given in Materials and methods.

Chemical nomenclature. The conventions of the International Union of Pure and Applied Chemistry, and the recommendations of the IUPAC-IUB Combined Commission on Biochemical Nomenclature should be applied.

References to the literature

Citation in the text:
Use the name and year system: Adam (1983) or (Adam, 1983). For two authors, use: Adam and Eve (1982), not Adam & Eve or Adam et Eve. For more than two authors, use et al.: Adam et al. (1982). Initials should be used in the case of personal communications (pers. comm.), which need not be repeated in the reference list. Reference can also be made to a particular page, table or figure in published work, as follows: Brown (1966: p. 182) or Brown (1966: p. 182, Fig. 2).

Citation in the list of references:
All publications cited in the text, and only these, should be listed alphabetically after first authors. If an author published several papers in the same year, they should appear as Adam, 1980a, 1980b, ... This also applies to citations in the text. If an author has published both alone and with (a) co-author(s), the papers which he authored alone should be ranked first, followed by the ones with one co-author alphabetically after the name of the co-authors (not chronologically), followed by the ones with two-co-authors, etc.

Initials of authors should always follow family names:
Casey RP, Lubitz JA, Benoit RJ, Weissman BJ, Chau H (1963) Mass culture of *Chlorella*. Food Technology 17: 85--89.

Prospective authors are urged to give attention to details of punctuation in this example.

Compound names: alphabetization by first word of the family name is preferred: Von Stroheim, Van Straelen should appear under V, De Ridder and Du Plessis under D. Authors should carefully check and conform to capitalization and spacing in such names. For non-European names, where the use of a family name is substituted by other systems (as in Arabic and several Asiatic cultures), authors are requested to indicate clearly on their manuscript which name they wish to use as the homologue to a family name. However, Chinese names should usually be written in full, starting with the family name (1-syllable name), followed by the given name (2-syllable name) written without hyphen and no capital for second syllable, e.g. Liu Chungchu.

Publications should always be cited in their original language, except if in a non-Latin alphabet. For the latter a Latin letter-by-letter transliteration is preferred, but an English translation of the title may be added with the original language indicated between square brackets at the end of the reference.

Papers which are unpublished or in press should be cited only if formally accepted for publication. Unpublished, internal reports are not acceptable in reference lists, unless they are available for general distribution.

Avoid the use of 'Anonymous'. If no author is ascertainable, list reference by name of sponsoring body, or name of editor.

In a continuous series of article citations from a single journal, do not use *ibid.* instead of the journal abbreviation.

Journal citations and abbreviations
If the title of a journal is a single word, *do not* abbreviate. Examples: Behaviour, BioScience, Biotechnology, Experienta, Growth, Hydrobiologia, Photosynthetica. Do not insert a comma between the name of the journal and the volume number.

Journals and book series that appear on a regular basis should be abbreviated (example 1). Several systems are in use but *Journal of Applied Phycology* uses a standard which is based on the 'World List of Scientific Periodicals', published by Butterworths, London, with certain simplifications.

Note that adjectives are only capitalized if they are the first word of a journal's title. Abbreviated words are followed by a period (Journal = J.), contracted words are not (Board = Bd, not Bd.; other examples of contractions are: Doctor = Dr, circa = ca.

Issue numbers should be added only (between brackets) if every issue starts with page one. Volume numbers should be expressed by Arabic numbers in all cases.

Edited symposia, special volumes or issues, etc., published in a periodical.
Author(s), year of publication. Title of paper. In editor(s), title of special volume, periodical (abbreviated, cf. supra), vol: pp. (example two).

Books
Author(s), year. Title. Publisher, city: pp. (example three).

Multi-author books
Author(s) of chapter, year, title of chapter. In editor(s), title of book. Publishers, city: pp.

Examples

- Edwards P (1980) The production of micro-algae on human wastes and their harvest by herbivorous fish. In Shelef G, Soeder CJ (eds), Algae Biomass. Production and Use. Elsevier/North Holland Biomedical Press, Amsterdam: 191--203.
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Final recommendations

Before mailing a manuscript to *Journal of Applied Phycology*, proofread the final version thoroughly and correct any left-over errors. In particular, check the spelling of all scientific terms, Latin names of animals and plants, figure captions and tables. Are all units S.I.? Is all lettering properly composed and will it be readable after reduction? Are all numerical values and mathematical symbols exact? Are locations of figures and tables indicated in the margin? Make certain that every reference is abbreviated correctly, and appears both in the text and reference list.

To those scientists who use English as a foreign language, we strongly recommend that their manuscript be read by a native English-speaking colleague.

Journal Abbreviations

Adv. ecol. Res.
Am. J. Bot.
Analyt. chim. Acta
Analyt. Chem.
Ann. Biol. lac.
Ann. Fac. Sci. Marseille
Ann. Hydrobiol.
Ann. Limnol.
Ann. appl. Biol.
Ann. Bot.
Appl. envir. Microbiol.
Aquat. Bot.
Arch. Biol.
Arch. envir. Contam. Toxicol.
Arch. Hydrobiol.
Arch. Mikrobiol.
Arch. Microbiol.
Aust. J. mar. Freshwat. Res.
Bact. Rev.
Biochem. J.
Biol. Bull.
Biol. Fert. Soils
Biol. Rev.
Biotech. Bull.
Bot. mar.
Bot. Notiser
Bot. Rev.
Bot. Tidsskr.
Br. J. exp. Biol.
Br. phycol. Bull.
Bull. envir. Contam. Toxicol.
Bull. Fish. Res. Bd Can.
Bull. mar. biol. Lab. Woods Hole
Bull. mar. Sci.
Bull. Mus. Hist. nat., Marseilles
Bull. Mus. natn. Hist. nat., Paris
Bull. natn. Sci. Mus., Tokyo

Bull. Soc. r. Bot., Belg.
Cah. Biol. mar.
Cah. O.R.S.T.O.M. ser. Hydrobiol.
Can. J. Bot.
Can. J. Earth Sci.
Can. J. Fish. aquat. Sci.
Can. J. Microbiol.
Comp. Biochem. Physiol.
C. r. Acad. Sci. Paris (= C.r. hebd. Seanc. Acad. Sci., Paris)
C. r. Trav. Lab. Carlsberg
Crit. Rev. Microbiol.
Curr. Sci.
Deep Sea Res.
Econ. Bot.
Ekol. pol.
Envir. Pollut.
Envir. Sci. Technol.
Estuar. coast. mar. Sci.
Evol. Biol.
Exp. Cell Res.
Folia limnol. scand.
Freshwat. Biol.
Gewaess. Abwaess.
Helgolaender wiss. Meeresunters.
Hyacinth Cont. J.
Hydrobiol. Bull.
Indian J. Limnol.
Int. Revue ges. Hydrobiol.
Int. Revue ges. Hydrobiol. Hydrogr.
Jap. J. Limnol.
Jap. J. Parasitol.
Jap. J. Wat. Pollut. Res.
J. agric. Sci., Peking
J. agric. Sci., Tokyo
J. am. Wat. Wks Ass.
J. appl. Ecol.
J. appl. Phycol.
J. Bact.
J. Bot., Paris
J. Cell Biol.
J. cell. comp. Physiol.
J. Cell Sci.
J. Cons. perm. int. Explor., Mer.
J. Ecol.
J. envir. Qual.
J. exp. mar. Biol. Ecol.
J. Fac. Sci. Hokkaido Univ.
J. Fish Res. Bd Can.
J. gen. Microbiol.
J. Great Lakes Res.

J. linn. Soc., Bot.
J. mar. biol. Ass., U.K.
J. mar. Res.
J. Palaeolimnol.
J. Parasitol.
J. Plankton Res.
J. r. microsc. Soc.
J. theor. Biol.
J. Wat. Pollut. Cont. Fed.
Kieler Meeresforsch.
Limnol. Oceanogr.
Mar. Biol.
Microb. Ecol.
Mitt. int. Ver. Limnol.
New Phytol.
New Scientist
Phil. Trans. r. Soc., Lond.
Physiol. Pl.
Physiol. Rev.
Pl. Cell Physiol.
Pol. Arch Hydrobiol.
Riv. Biol.
Schweiz. Z. Hydrol.
Trans. r. Soc. Edinb.
Verh. int. Ver. Limnol.
Wat. Air Soil Pollut.
Wat. Pollut. Contr.
Wat. Res.

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