ANTIMYCOBACTERIAL ACTIVITY OF THE RED ALGAE *GELIDIUM PRISTOIDES, PLOCAMIUM CORALLORHIZA AND POLYSIPHONIA VIRGATA*

by

Denise Saravanakumar

A thesis presented for the degree of

Doctor of Philosophy

University of Cape Town

March 2006

Approved by _____
Chairperson of Supervisory Committee

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Program Authorized
to Offer Degree _____

Date _____

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Denise Saravanakumar

DEPARTMENT OF MEDICINE, DIVISION OF PHARMACOLOGY

SUPERVISOR: Prof. P. Folb

CO-SUPERVISORS: Prof. P. Smith and Dr. W.E. Campbell

DEDICATION

I dedicate this project to my Lord and Saviour, Jesus Christ, my husband and best friend, P. Saravanakumar, and my parents. You enable me to live out my purpose and reach my divine destiny.

DECLARATION

I declare that ANTIMYCOBACTERIAL ACTIVITY OF THE RED ALGAE *GELIDIUM PRISTOIDES, PLOCAMIUM CORALLORHIZA AND POLYSIPHONIA VIRGATA* 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. All the experimental work reported in this thesis was conducted by the candidate with the exception some of the *in vitro* activity testing with the pathogen *Mycobacterium tuberculosis* and this is duly acknowledged in the thesis.

DENISE SARAVANAKUMAR MARCH 2006

Signed by candidate

SIGNED:...

KEYWORDS

ANTIMYCOBACTERIAL ACTIVITY OF THE RED ALGAE *GELIDIUM PRISTOIDES, PLOCAMIUM CORALLORHIZA AND POLYSIPHONIA VIRGATA*

Denise Saravanakumar

Antimycobacterial

Marine algae

Seaweeds

Algal extracts

Secondary metabolites

Marine natural products

Biological activity

Tuberculosis

ACKNOWLEDGEMENTS

My sincere gratitude extends to the following individuals and institutions:

Prof. P. Folb, Prof. P. Smith and Dr. W.E. Campbell who gave me the opportunity to do this project and whose supervision has enabled me to bring it to completion.

The National Research Foundation for financial assistance without which this project would not have been possible.

Ms. Vanessa January and Ms. Monica Chojnacki from the Microbiology Laboratory of the National Health Laboratory Services, which is based at Groote Schuur Hospital, for undertaking the anti-tuberculosis assessment of seaweed extracts in Chapter 4 and 5 of this thesis.

Ms. Carmen Lategan from the Division of Pharmacology in the Department of Medicine, UCT, for undertaking the cytotoxicity and anti-plasmodial activity determinations.

The Division of Pharmacology in the Department of Medicine.

Ms. Jean Mckenzie for the NMR spectra, Mr. Bertie Barnard and Dr. Stephan Louw for the LC-MS and GC-MS analysis from the Central Analytical Facility, University of Stellenbosch.

Ms. Tracy Seaman for her kind assistance and training with the direct bioautography assay and the Bactec-460 radiometric method that were used in the re-investigation of one of the seaweeds.

Mr. Mark Rothman and colleagues who collected and verified the algae.

Dr. Denzil Beukes from the Faculty of Pharmacy at Rhodes University for hosting a 2-week visit in which partial purification of a few fractions was achieved under his supervision.

Mr. Christo Whittle from the Department of Oceanography at the University of Cape Town for the chlorophyll-a concentration images obtained by using an ocean colour sensor.

My family, for their support and love.

ABSTRACT

In 1993, the World Health Organisation declared tuberculosis a global health emergency. Currently, efforts are underway to improve the way the disease is managed and to find more effective treatments that would combat the problem of long treatment periods, toxicity, drug-resistance and HIV-coinfection. In the process, natural product chemistry continues to play an important role in the search for new compounds to treat tuberculosis. Terrestrial plants have been investigated for antimycobacterial activity, while marine plants are yet to receive as much attention. In this project, three South African marine plants were drawn into the search for novel anti-tuberculosis compounds. One of the seaweeds is already part of the local seaweed industry, namely *Gelidium pristoides*, while *Plocamium corallorhiza* and *Polysiphonia virgata* have economic potential. These three red algae were extracted extensively and fractionated using preparative layer chromatography and preparative centrifugally accelerated radial thin-layer chromatography (Chromatotron). The crude extracts of the algae showed no inhibitory activity to growth of the causative agent of human tuberculosis, *Mycobacterium tuberculosis*. However, when the purified fractions were tested against *M. tuberculosis* in the BACTEC-460 radiometric method at a concentration of 125 µg/mL, fractions 322, 323 and 333 of *P. virgata* showed 100% inhibition, while two fractions of *G. pristoides* showed 91.7% and 79.2% inhibition, respectively. Two fractions of *P. corallorhiza* demonstrated 41.2% and 73.5% inhibition. The bioactive fractions of *P. virgata* were further purified and resulted in the isolation of a known compound namely, 2-methoxyethyl-2-methacrylate (MEMA). When MEMA was tested by radiometric assay against *M. tuberculosis,* it showed anti-tuberculosis activity at a MIC-value of 100 µg/mL and no cytotoxicity against Chinese hamster ovarian cells. However, in a re-investigation into the bioactive compounds of *P. virgata* it was established that MEMA was not the major bioactive compound. Long chain fatty acids were responsible for the antimycobacterial activity of the algal extract particularly oleic acid, linoleic acid, dodecanoic acid, and myristic acid. Oleic acid inhibited the growth of *M. tuberculosis* at and MIC-value of 25 µg/mL, while dodecanoic acid, myristic acid and linoleic acid all had MIC-values of 50 µg/mL. Stearic acid and palmitic acid was also isolated from the seaweed, but only moderate inhibition of *M. tuberculosis* was observed for at MIC-values of 50 µg/mL. Oleic acid showed moderate inhibition at 50 µg/mL against the multi-drug resistant isolate of *M. tuberculosis*, while myristic acid and dodecanoic acid showed significant inhibition against the same at 50 µg/mL and moderate inhibition at 25 µg/mL. Linoleic acid also inhibited the growth of the multi-drug resistant strain at 50 µg/mL. Oleic acid showed the most inhibition of the growth of *M. smegmatis* in direct bioautography with an MIC-value of 0.8 µg/mL, while linoleic acid and dodecanoic acid had MIC-values of 1.56 µg/mL and 3.125

µg/mL., respectively. Stearic acid, palmitic acid, and myristic acid did not inhibit the growth of *M. smegmatis*.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

INTRODUCTION

1.1 Tuberculosis

Tuberculosis is defined as "an infectious disease caused by the bacillus *Mycobacterium tuberculosis* and is characterized by the formation of nodular lesions (tubercles) in the tissues" (Concise Medical Dictionary[1]). Tuberculosis can involve many organs (Gregory and Tolman, 2000). In cattle, it is caused by *M. bovis*. *M. leprae* is the mycobacterial pathogen responsible for leprosy. Mycobacteria are either tuberculous or nontuberculous (atypical) (Petri, 2001). In addition, tuberculous mycobacteria are regarded as slowly growing mycobacteria (SGM) and nontuberculous as rapidly growing mycobacteria (RGM), based on their growth rate on solid medium (Howard and Byrd, 2000). Those that form visible colonies in less than 7 days are SGM while mycobacteria that do the same in more than seven days are RGM (Howard and Byrd, 2000). The major SGM are *M. tuberculosis*, *M. leprae* and *M. avium* complex (Howard and Byrd, 2000). *M. avium* complex causes infections in patients with AIDS (Petri, 2001). RGM involved in human disease include *M. abscessus* and *M. chelonae*, which are associated with skin and soft tissue disease (Howard and Byrd, 2000) and *M. fortuitum*, which are normally saprophytes, but can cause chronic lung disease and infections in skin and soft tissues (Petri, 2001). *M. kansasii* causes a disease that is similar to tuberculosis, but milder (Petri, 2001). Of all the various types of tuberculosis, pulmonary tuberculosis (TB) or tuberculosis of the lungs is the most common and will be the focus of this investigation. Other forms of tuberculosis caused by *M. tuberculosis* include lymphatic tuberculosis, pleural tuberculosis, tuberculous meningitis, genitourinary tuberculosis, etc. (Frieden *et al.*, 2003).

In pulmonary tuberculosis, *M. tuberculosis* settles in the lungs after inhalation and develops a primary tubercle from where it disseminates to the lymph nodes. Other mycobacteria that infect human lungs are the atypical or opportunistic mycobacteria that exploit a weakened immune system or prior lung damage. These include *M. kansasii*, *M. xenopi*, *M. malmoense*, *M. avium*, and *M. intracellulare.* Their infections are similar to pulmonary tuberculosis, but much more difficult to treat because of resistance to the primary tuberculosis drugs (Concise Medical Dictionary[2]).

1.2 Global perspective

Pulmonary tuberculosis is one of the most deadly infections of our time and in 1993, the World Health Organisation (WHO) declared it a global emergency (Gandy and Zumla, 2002). Annually, the WHO compiles a global TB control report after obtaining data of TB case notifications and/or treatment outcomes from governments of up to 201 countries. According to the 2003 WHO Report, 72% of TB-cases were reported under directly observed therapy short-course (DOTS) programmes in 2001 and 77% in 2002 (WHO Report, 2003). According to the 2004 WHO report on Global Tuberculosis Control, an estimated 8.8 million new cases of tuberculosis (TB) were reported in 2002, of which 3.9 million were confirmed by sputum smear microscopy or culture where resources were available (WHO Report, 2004). However, it is important to note that of the 2 billion individuals infected with *M. tuberculosis* only 10% developed active tuberculosis (Kaufmann, 2000). Approximately 3 million people die of tuberculosis each year while a third of the global population has dormant or latent tuberculosis infection (Gandy and Zumla, 2002). Furthermore, the global incidence rate of TB (per capita) has grown at approximately 1.1% per year (Figure 1.1) and the number of tuberculosis cases at 2.4% per year. The case notifications were faster in African countries and in Eastern Europe – mainly in the former Soviet Union (WHO Report, 2004). Sub-Saharan Africa countries are reported to have 290 infected individuals per 100 000 of the population, while the highly populated countries of Asia, such as India, China, Indonesia, Bangladesh and Pakistan have the largest number of tuberculosis cases, bearing more than half the global tuberculosis burden (Frieden *et al.*, 2003). Most of the disease occurs in people aged 15 to 49 years (Frieden *et al.*, 2003) and it is more common in the poor, and in people living in nursing homes, prisons, and homeless in inner city centers. Pulmonary tuberculosis is also often diagnosed in alcoholics, drug users, and patients recovering from gastrectomy, renal transplant, renal failure, or other weakening conditions (Gregory and Tolman, 2000). Another contributing factor to the increase in TB-notifications is the mass movements of people because of war or social disruptions resulting in crowding in inadequate housing and poor sanitation (Gandy and Zumla, 2002). Overall, the increase in tuberculosis incidence is the result of economic decline and deterioration in the management of tuberculosis and other health services (Frieden *et al.*, 2003). Furthermore, the spread of the HIV/AIDS epidemic and multi-drug resistant tuberculosis increasingly makes it more difficult to treat TB-infected individuals (Corbett *et al.*, 2003). With multi-drug resistant tuberculosis the treatment is individualized based on susceptibility testing (Kremer and Besra, 2002) and the second-line treatments required are more costly and in some cases more toxic (Fujiwara *et al.*, 2000).

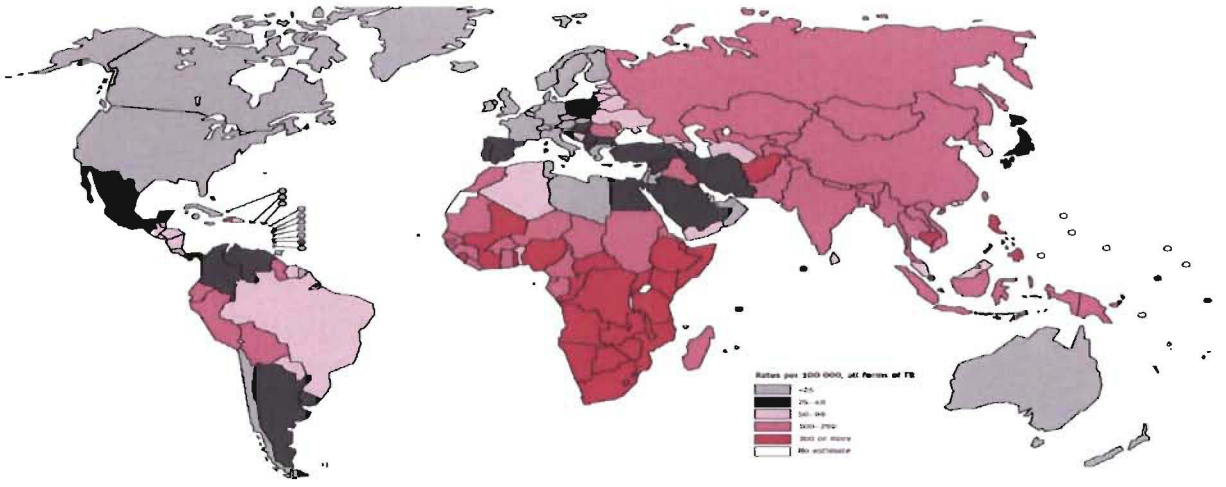


Figure 1.1. Estimated TB incidence rates for 2002 (WHO Report, 2004)

1.3 Tuberculosis in South Africa

South Africa is among the 22 countries that bear 80% of the global tuberculosis case burden (Ravigloine, 2002). Bradshaw *et al.* (2003) investigated the causes of mortality in South Africa in the year 2000 and estimated that tuberculosis was the third highest cause of death after HIV/AIDS and homicide. Another study published in the same year indicated that South Africa had the largest number of adults co-infected with HIV and tuberculosis worldwide, amounting to 2 million individuals (Corbett *et al.*, 2003). The 2004 WHO estimates showed that the 44 759 187 population showed a TB case incidence of 558/100 000 of the population, with 60% of adults cases being co-infected with HIV (WHO Report, 2004). A plan for TB-control was introduced in 2001 and is set to run to 2005; one of the first steps involves the appointment of TB-coordinators for all the provinces with the expectation that each province would function within a provincial TB-budget (WHO Report, 2003). It is hoped that greater political commitment to the eradication of TB would ensure more effective TB-control.

1.4 Modern Medicine

1.4.1 Traditional Medicine in TB-treatment

Despite the tremendous advances in modern medicine, many people continue to rely on traditional remedies as their supply of medication (WHO, 2003). It is estimated that approximately 60% of the world population use traditional medicines to treat their illnesses (Harvey, 2000). In China, 30 – 50% of medicines are comprised of traditional herbal preparations, while in Europe, North America, and other industrialized countries 50% of the population used complementary or alternative medicine at least once. The global market for herbal medicines is estimated at about US$ 60 billion (WHO, 2003). These

traditional remedies are mainly of botanical origin. Historically, plants have played an important role in the medicines of the great civilisations of the ancient Chinese, Indians and North Africans (Phillipson, 2001). Today, some people in Britain make use of the Asian plant *Centella asiatica* and in the West a tonic is prepared from the bark of the Chinese herb Lycium chinense Miller (Evans, 1996a) to treat tuberculosis.

Since the nineteenth century, scientists have isolated the active compounds of medicinal plants; for example, the isolation of quinine from Cinchona bark (Phillipson, 2001). Today, the scientific investigations of plants used in traditional medicines continue vigorously as is reflected in the many publications about medicinal plants; for example, Trease and Evans' Pharmacognosy (Evans, 1996a), various pharmacopoeias (Evans, 1996a), and the many reviews in journals, such as, Natural Product Reports, Phytotherapy Research and Phytochemistry. The battle against tuberculosis has not been excluded in the search for new bioactive compounds as is reflected by the number of reviews on antimycobacterial compounds published recently, such as, those by Newton *et al.* (2000), Cantrell *et al.* (2001), Copp (2003), and Okunade *et al.* (2004).

Newton *et al.* (2002) investigated the antimycobacterial activity of 43 plant species known to be used in the treatment of tuberculosis and leprosy or various symptoms of these diseases. The plants originated from countries worldwide and were screened against *Mycobacterium smegmatis* and *M. aurum*. In another investigation, Lall and Meyer (1999) screened 20 South African plants used in traditional medicine to treat TB or its symptoms and found more than 50% of the plants selected to have significant antimycobacterial activity. Seidel and Taylor (2004) screened two Pelargonium species for antimycobacterial activity because the extracts of its roots were given to Charles Stevens by a South African healer and he was cured of tuberculosis. The results served to justify further investigation of the plant extracts against pathogenic mycobacteria. These are a few examples of the importance of traditional medicines in the search for new anti-tuberculosis drugs.

1.4.2 Chemotherapy

The year 1944 marked the beginning of chemotherapy of tuberculosis with the introduction of streptomycin (Fujiwara *et al.*, 2000). Since then, many other anti-tuberculosis drugs have been introduced and are still in use. There has been much investigation into the efficacy and correct dose of these drugs to optimise their effectiveness and to combat drug resistance. The drugs currently used in the treatment of tuberculosis are divided into first- and second-line agents. The first-line agents are isoniazid, rifampicin, ethambutol,

streptomycin and pyrazinamide (Figure 1.2). Patients infected with drug-sensitive *M. tuberculosis* are treated successfully with the first-line therapy for a period of 6 months. During the first 2 months, first-line drugs are given followed by a 4 months long treatment of isoniazid and rifampicin only (Petri, 2001). Alternatively, a combination of rifampicin and isoniazid over a period of 9 months also proved effective. However, where there is resistance to isoniazid, therapy begins with the 4-drug combination with either streptomycin or ethambutol. Second-line drugs come into play if the patient has developed resistance to the first-line treatment or becomes drug intolerant (Fujiwara *et al.*, 2000). They are less potent and more toxic than first-line treatment and include cycloserine, ethionamide, para-aminosalicylic acid, capreomycin, kanamycin/amikacin, and the fluoroquinolones ciprofloxacin, ofloxacin, levofloxacin, and sparfloxacin (Fujiwara *et al.*, 2000). In the treatment of tuberculosis, anti-tuberculosis drugs are just half of the battle won. The rest of victory depends on adherence, which may be difficult as many drugs have to be taken over a long period and the cost of treatment can be unaffordable (Fujiwara *et al.*, 2000). In 1999, the global impact of tuberculosis complicated by multi-drug resistance and HIV-infection encouraged the WHO to adopt a new strategy referred to as directly observed treatment short course (DOTS) (Fujiwara *et al.*, 2000). This strategy was not accepted by all countries, but the number of participating countries has increased gradually since. By 2002, 180 of the 210 countries worldwide had implemented the DOTS strategy (WHO Report, 2004). According to the same report, 69% of the world's population live in countries or parts of countries covered by DOTS. The success of DOTS relies on the commitment of governments around the world to work towards the eradication of tuberculosis. The strategy includes the diagnosis of tuberculosis by smear microscopy or culture growths where possible, a standardized course of chemotherapy that is taken under observation for at least 2 months, a supply of safe and high-quality drugs, and regular, effective reporting of treatment outcomes. Through the DOTS programmes, 3 million new TB cases have been notified, of which 1.4 million were smear-positive. WHO aims to achieve 70% case detection of TB cases worldwide by 2005. Currently, treatment success under DOTS for the years 2000 and 2001 has been 82%.

When patients cannot be observed directly, one of the keys to the success of tuberculosis chemotherapy lies in combination of antimycobacterial drugs, specifically fixed-dose combinations to prevent monotherapy that rapidly leads to development of drug resistance. Combinations of isoniazid and rifampicin (Rifamate) and isoniazid, rifampicin and pyrazinamide (Rifater) are used in the United States of America (Fujiwara *et al.*, 2000), while combination tablets of isoniazid and thioacetazone or isoniazid and ethambutol are available elsewhere.

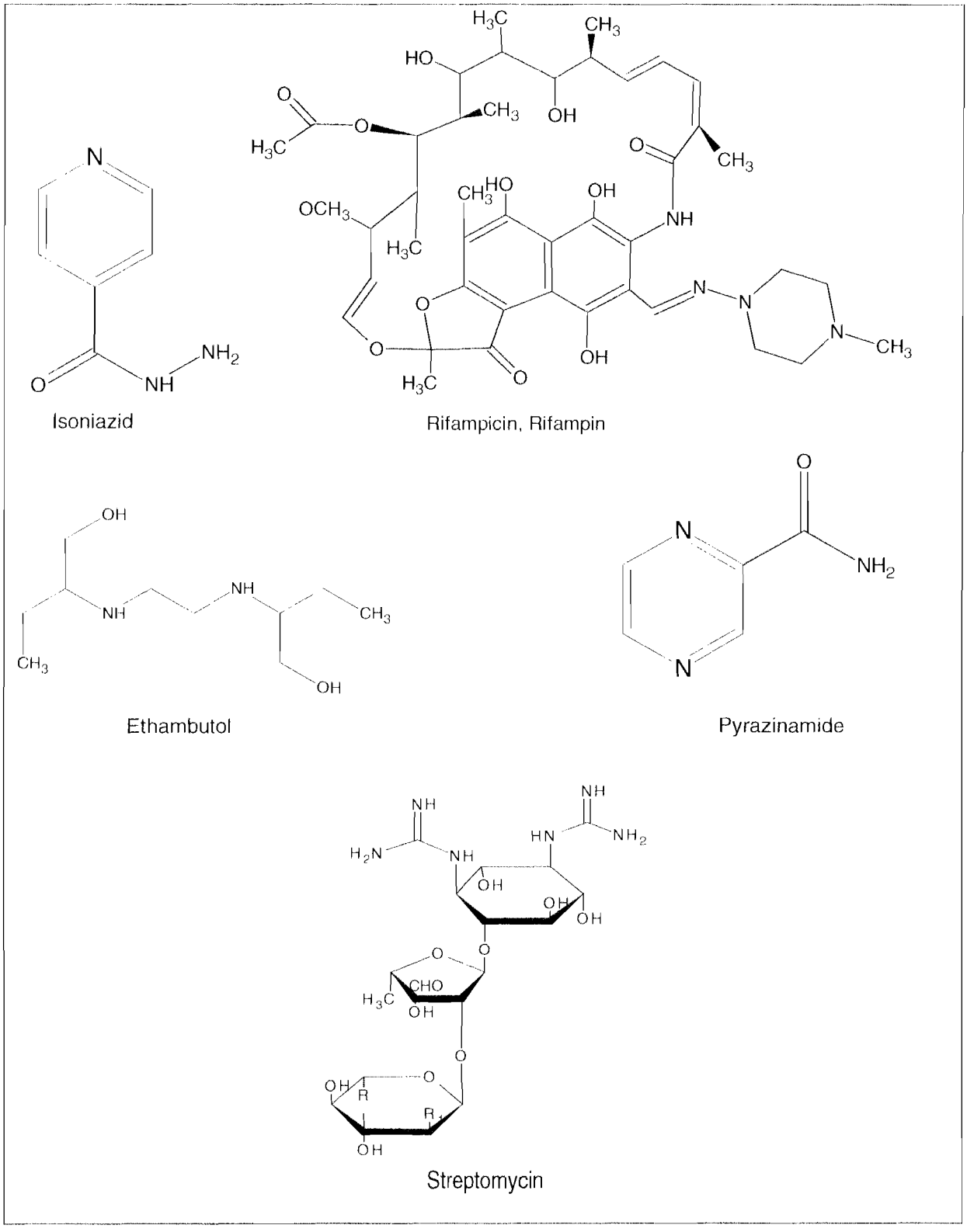


Figure 1.2. First line anti-tuberculosis drugs: isoniazid, rifampicin, ethambutol, pyrazinamide, and streptomycin were reproduced from Petri (2001) and Chambers (2001).

1.4.2.1 Multi-drug resistant tuberculosis (MDR-TB)

A strain of *M. tuberculosis* is defined as being resistant if it shows resistance to isoniazid and rifampicin (Mukherjee *et al.*, 2004). Fujiwara *et al.* (2000) equate multi-drug resistance with poorly managed tuberculosis control programmes in which either patients do not adhere to treatment or health-care providers do not prescribe correctly. Before initiating treatment for tuberculosis caused by drug-resistant *M. tuberculosis*, it is important to establish to which drugs the strain is resistant and sensitive. Isoniazid-resistant strains should be treated with a fixed dose combination of rifampicin, ethambutol, and pyrazinamide over a period of six months. Should the strain be resistant to more than one drug, the regimen becomes more complicated as it might not be possible to tell which drug would be more efficient. Where second-line drugs are resorted to, the side effects may make it impossible for the patient to continue treatment for the recommended period. Infections caused by strains resistant to both rifampicin and isoniazid are the most difficult to treat (Fujiwara *et al.*, 2000).

1.4.2.2 HIV/AIDS co-infection

Worldwide, it is estimated that 11% of new adult tuberculosis cases reported in 2000 were co-infected with HIV. This included 38% of new cases in sub-Saharan Africa, 14% in developed countries, and 1% in the Western Pacific (Frieden *et al.*, 2003). Of the 2 million people that died of tuberculosis in 2000, approximately 13% were co-infected with HIV (Frieden *et al.*, 2003). In Botswana, South Africa, Zambia and Zimbabwe approximately 60% of tuberculosis patients are reported also infected with HIV (Frieden *et al.*, 2003). Although pulmonary tuberculosis is the most common form of *M. tuberculosis* infection in HIV-positive patients, 20% of cases with HIV/AIDS co-infection have extrapulmonary tuberculosis in HIV-seronegative people and it is very common in those that are HIV-seropositive (Frieden *et al.*, 2003). In the treatment of HIV-infection, protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) form an integral part of the multi-drug regimens in AIDS patients (Fujiwara *et al.*, 2000). However, these medications have serious consequences for the treatment of tuberculosis. They interact with the rifamycins, the most important chemotherapeutic drug in the treatment of tuberculosis, namely rifampicin and rifabutin, but more so with rifampicin. Both PIs and rifamycins are metabolized by the hepatic cytochrome P450 system – rifamycins induce the cytochrome P450 system resulting in increased metabolism and decreased levels of PIs, while PIs inhibit the cytochrome P450 system resulting in toxic level of rifamycins (Fujiwara *et al.*, 2000). Thus, NNRTIs nevirapine and delavirdine should not be used with rifampicin, but nevirapine and rifabutin can be safely used together.

1.4.3 Vaccines

Currently, the Bacillus Calmette-Guérin (BCG) vaccine is the only vaccine routinely used against tuberculosis and BCG has been given to more people than any other vaccine (Kaufmann, 2000). Its side effects are minimal and it is believed to prevent miliary and meningeal tuberculosis in children, but it is not effective prevention against pulmonary tuberculosis (Kaufmann, 2000; Kremer and Besra, 2002).

1.5 Demand for new anti-TB drugs

Current tuberculosis drugs must be taken over a long period of time and in combinations to avoid the development of drug resistance (Kaufmann, 2000). The adverse reactions experienced by many patients lead to poor adherence and treatment failure (O'Brien, 2001). The drugs used to treat multi-drug resistant tuberculosis are even more expensive and toxic (O'Brien, 2001). Therefore, new TB-drug treatments are needed that ideally would shorten the treatment period, be effective against MDR-TB, elimnate latent TB infection (O'Brien, 2001), and be affordable to all.

1.6 Initiatives in the search for new drugs: TB-drug development

The Global Alliance for TB Drug Development (GATB), based in New York, Brussels and Cape Town together with their partners, stakeholders and allies worldwide are spearheading the development of new and improved drugs for the treatment of tuberculosis (http://www.tballiance.org). Within its broad network the Alliance has compounds such as nitroimidazole analogs, carboxylates, quinolones, macrolides, Inh A inhibitors, isocitrate Lyase inhibitors and pleuromutilins in the discovery phase of its portfolio. Recently it teamed up with Bayer, a German pharmaceutical group to submit a patented Bayer antibiotic, moxifloxacin, to global clinical trials in the treatment of tuberculosis (http://www.tballiance.org). Another initiative involves the synthetic compound nitroimidazopyran, PA-824, which is also in Phase 1 clinical trials (http://www.tballiance.org). A comprehensive approach to developing new drugs for the treatment of tuberculosis is ideal although the initial steps in the discovery process are taken in one of the many disciplines from which new drugs could be obtained, such as natural product chemistry and combinatorial chemistry.

1.6.1 Natural product chemistry

Natural products continue to play a vital role in drug discovery. On considering, that only 10% of the world's biodiversity has been investigated so far it is clear that humankind has only just begun to tap these remarkable resources (Harvey, 2000). Many organisms remain as new galaxies of natural products to be explored for example, microorganisms of which approximately 6000 bacterial species have been identified

(Harvey 2000). Recent drugs that were added to the medicinal armamentarium from natural products are the antimalarial drug artemisinin, isolated from the antimalarial herb *Artemisia annua* (Harvey, 2000; Phillipson, 2001), the anticancer drugs taxol, obtained from the bark of the Western Pacific Yew, *Taxus brevifolia*, (Phillipson, 2001) and camptothecin isolated from the tree *Camptotheca acuminata* (Wall *et al.*, 1966; Wani *et al.*, 1971; Harvey, 2000; Oberlies and Kroll, 2004).

Small-scale screening programmes are the most common form of natural product research that are being done at academic institutions, reflected in the many publications produced yearly in the search for new antimycobacterial compounds. It continues to be the main thrust in natural product chemistry and a focus for the training of students. Natural products screens test the extracts or isolated compounds in a single one bioassay against one organism or in various bioassays against many different organisms. When extracts were tested in more than one assay, unexpected bioactivities have been detected. In an investigation on seven plants species from The Republic of Congo, the plants were selected because they were found in a malaria endemic area and were used in the traditional medicine of the local people in the treatment of malaria. The extracts were tested for antimalarial activity, as well as, against *M. tuberculosis*, *Trypanosoma cruzi*, *Bacillus megaterium*, the fungus *Microbotryum violaceum*, etc (Tshibangu *et al.*, 2002). Five species showed considerable antiplasmodial activity and one species had noteworthy antimycobacterial and antitrypanosomal activity. Large-scale screening programmes have emerged in recent years, particularly after the 1992 Convention of Biodiversity (Harvey, 2000) with collaborations between research institutions, academia, the pharmaceutical industry, and indigenous communities. An example of such a programme is one where Peruvian medicinal plants were investigated as sources of new pharmaceuticals (Lewis *et al.*, 1999). A collection of 492 plants was screened against *M. tuberculosis* and antimycobacterial active extracts were identified. In such large-scale programmes, many plant and animals species are investigated for a range of pharmacological activities. In the Peruvian study, indigenous peoples of the study area were actively involved and stood to gain financially should any drug be developed from the plants investigated. The networks established in this manner greatly advance the number of species that are screened and increase the number of bioassays for which an extract can be tested.

1.6.2 Chemical modifications of known drugs, genomics and combinatorial chemistry

Currently, efforts are underway to employ chemical modification to improve the properties of fluoroquinolones such as, norfloxacin and macrolides such as clarithromycin and rifamycin derivatives such as rifabutin and rifapentine (Tomioka, 2000). These modified compounds are showing great potential with characteristics such as good absorption, favourable pharmacokinetics, and host macrophage

penetration to mention a few, but further research is required before they can be regarded as suitable for clinical trials (Tornioka, 2000).

With the sequencing of the genome of *M. tuberculosis* (Cole *et al.*, 1998) it is hoped that new drug targets will be easily established and new effective anti-tuberculosis drugs developed (Barry *et al.*, 2000). Together with combinatorial chemistry, in which ideal and diverse drug structures might be optimised and compiled in a library from which a drug candidate could be selected, it has transformed drug discovery (Barry *et al.*, 2000).

1.7 Marine natural products

Since the introduction of the self-contained underwater breathing apparatus (SCUBA) a few decades ago, marine organisms have increasingly become the objects of investigation of natural product chemists, intrigued by the diversity of their chemical composition. In the last decade, many investigations on marine organisms have been conducted as are reflected in the number of reviews available in the scientific arena. Faulkner has published yearly reviews on marine natural products, with many reports of isolated compounds and their biological activities (Faulkner, 2000). Fenical has been involved in many investigations in which marine animals and plants have been screened for novel compounds (Fenical, 1974; Howard *et al.*, 1977; Spyere *et al.*, 2003) and bioactive agents (Lindquist *et al.*, 1988; Lee *et al.*, 1997). He predicted that, "marine drug discovery has an exceedingly bright future" (Fenical, 1997). In 2002, the review by Mayer and Hamann (2002) on marine pharmacology demonstrated the huge contribution marine organisms are making in the search for new therapeutic agents.

The search for drugs from marine natural products has produced a significant number of drug candidates over the last few years (Proksch *et al.*, 2002; Haefner, 2003). The venoms of marine cone snails have been found to contain conotoxins that inhibit various receptors. A particular conotoxin that has been synthesized and approved in the United States by the Food and Drug Administration is ω-conotoxin MVIIA of which the synthetic form is called Ziconotide. It is a neuron-specific, N-type calcium-channel blocker (Haefner, 2003). Another ω-conotoxin is in clinical development at AMRAD, one of four companies involved in conotoxin commercialisation (Haefner, 2003). The investigations of the nemertine worm *Amphiporus lactifloreus* have yielded a compound, 3-(2,4-dimethoxybenzylidene)-anabaseine, that is being investigated for efficacy in the treatment of Alzheimer's disease and schizophrenia (Haefner, 2003). Screens of bryozoans have resulted in discovery of a potent anti-tumour compound, bryostatin (Haefner, 2003). Since then, other bryostatins have also been isolated and bryozoans harvested commercially as there are difficulties in synthesising the highly potent anti-cancer agent (Haefner, 2003).

A closer look at the bioactivity of marine natural products resulted in the publication of many papers recording bioactivities, such as anti-angiogenic (Koyanagi *et al.*, 2003), anti-tumour (Hiroishi *et al.*, 2001; Zhou *et al.*, 2004), anticoagulant (Chevolot *et al.*, 1999), immunomodulating (Shan *et al.*, 1999), antioxidant (Ahn *et al.*, 2004; Zhang *et al.*, 2004), antiviral (Boyd *et al.*, 1997; Carlucci *et al.*, 1999; Huheihel *et al.*, 2002; Ahn *et al.*, 2002), anti-inflammatory (Okai and Higashi-Okai, 1997; Jiang *et al.*, 1999) antimicrobial (Donia and Hamann, 2003; Haefner, 2003), hemaglutination (Kakita *et al.*, 1999), and many more. The review by Donia and Hamann (2003) gave renewed appreciation for the diversity of marine organisms such as sponges, gorgonian, soft corals, bryozoans and other invertebrates, marine microorganisms and algae, and their isolates, as they highlighted bioactivities such as antitumour, antimycobacterial, antihelmintic, antifungal, and antiprotozoal. Marine cyanobacteria are mentioned separately as these blue-green algae have great potential for future drug discovery. Unique secondary metabolites with diverse pharmacological activities have been isolated from these marine organisms (Burja *et al.*, 2001). From South Africa, Michael Davies-Coleman and some other researchers have investigated marine animals for novel compounds, e.g. the sea hare *Aplysia dactylomela* (Copley *et al.*, 2002), a nudibranch (McPhail *et al.*, 2001) and gastropod (Brecknell *et al.*, 2000), to mention a few.

Marine plants have not been excluded from marine natural product chemistry as they share the same environment with marine animals (Table 1.1). Certain algae have received much more attention than others either because they possess interesting novel compounds or they have economic value. Of the many marine algae investigated, the red alga *Laurencia* species has been the object of many screens resulting in numerous reports of their secondary metabolites (Brito *et al.*, 2002; De Carvalho *et al.*, 2003; Iliopoulou *et al.*, 2003) and bioactive compounds, such as halogenated metabolites with antibacterial activity (Vairappan *et al.*, 2001; Vairappan, 2003). Other reports of halogenated marine natural products have been published recently, e.g. antibacterial bromophenols from the red alga *Rhodomela confervoides* (Xu *et al.*, 2003). Antiviral activity of Korean seaweeds (Hudson *et al.*, 1999, Ahn *et al.*, 2002) has been clearly demonstrated in recent years (Huheihel *et al.*, 2002). Of all the algae, contributing to marine natural product chemistry it appears that red algae have made the largest contribution and many of their bioactivities have been reported (Table 1.2).

Table 1.1 Seaweeds and the compounds isolated from them.

SEAWEEDS	ISOLATED COMPOUNDS	REFERENCES
BROWN SEAWEEDS		
Ascophyllum nodosum	Fucans	Chevolot *et al.*, 1999
Ascophyllum nodosum	Fucans (sulphated polysaccharides)	Ellouali *et al.*, 1993
Bifurcaria bifurcata	Diterpenes	Hougaard *et al.*, 1991a
Bifurcaria bifurcata	Diterpenes	Hougaard *et al.*, 1991b
Carpophyllum angustifolium	Trihydroxyphlorethols	Glombitza and Schmidt, 1999
Cladosiphon okamuranus	Fucoidan	Haneji *et al.*, 2005
Cystophora retroflexa	Phlorotannins	Sailler and Glombitza, 1999
Cystoseira tamariscifolia	Meroditerpenoid	Bennamara *et al.*, 1999
Dictyopteris undulata	Cyclozonarone (sesquiterpene-substituted benzoquinone)	Kurata *et al.*, 1996
Dilophus okamurae	Dictyterpenoids A and B	Suzuki *et al.*, 2002
Hijikia fusiformis	Fucoxanthin	Yan *et al.*, 1999
Laminaria sinclarii	Divinyl ether fatty acids	Proteau and Gerwick, 1993
Notheia anomala	Epoxy lipids	Murray *et al.*, 1991
Sargassum spinuligerum	Fucophlorethols	Glombitza and Keusgen, 1995
Sargassum spinuligerum	Fuhalols, phlorethols	Glombitza *et al.*, 1997
Spatoglossum schroederi	Sulphated fucan	Rocha *et al.*, 2005
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
Stypopodium flabelliforme	Meroditerpenoids	Sabry *et al.*, 2005
GREEN SEAWEEDS		Kuniyoshi *et al.*, 1985
Caulerpa prolifera	Acetylene sesquiterpenoid esters	Smyrniotopoulos *et al.*, 2003
Chroomonas salina	Betaine lipids	Eichenberger *et al.*, 1996
Codium decorticatum	Sterols, alcohols	Ahmad *et al.*, 1994
Ulva lactuca	Lectin	Sampaio *et al.*, 1998
Ulva pertusa	Long-chain aldehydes	Akakabe *et al.*, 2005
RED SEAWEEDS		
Acantophora spicifera	cholest-4-ene-3α, 6β-diol and cholest-4-ene-3-one, lauric acid	Wahidulla *et al.*, 1998
Agardhiella subulata	Agardhilactone	Graber and Gerwick, 1996
Alsidium helminthocorton	Kainic acid	Calaf *et al.*, 1989
Ceramium tenuicome	Hydroxylated and methoxylated brominated diphenyl ethers	Malmvärn *et al.*, 2005
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
Gelidium pristoides	Galactosyl glycerol	Nunn and Von Holdt, 1955
Gracilaria asiatica	Eicosanoids	Sajiki and Kakimi, 1998
Gracilaria confervoides	Galactosyl glycerol	Nunn and Von Holdt, 1955
Gracilaria coronopifolia	Malyngamides M and 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
Gracilaria verrucosa	Chlorinated C12 fatty acids	Shoeb and Jaspars, 2003
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 sp.	1(S)-bromo-4-(R)-hydroxy-(-)-selin-7-ene	Howard and Fenical, 1977
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
Laurencia perforata	Sesquiterpenes	Wright *et al.*, 2003
Laurencia rigida	Sesquiterpenes	König and Wright, 1997
Laurencia papillosa	*p*-hydroxy-benzaldehyde, *p*-methoxy benzyl alcohol	Wright and König, 1996
Laurencia saitoi	Brominated and non-brominated diterpenes	Kurata *et al.*, 1998
Odonthalia corymbifera	Diarylmethane-type bromophenol	Kurata *et al.*, 1997
Odonthalia dentate	Bromophenol	Craigie and Gruenig, 1967

SEAWEEDS	ISOLATED COMPOUNDS	REFERENCES
Pantoneura plocamioides	Terpenes	Cueto *et al.*, 1998
Plocamium cartilagineum	Halogenated monoterpene	Argandoña *et al.*, 2002
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 corallohiza	Polyhalogenated monoterpenes	Knott *et al.*, 2005
Plocamium costatum	Polysaccharide	Miller, 1999
Plocamium hamatum	Terpenes	König *et al.*, 1999b
Plocamium leptophyllum	Halogenated terpenes	Sakata *et al.*, 1991
Plocamium sp.	Terpene	Whitney *et al.*, 1997
Polysiphonia lanosa	Galactan sulphate	Batey and Turvey, 1975
Polysiphonia paniculata	Dimethylsulfoniopropionate	Nishiguchi and Goff, 1995
Polysiphonia urceolata	Acrylic acid	Glombitza, 1970a; Glombitza, 1970b
Porphyra capensis	Polysaccharide	Zhang *et al.*, 2005
Porphyra haitanensis	Sulfated galactan	Zhang *et al.*, 2004
Ptilota filicina	Lectin	Sampaio *et al.*, 1998
Rhodomela confervoides	Bromophenol	Craigie and Gruenig, 1967
Rhodomela confervoides	Bromophenols	Xu *et al.*, 2003
Schizymenia dubyi	Methoxy fatty acids	Barnathan *et al.*, 1998
Tichocarpus crinitus	Fatty acids	Khotimchenko and Yakovleva, 2005
Vidalia obtusaloba	Vidalols A and B	Wiemer *et al.*, 1991
Vidalia sp.	Vidalenolone	Yoo *et al.*, 2002

Table 1.2 Red seaweeds and the bioactivities reported for their extracts or isolates.

SEAWEED	BIOACTIVITY	COMPOUND	REFERENCES
Amphiroa ephedraea	Antimicrobial		Vlachos *et al.*, 1997
Arthrocardia carinata	Antimicrobial		Vlachos *et al.*, 1997
Asparagopsis taxilormis	Antibacterial		Horikawa *et al.*, 1999
Beckerella pinnatifida	Antimicrobial		Vlachos *et al.*, 1997
Botryocladia botryoides	Antiviral		Caccamese *et al.*, 1980
Callithamnion pikeanum	Antiviral		Kim *et al.*, 1997
Callophycus serratus	Cytotoxic	Bromophycolide A	Kubanek *et al.*, 2005
Callophylis sp.	Antimicrobial		Heríquez *et al.*, 1979
Callophyllis japonica	Antioxidant		Kang *et al.*, 2005
Callophyllis megalocarpa	Antibacterial		Pratt *et al.*, 1951
Campylaephora hypnaeoides	Antiviral		Ohigashi *et al.*, 1992
Carpoblepharis flaccida	Anti-inflammatory		Stirk *et al.*, 1996
Cheilosporum sagittatum	Antimicrobial		Vlachos *et al.*, 1997
Chondria armata	Hypotensive		Solimabi *et al.*, 1980
Chondria atropurpurea	Anthelminthic	Chondriamides A, B, C	Davyt *et al.*, 1998
Chondria atropurpurea	Anthelminthic	3-indoleacrylic acid	Davyt *et al.*, 1998
Chondria coerulescens	Antimicrobial		Caccamese *et al.*, 1985
Chondria dasyphylla	Antimicrobial		Hornsey and Hide, 1974
Chondrus crispus	Antimicrobial		Hornsey and Hide, 1976
Chondrus ocellatus	Antiviral		Hudson *et al.*, 1999
Corallina elongata	Anti-inflammatory		Bustos *et al.*, 1992
Corallina pilulifera	Antiviral		Kim *et al.*, 1997
Corallina pilulifera	Antiviral		Hudson *et al.*, 1999
Coraltina vancouveriensis	Antiviral		Kim *et al.*, 1997
Delesseriacean sp.	Antimicrobial	Almazole D	N'Diaye *et al.*, 1996
Delisea fimbriata	Antimicrobial	Fimbrolides	Reichelt and Borowitzka, 1984
Delisea hypnoides	Antimicrobial		Reichelt and Borowitzka, 1984
Digenea simplex	Antiviral		Sekine *et al.*, 1995
Falkenbergia rufolanosa	Antibacterial		Ballesteros *et al.*, 1992
Galaxaura marginata	Cytotoxic	Desmosterols	König *et al.*, 1994
Galaxaura marginata	Cytotoxic	Oxygenated desmosterols	Sheu *et al.*, 1997
Galaxaura oblongata	Anti-inflammatory		Bustos *et al.*, 1992

SEAWEED	BIOACTIVITY	COMPOUND	REFERENCES
Galaxaura oblongata	Anti-inflammatory		Payá *et al.*, 1990
Gelidiella acerosa	Antifertility		Solimabi *et al.*, 1980
Gelidium abbottiorum	Antimicrobial		Vlachos *et al.*, 1997
Gelidium amansii	Cytotoxic		Numata *et al.*, 1991
Gigartina acicularis	Antimitotic		Chenieux *et al.*, 1980
Gigartina intermedia	Antiviral		Nakamura *et al.*, 1994
Gigartina tenella	Anti-tumour		Nakamura *et al.*, 1997
Gigartina skottsbergii	Antiviral	Carrageenan	Carlucci *et al.*, 1999
Gloiosiphonia verticillaris	Antimicrobial	Dimethyl gloiosiphone	Sturino *et al.*, 1997
Gracilaria corticata	Antibacterial		Sastry and Rao, 1994
Gracilaria domingensis	Antimicrobial		De Campos-Takaki *et al.*, 1988
Gracilaria pacifica	Antiviral		Kim *et al.*, 1997
Gracilaria sjoestedtii	Antimicrobial		De Campos-Takaki *et al.*, 1988
Gracilaria textorii	Cytotoxic		Numata *et al.*, 1991
Grateloupia sp.	Antimicrobial		Heríquez *et al.*, 1979
Grateloupia turuturu	Antiviral		Kim *et al.*, 1997
Grateloupia turuturu	Antiviral		Hudson *et al.*, 1999
Haliptylon sp.	Antimicrobial		Heríquez *et al.*, 1979
Halosaccion glandiforme	Antibacterial		Pratt *et al.*, 1951
Hypnea musciformis	Antimicrobial		De Campos-Takaki *et al.*, 1988
Hypnea musciformis	Antimicrobial		Melo *et al.*, 1997
Hypnea musciformis	Diuretic		Solimabi *et al.*, 1980
Hypnea musciformis	Anti-inflammatory		Payá *et al.*, 1990
Iridaea membranacea	Antimicrobial		Heríquez *et al.*, 1979
Iridophycus flaccidum	Antibacterial		Pratt *et al.*, 1951
Laurencia brongniartii	Antibacterial		Horikawa *et al.*, 1999
Laurencia complanata	Antimicrobial		Vlachos *et al.*, 1997
Laurencia hybrida	Antimicrobial		Hornsey and Hide, 1974
Laurencia implicata	Cytotoxic	Acetogenins	König *et al.*, 1994
Laurencia intermedia	Antiviral		Ohigashi *et al.*, 1992
Laurencia majuscula	Antibacterial	Elatol, iso-obtusol	Vairappan, 2003
Laurencia obtuse	Antibacterial	Laurencienyne	Caccamese *et al.*, 1981
Laurencia obtuse	Antibacterial		Mahasneh *et al.*, 1995
Laurencia obtuse	Antimicrobial		Caccamese *et al.*, 1980
Laurencia obtuse	Antiviral		Caccamese *et al.*, 1980
Laurencia obtuse	Anti-inflammatory		Bustos *et al.*, 1992
Laurencia okamurae	Antibacterial		Horikawa *et al.*, 1999
Laurencia papillosa	Antibacterial		Mahasneh *et al.*, 1995
Laurencia papillosa	Antimalarial		Wright and König, 1996
Laurencia pinnatifida	Antimicrobial		Hornsey and Hide, 1976
Laurencia sp.	Antibacterial		Vairappan *et al.*, 2001
Mazzaella capensis	Antimicrobial		Vlachos *et al.*, 1997
Mazzaella cornucopiae	Antiviral		Kim *et al.*, 1997
Mazzaella cornucopiae	Cytotoxic		Kim *et al.*, 1997
Nothogenia fastigiata	Antiviral	Xylomannans	Kolender *et al.*, 1997
Nothogenia fastigiata	Antiviral		Witvrouw and De Clercq (1997)
Nothogenia fastigiata	Antiviral		Damonte *et al.*, 1994
Odonthalia corymbifera	Antifouling	Bromophenol	Kurata *et al.*, 1997
Odonthalia corymbifera	Antibacterial		Horikawa *et al.*, 1999
Odonthalia floccosa	Antiviral		Kim *et al.*, 1997
Pachymeniopsis elliptica	Antiviral		Kim *et al.*, 1997
Peyssonnelia rosa-marina	Cytotoxic		Ballesteros *et al.*, 1992
Peyssonnelia rosa-marina	Antimitotic		Ballesteros *et al.*, 1992
Plocamium cartilagineum	Antifeedant		Argandoña *et al.*, 2002
Plocamium costatum	Antifouling		König *et al.*, 1999a
Plocamium hamatum	Antibacterial		König *et al.*, 1999b
Plocamium hamatum	Anti-algal		König *et al.*, 1999b
Plocamium leptophyllum	Antifouling		Sakata *et al.*, 1991
Plocamium rigidum	Antimicrobial		Vlachos *et al.*, 1997

SEAWEED	BIOACTIVITY	COMPOUND	REFERENCES
Plocamium telfairiae	Insecticidal	Telfairine, Aplysiaterpenoid a	Watanabe *et al.*, 1990
Polysiphonia furcellata	Antimicrobial		Caccamese *et al.*, 1985
Polysiphonia hendryi	Antiviral		Kim *et al.*, 1997
Polysiphonia lanosa	Antimicrobial		Hornsey and Hide, 1976
Polysiphonia lanosa	Cytotoxic	Bromophenols	Shoeib *et al.*, 2004
Polysiphonia urceolata	Antimicrobial	Acrylic acid	Glombitza, 1970a; Glombitza, 1970b
Polysiphonia virgata	Antimicrobial		Vlachos *et al.*, 1997
Porphyra haitanensis	Antioxidant	Sulphated galactan	Zhang *et al.*, 2004
Portieria hornemannii	Antitumor	Halomon, halogenated monoterpenes	Fuller *et al.*, 1994
Portierra hornemannii	Antimicrobial		Vlachos *et al.*, 1997
Pterocladia capillacea	Antimicrobial		Khaleafa *et al.*, 1975
Ptilonia australasica	Antimicrobial	Pentabromopyrone	Reichelt and Borowitzka (1984)
Rhodomela confervoides	Antibacterial	Bromophenols	Xu *et al.*, 2003
Rhodomela teres	Antibacterial		Horikawa *et al.*, 1999
Sarcothalia stiriata	Anti-inflammatory		Stirk *et al.*, 1996
Schizymenia binderi	Antiviral	Sulphated galactan	Matsuhiro *et al.*, 2005
Schizymenia pacifica	Antiviral		Witvrouw and De Clercq (1997)
Scinaia japonica	Cytotoxic		Numata *et al.*, 1991
Symphyocladia latiuscula	Antiviral		Hudson *et al.*, 1999
Symphyocladia latiuscula	Antiviral	2,3,6-tribromo-4,5-dihydroxybenzyl methyl ester	Park *et al.*, 2005
Symphyocladia marchantioides	Antiviral		Hudson *et al.*, 1999
Symphyocladia spp	Antiviral		Kim *et al.*, 1997
Trematocarpus dichotomus	Antimicrobial		Heríquez *et al.*, 1979
Vidalia obtusaloba	Anti-inflammatory		Wiemer *et al.*, 1991

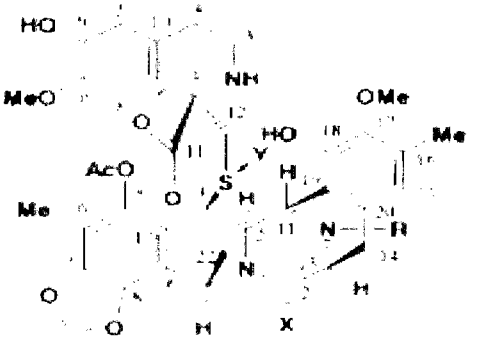
An important aspect of marine algae worth mentioning is that a few species are part of a global industry. The algal aquaculture industry is estimated yearly at US $5-6 billion worldwide (Wikfors and Ohno, 2001). The natural products currently supporting this industry include the algal phycocolloids, such as agar, carrageenan and alginates, the pigments beta-carotene, astaxanthin and the fatty acid docosahexaenoic acid (DHA). The phycocolloids are of particular interest with their ability to modify the viscosity or texture of food products. Whole algae are added to food for their nutritional value or their extracts are used as nutritional supplements as with the cyanobacterium Spirulina (Wikfors and Ohno, 2001). The bioactivities of phycocolloids have also been investigated. Carrageenans isolated from the red seaweed *Gigartina skottsbergii* have shown potent inhibition of herpes simplex virus types 1 and 2 (Carlucci *et al.*, 1999). These algae and many more should be investigated extensively for bioactivities; if significant findings are made, they could have considerable commercial value. In addition, building on an established harvesting culture the availability of this resource is a great advantage compared to terrestrial plants where the agriculture has yet to be established.

1.8 Marine antimycobacterial compounds

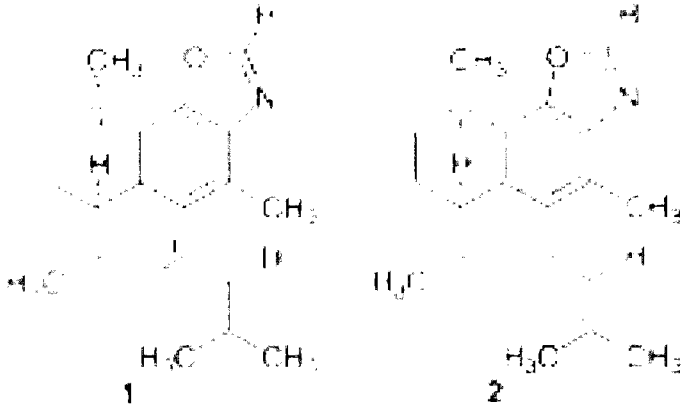
In the search for antimycobacterial compounds from marine organisms such as sponges, gorgonia, and soft corals, compounds with significant antimycobacterial activity have been found (Donia and Hamann,

2003). One example is the isolation of two novel alkaloids manadomanzamines A and B (Figure 1.3) from the Indonesian sponge, *Acanthostrongylophora sp.* (Peng *et al.*, 2003) which inhibited the growth of *M. tuberculosis* with MIC-values of 1.9 and 1.5 µg/mL, respectively. In another investigation Rodríguez and Ramírez (2001) isolated antimycobacterial serrulatane diterpenes (Figure 1.3) which caused up to 97% inhibition of growth of *M. tuberculosis*. Suwanborirux *et al.* (2002) isolated ecteinascidins 770 and 786 (Figure 1.3) from a Thai tunicate *Ecteinascidia thurstoni* each of which showed antimycobacterial activity against *M. tuberculosis*. In 2000, König *et al.* screened 39 marine derived natural products for antimycobacterial activity against *Mycobacterium tuberculosis* and *M. avium* (König *et al.*, 2000). The compounds represented various structural classes, including terpenes, aliphatics, aromatics, alkaloids, and sterols. Fifteen compounds were from red algae, one from a brown alga, three from cyanobacteria, ten were derived from sponges, one from a soft coral, three from a gorgonian, three from lichens and three were semi-synthetic. The seven sponge-derived compounds were the most antimycobacterial against *M. tuberculosis*, with MIC-values below 10 µg/mL. The compounds isolated from the red algae *Laurencia obtusa*, *L. flexilis*, *L. rigida* and *Plocamium cartilagineum* possessed antimycobacterial activity ranging from MIC-values of 16 to 64 µg/mL. Two compounds isolated from blue-green algae also showed antimycobacterial activity. The lichen derived compounds had antimycobacterial activity, as did the compound isolated from a soft coral. In 2003, Nicholas *et al.* screened approximately 1500 crude organic extracts for inhibition of natural *Mycobacterium smegmatis* mycothiol-S-conjugate amidase (MCA) and recombinant *M. tuberculosis* MCA. An impressive 1200 extracts were obtained from marine plants and invertebrates and another 300 came from terrestrial fungi (Nicholas *et al.*, 2003). The initial screen presented only 20 active extracts, of which 10 were further purified, and of the active compounds isolated 13 active; 10 came from marine sponges and the remaining three from fungi. These studies demonstrate the antimycobacterial capabilities of marine-derived compounds and give strong support to their inclusion in any drug development programme.

Manadomanzamine A(1): 22β-H
reproduced from Peng *et al.*, 2003



Ecteinascidins 1, reproduced from Suwanborirux *et al.*, 2002



Serrulatane diterpenes reproduced from Rodríguez and Ramírez, 2001

Figure 1.3. A few antimycobacterial compounds isolated from marine organisms.

1.9 Rationale for this investigation

In a previous investigation done by Cameron *et al.* (2001), seventeen South African marine red seaweeds were screened for antimicrobial activity following the findings of marine chemists such as Caccamese *et al.* (1985) that red seaweeds are potential sources of antibiotics. The algae were tested against *Staphylococcus aureus*, *Candida albicans*, and *Mycobacterium smegmatis* using the agar overlay bioautography method. The purified fractions of eight algae revealed the presence of antimicrobial

compounds. Most of the antimicrobial activities of both the crude and the fractions of these algal extracts inhibited growth of *S. aureus* and *M. smegmatis*. The extracts and fractions of *Plocamium corallorhiza* and *Polysiphonia virgata* produced the largest inhibition zones. *Gelidium pristoides* was among the eight species that showed antimicrobial activity. This made the selection of algae for this present study easy as *P. corallorhiza* and *P. virgata* have economic potential in addition to their reported antimicrobial activity and *G. pristoides* already has commercial value. Should any significant antimycobacterial compounds be present in these algal extracts, the source material will not be difficult to collect and the results of this investigation would serve to strengthen the motivation for a local seaweed pharmaceutical industry.

1.10 Aim and Objectives

The aim of this investigation has been to establish whether the algal extracts of *P. corallorhiza*, *G. pristoides,* and *P. virgata* contain compounds that inhibit the growth of *Mycobacterium tuberculosis*. Therefore, the objectives of this project were the following:

- To extract the secondary metabolites of the three red algae and to purify the crude extracts.
- To test the extracts and fractions against a sensitive strain of *M. tuberculosis* using the BACTEC-460 radiometric method.
- To isolate and characterize the bioactive compounds.

In the course of this project, the in house availability of an antimalarial assay made it easy to test the antimalarial activity of some of the algal extracts. The importance of evaluating plant extracts for antimalarial activity needs no motivation, as malaria is one of the leading causes of mortality in the world causing about 1 million deaths annually of which 90% are African children (WHO Health Report, 2005). A few of the algal extracts were therefore tested in a parasite lactate dehydrogenase assay for *Plasmodium falciparum* sensitivity.

The seaweeds are described in detail by elaborating on their uses and where they are found in South Africa, their phytochemistry as reported in scientific publications, and the biological activities obtained for them from NAPRALERT and journal articles. The various methods used in this investigation are described and the results obtained in the various assays are reported. The isolation and characterization of the isolated compounds and the antimycobacterial activities of the isolates are described and the findings reported into context. The overall assumptions of this investigation are stated and the implications for the future are reported.

Chapter 2

SOUTH AFRICAN SEAWEEDS: *PLOCAMIUM CORALLORHIZA*, *GELIDIUM PRISTOIDES*, AND *POLYSIPHONIA VIRGATA*

2.1. South African seaweed industry

South Africa is home to a diverse seaweed population which is divided into four; namely, 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). It is estimated that there are altogether 800 species (Stegenga *et al.*, 1997). Red seaweeds constitute most of the 800 species and more red than brown and green seaweeds are considered potentially harvestable and/or economically important (Critchley *et al.*, 1998). This is, however, not reflected in the current status of South Africa's seaweed industry that uses two kelps (brown algae) and three species of Gelidium and Gracilaria/Gracilariopsis, all red algae (Anderson *et al.*, 2003). The beach-cast kelps, *Ecklonia maxima* and *Laminaria pallida* are sun-dried, milled and exported for the extraction of alginate. Powdered kelp is exported to Japan for use in formulated fish-feed. Fresh kelp is used in commercial abalone farming as feed and harvested to produce a liquid plant-growth stimulant, which contains cytokinins (Anderson *et al.*, 2003). In the Cape Floristic Region alone an estimated 1800 ton of kelp is harvested annually (Turpie *et al.*, 2003). Other seaweeds used in abalone farming include the green alga Ulva and species of the red algae Gracilaria and Plocamium. Gracilaria's commercial value comes from the demand from countries, such as, Japan and Korea. Its beach-cast material is collected in Saldanha Bay, dried, sorted and finally exported. Gracilariopsis makes a similar contribution although it is collected on a much smaller scale in St. Helena Bay (Anderson *et al.*, 2003). For both these seaweeds, attempts are underway to establish suspended cultivation. Lastly, but not least, various species of Gelidium, namely, *Gelidium pristoides*, *G. abbottiorum*, *G. pteridifolium* and *G. capense* are harvested in the Eastern Cape and exported mainly to Japan and Korea for agar extraction (Anderson *et al.*, 2003). Other seaweeds with potential commercial value include *Gigartina polycarpa*, *Sarcothalia stiriata*, *Mazzaella capensis*, *Aeodes orbitosa*, *Hypnea spicifera,* and *Porphyra capensis*. Despite all this commercial activity, there is no extraction industry in South Africa (Melo, 1998) – research into the pharmaceutical potential of these algae could support the establishment of such an industry, which might also benefit other countries in our region, such as

Mozambique, Angola and Namibia that have commercial seaweeds. The latter has an agar factory that ceased production in 1995 (Anderson *et al.*, 2003).

2.2 Biological activities of South African algae

Considering the vast amounts of seaweeds available along the South African coast, there has been little investigation into their pharmaceutical potential. Vlachos *et al.* (1997) screened 56 seaweeds for their antimicrobial activity against 16 microorganisms, including, *Bacillus subtilis* EL 39, *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Salmonella enteritidis*, *Penicillium sp.*, and *Candida albicans*. The brown alga, *Zonaria subarticulata*, showed the highest amount of inhibition against all the microorganisms tested. Stirk *et al.* (1996) tested the crude algal extracts of 35 seaweed species for inhibitory activity to prostaglandin synthesis and found species from the red, brown, and green seaweeds with potential novel anti-inflammatory activity. Stirk and Van Staden (1997) screened 20 algal species for cytokinin-like activity and found it present in most of the extracts.

2.3 Seaweeds screened in this investigation

All three seaweeds researched in this thesis are red and belong to the class Rhodophyceae. They possess chlorophyll, phycobiliproteins (allophycocyanin, phycocyanin and phycoerythrin), and carotenoids. The algae can be red, violet, brown, black, or blue in colour with floridean as the storage product and the cell walls are composed of cellulose or xylans and galactans. The Rhodophyceae have the largest variety of toxic secondary metabolites and they include compounds such as halogenated acetates, acrylates, and ketones (South and Whittick, 1987).

Of the genera screened in this research, Plocamium species have been studied the most for their natural products and biological activities (Table 2.1). Gelidium has received much attention for its agar content, the chemical component largely responsible for its commercial value (Renn, 1997; Evans, 1996a) and for its management as a wild resource (Tronchin *et al.*, 2003; Santos *et al.*, 2003). Polysiphonia have been part of screens in which antimicrobial activities were tested (Caccamese *et al.*, 1985; Vlachos *et al.*, 1997).

Table 2.1 Red algal species of the genus Gelidium, Plocamium and Polysiphonia, their isolated compounds and biological activities reported for their extracts or isolates.

Species	Biological activity/Isolated compounds	Reference
Gelidium amansii	Cytotoxic activity	Harada *et al*., 1997
Gelidium amansii	Antitumour activity	Ohigashi *et al*., 1992
Gelidium amansii	Cytotoxic activity	Numata *et al*., 1991
Gelidium attenuatum	Antimitotic activity	Chenieux *et al*., 1980
Gelidium cartilagineum	Antiviral activity	Gerber *et al*., 1958
Gelidium divaricatum	Antioxidant activity	Fujimoto *et al*., 1985
Gelidium elegans	Antitumour activity	Nakamura *et al*., 1997
Gelidium pristoides	Galactosyl glycerol	Nunn and Von Holdt, 1955
Gelidium pusillum	Antitrypanosomal activity	Gonzalez *et al*., 1990
Gelidium sesquipedale	Antimitotic activity	Chenieux *et al*., 1980
Plocamium angustatum	Bromophenols	Whitfield *et al*., 1999
Plocamium cartilagineum	Polyhalogenated cyclic monoterpenes	Stierle and Sims (1979)
Plocamium cartilagineum	Terpenes	Abreu and Galindro (1996)
Plocamium cartilagineum	Poly(β-hydroxybutyrate), Floridoside	Abreu *et al*., 1997
Plocamium cartilagineum	Terpenes	Cueto *et al*., 1998
Plocamium cartilagineum	Acyclic halogenated monoterpenes	Faulkner, 1998
Plocamium cartilagineum	Linear monoterpene	Faulkner, 2000
Plocamium cartilagineum	Plocamapyranoid	Faulkner, 2000
Plocamium cartilagineum	4-bromo-5-chloro-2-(*E*)-chlorovinyl-1,5-dimethyl-1,2-epoxycyclohexane	Faulkner, 2000
Plocamium cartilagineum	(1*S*,2*R*,4*R*,5*S*,1A*E*)-2,4,5-trichloro-1-(2Achloroethynyl)-1,5-dimethylcyclohexane	Faulkner, 2000
Plocamium cartilagineum	Polyhalogenated monoterpenes	Jongaramruong and Blackman ,2000
Plocamium cartilagineum	Polyhalogenated monoterpenes	Darias *et al*., 2001
Plocamium cartilagineum	Polyhalogenated homosesquiterpenic fatty acids	Řezanka and Dembitsky, 2001
Plocamium cartilagineum	Polyhalogenated homosesquiterpenic fatty acids	Dembitsky and Srebnik, 2002
Plocamium cartilagineum	Tetrahydrofuran halogenated monoterpene(1), acyclic polyhalogenated monoterpenes(2)(3) (4) Furoplocamioid (5) Plocamenol	Díaz-Marrero *et al*., 2002a
Plocamium cartilagineum	Polyhalohydroxylated monoterpenes, plocamenols A – C	Díaz-Marrero *et al*., 2002b
Plocamium cartilagineum	Polyhalogenated monoterpenes	Faulkner, 2002
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 cartilagineum	Halogenated monoterpens	Argandoña *et al*., 2002
Plocamium coccineum	Antimitotic activity	Chenieux *et al*., 1980
Plocamium coccineum	Antibacterial activity	Usmanghani *et al*., 1984
Plocamium corrallorhiza	Polyhalogenated monoterpenes	Knott *et al*., 2005
Plocamium costatum	Antifouling	König *et al*., 1999a
Plocamium costatum	Polysaccharide	Miller, 1999
Plocamium costatum	Polysaccharide	Miller *et al*., 1996
Plocamium costatum	Antifouling activity	König *et al*., 1999a
Plocamium hamatum	Halogenated monoterpene	König *et al*., 1994
Plocamium hamatum	Terpenes Compound 11 – weakly antimicrobial, Compound 4 – anti-algal against alga *Chlorella fusca*, Compound 3 – antimycobacterial, Compound 4 – antialgal, cytotoxic, antimycobacterial, Compound 6 – antimycobacterial	König *et al*., 1999b
Plocamium hamatum	Cyclic monoterpene	Faulkner, 1999
Plocamium hamatum	Terpenes	König *et al*., 1999b
Plocamium leptophyllum	Antifouling	Sakata *et al*., 1991
Plocamium leptophyllum	Terpenes	Sakata *et al*., 1991
Plocamium oregonum	Halogenated monoterpene	Crews *et al*., 1984

Species	Biological activity/Isolated compounds	Reference
Plocamium rigidum	Antimicrobial	Vlachos *et al.*, 1997
Plocamium sp.	Terpene	Whitney *et al.*, 1997
Plocamium telfairiae	Cytotoxic activity	Harada *et al.*, 1997
Plocamium violaceum Farlow	Oleic acid, palmitic acid, cis-vaccenic acid, dihomo-γ-linolenic acid, arachidonic acid, eicosapentaenoic acid	Khotimchenko *et al.*, 2002
Plocamium violaceum	Cyclic monoterpene	Crews and Kho, 1975
Plocamium violaceum	Acyclic polyhalogenated monoterpenes	Crews and Kho-Wiseman, 1977
Plocamium violaceum	Larvicidal activity	Crews *et al.*, 1984
Polysiphonia brodiaei	Brominated phenols	Pedersén *et al.*, 1974
Polysiphonia nigrescens	Bromochlorophenols	Pedersen, 1978
Polysiphonia paniculata	Dimethylsulfoniopropionate	Nishiguchi and Goff, 1995
Polysiphonia sphaerocarpa	Brominated anisoles and cresols	Flodin and Whitfield, 2000
Polysiphonia urceolata	Polyunsaturated fatty acids	Li *et al.*, 2002
Polysiphonia denudate	Antiviral activity	Serkedjieva, 2000
Polysiphonia fruticulosa	Antimitotic activity	Chenieux *et al.*, 1980
Polysiphonia hendryi var Gardneri	Antiviral activity	Kim *et al.*, 1997
Polysiphonia lanosa	Galactan sulphate	Batey and Turvey, 1975
Polysiphonia lanosa	Antimitotic activity	Chenieux *et al.*, 1980
Polysiphonia lanosa	Antibacterial activity	Lustigman *et al.*, 1992
Polysiphonia subtilissima	Antibacterial activity	Perez *et al.*, 1990
Polysiphonia urceolata	Antioxidant activity	Fujimoto *et al.*, 1985
Polysiphonia urceolata	Carotenoids	Bjørland and Aguilar-Martinez, 1976
Polysiphonia urceolata	Acrylic acid	Glombitza, 1970a; Glombitza, 1970b

2.3.1 *Gelidium pristoides* (Turner) Kuetzing

2.3.1.1 Description, ecology, distribution and uses

Figure 2.1. *Gelidium pristoides*

This is a bushy dark olive brown to black plant with a tough texture and it grows to approximately 20 cm (Stegenga *et al.*, 1997). It is endemic to South Africa and commonly found growing on rocks and the shells of the limpet *Patella* in the mid and lower intertidal zones from Sea Point eastward, across the southern Cape and finally the eastern Cape and Transkei where its biomass is the greatest and it is harvested commercially (Stegenga *et al.*, 1997). The phycocolloids extracted from species of *Gelidium*, amongst other algae, are used to prepare bacteriological and tissue culture media (South and Whittick, 1987) since

they are not metabolised by microorganisms, such as bacteria and fungi (Stegenga *et al.*, 1997). In addition, they are used as a gelling agent for fruit and vegetable jellies (Nagaoko *et al.*, 2000), added to bakery products, confectionery (Stegenga *et al.*, 1997) and pharmaceuticals (Renn, 1997) where they are used as a disintegrant in tablets and in the preparation of slow-release capsules (Nagaoko *et al.*, 2000). Whole plants species of *Gelidium* are eaten in the Orient and Pacific Islands (South and Whittick, 1987) while in the West they are found in health stores or as additives in health products (Nagaoka *et al.*, 2000) together with other seaweeds. Various ecological studies of *Gelidium* species have been undertaken, including investigation into the effects of grazing on the shore distribution of algal communities (Boaventura *et al.*, 2002; Tronchin *et al.*, 2003), algal population dynamic and productivity (Duarte and Ferreira, 1997), and its commercial exploitation worldwide (Melo, 1998).

2.3.1.2 Phytochemistry

Gelidium species are known for their high agar content (Evans, 1996a) - polysaccharides composed of sulphated and pyruvated galactoses (South and Whittick, 1987). Fatty acids have been reported in an investigation of *Gelidium amansii* (Li *et al.*, 2002). One of the reasons why this alga is included in the diet of people in the Orient and elsewhere is because it is rich in protein and vitamins such as C, A, B1, B12, E, riboflavin, niacin, panthothenic acid and folic acid (South and Whittick, 1987).

2.3.1.3 Pharmacological studies

A crude extract of *Gelidium abbottiorum* has been reported to contain antimicrobial compounds (Vlachos *et al.*, 1997). *Gelidium amansii* has cytotoxic constituents (Numata *et al.*, 1991) and its agar has been reported to be effective in treating obesity, constipation, irritable colon, diverticulitis and gastritis (Moro and Basile, 2000). Other bioactivities are referred to in Table 2.1.

2.3.2 *Plocamium corallorhiza* (Turner) Harvey

2.3.2.1 Description, distribution and uses

Figure 2.2. *Plocamium corallorhiza*

These plants are robust and grow to 30 cm. It has a bright red thallus and at times appears to have a bluish iridescence under water. It is endemic to southern Africa, and is commonly found in the sublittoral zone in Yzerfontein eastward to Natal and in northern Namibia. It is reported to be lacking from most of the Western Cape coast (Stegenga *et al.*, 1997) and it is used in feeding abalone (Anderson *et al.*, 2003).

2.3.2.2 Phytochemistry

Many halogenated monoterpenes, cresols, anisoles, fatty acids, and polysaccharides have been isolated from various species of algae belonging to this genus (Table 2.1).

2.3.2.3 Pharmacological studies

When compared to the genus Gelidium and Polysiphonia, the genus of Plocamium has been screened in many investigations. Anti-algal, antimicrobial and antimycobacterial activity has been reported for the algal extracts of various species (Table 2.1).

2.3.3 *Polysiphonia virgata* (C. Agardh) Sprengel

2.3.3.1 Description, distribution and uses

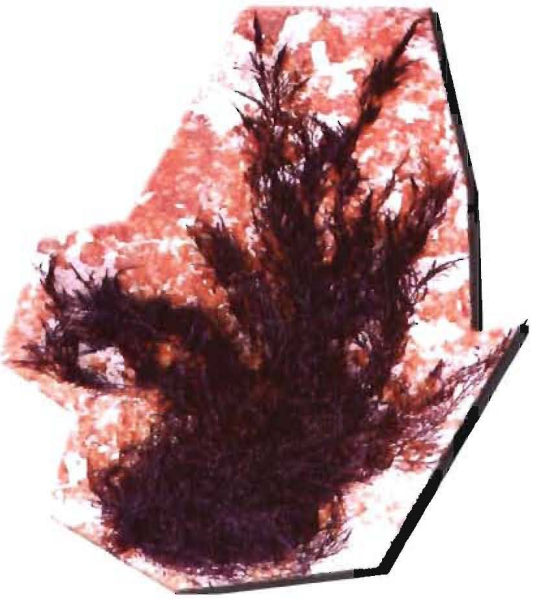


Figure 2.3. *Polysiphonia virgata*

This dark red to blackish plant grows to 30 cm or more. It often grows within the tissues of other plants, but is not necessarily parasitic, e.g. as an epiphyte on the kelp *Ecklonia maxima* (Stegenga *et al.*, 1997). *Polysiphonia virgata* is endemic to southern Africa and is found from the coast of Namibia to Brandfontein near Cape Agulhas.

2.3.3.2 Phytochemistry

From *Polysiphonia paniculata* dimethylsulfoniopropionate has been isolated (Nishiguchi and Goff, 1995) and *P. sphaerocarpa* contains brominated anisoles and cresols (Faulkner, 2000). The volatile compound dimethyl sulfide is given off spontaneously by *P. fastigiata* and *P. nigrescens* when they are exposed to air (Moore, 1977) and could be smelt in the laboratory while processing *P. virgata* in this investigation.

2.3.3.3 Pharmacological studies

Polysiphonia furcellata (Caccamese *et al.*, 1985), *P. lanosa*, *P. elongata*, *P. nigra*, *P. nigrescens*, *P. urceolata* (Hornsey and Hide, 1976) and *P. virgata* (Vlachos *et al.*, 1997) have been reported to contain antimicrobial compounds and *P. hendryi* has antiviral compounds (Kim *et al.*, 1997). Other bioactivities are referred to in Table 2.1.

Chapter 3

MATERIALS AND METHODS

3.1 Sample collection

Two seaweed species, namely *Gelidium pristoides* and *Plocamium corallorhiza* were collected on 28 February 2002 at Glen Cairn, False Bay, Cape Town (Figure 3.1). On 24 April 2002, a sample of *Polysiphonia virgata* was collected at Sweet Water, a little south of Kommetjie, Cape Town. For the re-investigation of *P. virgata* a second sample was collected at Sweet Water on 4 July 2005. All the algae were collected and verified by Mr Mark Rothman and Mr Chris Boothroyd from the Seaweed Research Unit of the Department of Environmental Affairs and Tourism: Marine and Coastal Management, which is based in the Botany Department at the University of Cape Town. Voucher specimens of the seaweeds [*G. pristoides*: Bolus Herbarium (BH) 99074; *P. corallorhiza*: BH 99075 and *P. virgata*: BH 99073] were deposited in the Bolus Herbarium at the Botany Department of the University of Cape Town (UCT). The herbarium specimen no. of the second *P. virgata* sample is: BH 126936. In addition, five liters of seawater samples were collected at Glen Cairn, Simonstown Harbour (2.5 L at the boathouse and 2.5 L at East Gate), and Sweet Water on 10 February 2006.

3.1.1 Background of collection sites

Glen Cairn is protected from heavy wave action in False Bay and is close to Simonstown harbour. It is home to a kelp community where *Plocamium corallorhiza* grows on the holdfast area from where divers removed it. *Gelidium pristoides* was found on the exposed rocks at lowtide. Environmental events that could influence the quality of the algae at a point in time are algal blooms and chemical waste from the harbour area or outfalls. In contrast, at Sweet Water the coastline is exposed to the waves of the Atlantic Ocean. The first sample of *Polysiphonia virgata* that was collected in February 2002 was found scattered about in the tidal pools at low tide probably as a result from a storm the previous night. At the second collection, the alga was cut from the kelp forest that is commercially grown at Sweet Water. Environmental events that could influence the quality of algae include upwelling in southern Benguela, which is in season from September to March. During this season the water temperatures of newly upwelled water is 14° to 16°C (Verheye *et al.*, 1992) as opposed to the normal 10° to 12°C. Upwelling brings about an increase in mesozooplankton (Verheye *et al.*, 1992) and so grazing of the algae in the effected area.

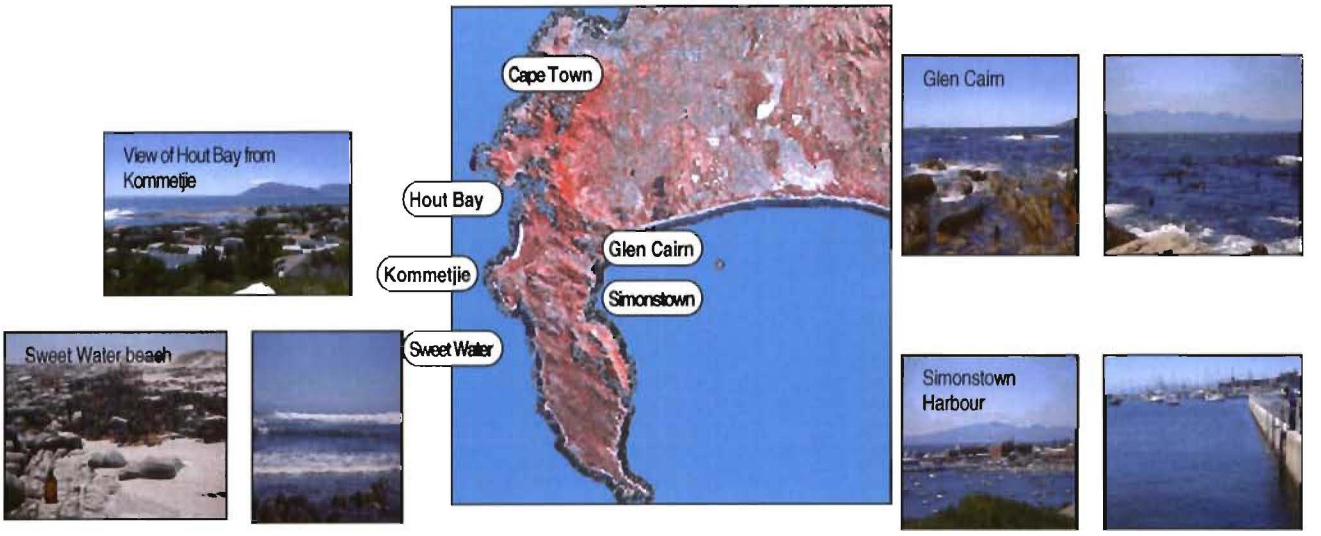


Figure 3.1. Map of False Bay indicating the positions of Glen Cairn and Kommetjie. Source is unknown. The photos were taken by Ms. Carmen Lategan

3.2 Extraction

3.2.1 Preparation

The seaweed samples were placed into plastic bags and kept cool on ice. In the laboratory, the algae were washed with distilled water to remove surface salts, sand, and epiphytes. The seaweeds were dabbed with paper towel to remove excess water and cut into small pieces. Samples were weighed and covered with volumes of dichloromethane (DCM)-methanol (MeOH) (2:1) solution. With the second collection of *P. virgata* the cleaned seaweed was weighed (6718.40 g) and placed in an oven at 50°C. A small portion of the wet sample was reserved to determine whether methoxyethyl methacrylate was adsorbed onto the algal surface or present inside the thallus. This was done by dipping the alga into 200 mL of ethanol, followed by 200 mL of dichloromethane (DCM) and finally it was left to soak in DCM overnight. The resultant solutions were evaluated using a LC-MS.

3.2.2 Solvent extraction

The seaweed samples were homogenized using a Kinematica homogenizer, stirred for 36 hours and filtered using a Büchner funnel with Whatman no. 541 filter paper. The seaweeds were extracted thrice with a solution of DCM-MeOH (2:1). This solvent was selected because it was recommended for the extraction of algal secondary metabolites by Cronin *et al.* (1995) and proved excellent in a previous study done by Cameron *et al.* (2001). Cronin *et al.* (1995) extracted two brown algae under various conditions and with different solvents. They found that when the seaweeds were extracted with methanol alone artifacts formed during the extraction procedure. When dichloromethane (DCM) was added to the

methanol in a 2:1 ratio, artifacts were reduced and the concentration of lipophilic secondary metabolites increased because of the less polar DCM. Other factors that also influenced the amount of secondary metabolites obtained included biochemical activity, light, vacuum, the drying procedure and the duration of extraction. These were all kept to a minimum during this extraction procedure. Therefore, the samples were extracted with a 2:1 mixture of DCM:MeOH. The samples that could not be processed on a particular day were stored in darkness in the freezer at –20°C. The filtrates were evaporated under vacuum in a Büchi Rotavapor R-205 at not more than 50°C. The dried extracts were also stored in a freezer at approximately -20°C. For the preparative Centrifugal Layer Chromatograph (Chromatotron) it was determined that the more polar compounds were difficult to remove from the silica plates and therefore the DCM-MeOH (2:1) crude extract was partitioned between dichloromethane and water using a separating funnel. The dichloromethane portion was dried with anhydrous magnesium sulphate. These filtrates were concentrated under vacuum at 50°C, while the water portion was concentrated by freeze-drying. The second collected sample of *P. virgata* was dried (782.50 g), milled using a blender, and soaked in cold methanol for 2 hours at 4°C. The methanol was filtered off and the algal material steeped in 2 L dichloromethane (DCM) overnight at 4°C. The cold DCM was filtered from the plant material and replaced with more DCM (5 x 2000 mL) at room temperature. The extracts were concentrated under vacuum using a Büchi Rotavapor R-205 at 50°C.

3.3 Isolation and Purification

3.3.1 Preparative Layer Chromatography (PLC)

Analytical thin-layer chromatography and PLC were carried out on Merck' pre-coated glass-backed, silica gel 60 F_{254} plates (0.25 mm and 2 mm or 1 mm thickness, respectively). Dichloromethane sample solutions were applied manually in approximately 17 cm long bands using a 5 mL glass pipette to PLC plates of the following specification: 1 mm thick 20 x 20 cm glass-backed silica 60 F_{254}. Chromatogram development was achieved by introducing the PLC-plates into chambers saturated with the mobile phase hexane-chloroform-methanol (7:2:1) which was determined from many different solvent combinations to give the best component separation. Five hundred milligram of the algal crude sample was applied to each PLC-plate and six plates were prepared per sample adding up to 3000 mg for each sample. After the solvent front was allowed to travel to about 0.5 cm from the edge of the plate and the solvents allowed to evaporate, six distinct bands were identified under ultraviolet light of wavelength 254 and 366 nm. These fraction bands were marked and the silica removed from the glass plates by scraping with a spatula. The band numbers allocated increased from the least polar (B1) to the most polar (B6). For each sample, bands of silica with similar numbers were combined and powdered using mortar and pestle. Desorption

was achieved by washing the silica extensively with DCM followed by MeOH under vacuum using a sintered glass Büchner funnel no.4. The washings were combined and evaporated under vacuum at 50°C in a Büchi Rotavapor R-205.

3.3.2 Preparative centrifugally accelerated radial layer chromatography (Chromatotron)

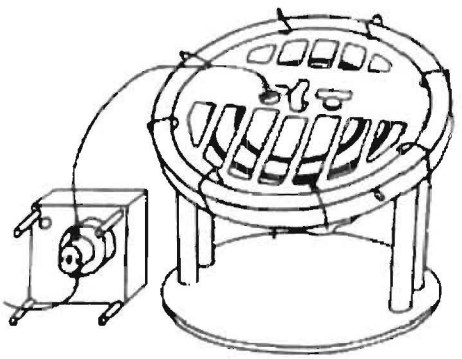


Figure 3.2. The Chromatotron model 8924. This picture was reproduced from the product manual.

Centrifugal preparative layer chromatography was performed using a Chromatotron model 8924 from Harrison Research Inc., USA (Figure 3.2). The dichloromethane-methanol (2:1) extract of the fresh and w*et al*gae were partitioned between water and dichloromethane in a separating funnel. The water-soluble portion was frozen and lyophilised while the DCM portion was concentrated under vacuum at 50°C in a Büchi Rotavapor R-205.

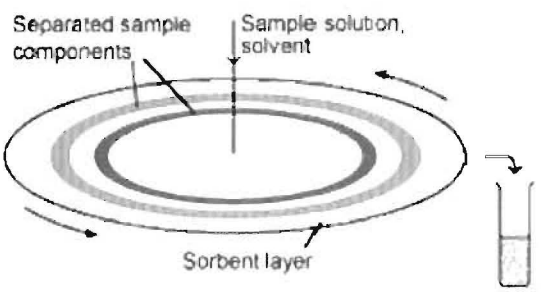


Figure 3.3 Purification of crude extract obtained using the Chromatotron. This image was reproduced from the product manual.

Only the DCM-portion was fractionated on the Chromatotron using silica gel 60 PF-254 coated plates (2 or 1 mm thickness) and hexane-DCM-MeOH (7:2:1) as solvent (Figure 3.3). The polarity of the solvent system was gradually increased to remove the more polar fractions from the rotor until only MeOH was effective. The less polar fractions were further fractionated using hexane-DCM-MeOH (5:3:2) or DCM-

MeOH (5:5), while the polar fractions resolved well with either toluene alone or hexane: DCM:MeOH (7:2:1). In some cases, better separation was achieved by starting with hexane and introducing more polar solvents enabling the bands to elute from the silica plate. The fractions were observed under ultraviolet light of 254 and 366 nm.

3.3.3 Flash Chromatography

Flash silica gel chromatography of the resultant sample material (7.074 g) was done with hexane and increasing proportions of ethyl acetate (EtOAc) as eluant and resulted in 44 main fractions (Appendix 1). Fraction 45 was obtained by washing the silica column with absolution ethanol until the eluant became colorless. The fractions were concentrated under vacuum using a Büchi Rotavapor R-205 at 50°C and dried completely under a stream of nitrogen.

3.4 Preparation and Isolation of Fatty Acid Derivatives

The sample was reacted with methanolic HCl, 1.5 N to obtain the methyl esters of the lipids present. This was done by refluxing 10.0 mg of sample C1730 and 6.4 mg of sample 1730/11 in 5 mL of methanolic HCl for 2 hours. The reaction mixture was evaporated to dryness under vacuum. The fatty acid methyl esters were purified by silica gel column chromatography using a Pasteur pipette and eluting with hexane/ether (8:2 v/v). The solvent was evaporated to dryness and the methyl esters dissolved in hexane and analyzed by gas chromatography-mass spectrometry (GC-MS).

3.5 Solvent partitioning of seawater samples

The water samples were kept at 4°C until the lipids were removed by partitioning between chloroform and water. The chloroform portion was dried with magnesium sulphate and reduced under vacuum using a Büchi Rotavapor R-205 at 50°C. It yielded 3.4 mg and 6.3 mg for the samples taken in Simonstown harbour at the East Gate and Boathouse, respectively. Sweet Water extract amounted to 8.6 mg and the seawater extract obtained from Glen Cairn to 2.4 mg.

3.6 Bioassay procedures

3.6.1 The agar-overlay bioautography method

This method was used to determine the antimicrobial activities of sample solutions. The microorganism used for testing was supplied by the Medical Microbiology Department of the University of the Cape Town. *Staphylococcus aureus* ATCC-25923 was cultured on Nutrient agar (Oxoid iso-sensitest) and later a

suspension was prepared in Müller-Hinton medium. The developed and glass-backed analytical TLC-plates as recommended by Gibbons and Gray (1998) 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 mixture and the microorganism were mixed thoroughly. The plates were removed from the incubator as soon as the agar mixtures were prepared. Forty milliliters of the agar mixtures was 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 incubated at 37°C for 24 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 incubated for 1 hour at 37°C, after which the inhibition zones were recorded.

3.6.2 Direct Bioautography

Five hundred micrograms of each fraction (see Appendix 1) was applied to an analytical thin-layer chromatography (TLC) 20 x 20 cm glass-backed plate with a fluorescent indicator and allowed to develop to the centre of the plate (about 9 cm) using hexane: chloroform: methanol (7:2:1) as the mobile phase. This permitted 18 fractions to be evaluated per plate. After development, the solvents were allowed to evaporate completely. Antimycobacterial activity was assessed using *Mycobacterium aurum* and *M. smegmatis*. These mycobacteria were cultured in Middlebrook-7H9 broth. The developed TLC-plates were dabbed with the bacterial suspension using sterile cottonballs and incubated in a moist atmosphere at 37°C. Plates with *M. aurum* were incubated for 3 days and *M.smegmatis* for 2 days. At the end of the incubation period, the plates were sprayed with a *p*-iodonitrotetrazolium violet solution (0.4 mg/mL) that converted areas with bacterial growth into a pink colour. The sprayed plates were incubated for 1 hour at 37°C, after which the clear zones were recorded as inhibition zones. R_f-value = Inhibition zone distance from origin (midpoint) in (mm)/Solvent front distance from origin in (mm). Purified fractions (500 µg) were applied to the plates, but not allowed to develop in a solvent system, thus only inhibition zones sizes were measured. Minimum inhibition concentration values (MIC-values) of samples were determined by applying sample sizes ranging from 500 µg to 0.4 µg to the tlc-plate. Ciprofloxacin (12 µL of 1 mg/mL solution, i.e. 12 µg) was used as a drug control.

3.6.3 Parasite lactate dehydrogenase assay for *Plasmodium falciparum* sensitivity

The *in vitro* antimalarial activity was determined by using a modified method of the parasite lactate dehydrogenase assay as described by Makler *et al.* (1993). It is a colorimetric enzymatic method, which capitalizes on the distinction between parasite lactate dehydrogenase (pLDH) activity and host LDH activity, by using the 3-acetyl pyridine adenine dinucleotide (APAD) analogue of nicotinamide adenine dinucleotide (NAD). Pyruvate is converted to lactate by pLDH using APAD as a coenzyme. In the presence of the reduced APADH, the yellow coloured nitro blue tetrazolium (NBT) is converted to purple formazan salt. The concentration causing 50% growth inhibition of *P. falciparum* was measured by observing the colour conversion from yellow to blue caused by surviving parasites.

3.6.3.1 Cultivation of parasites

A culture adapted chloroquine-sensitive (CQ^s) cloned (Papua New Guinea D10) strain of *P. falciparum* that was donated by Dr. A. Cowman, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia was used in this assay. Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen (1976). The parasite cultures were maintained in complete tissue culture medium containing 10.4 g/L RPMI 1640 with glutamine, 4 g/L glucose, 6 g/L Hepes buffer, 0.088 g/L hypoxamine, 5 g/L Albumax and 1.2 mL/L (0.05 g/L) gentamicin. Type O-positive human red blood cells obtained from the Western Province Blood Transfusion Service at Groote Schuur Hospital in Cape Town were washed with wash medium [10.4 g/L RPMI 1640 with glutamine, 4 g/L glucose, 6 g/L Hepes buffer, 0.088 g/L hypoxamine, and 1.2 mL/L gentamicin] and centrifuged. Sorbitol was used to synchronise the parasitized-infected red blood cells (PRBC) in the ring stage. The PRBC were grown in sterile tissue culture flasks (Greiner Bio-One), which were flushed with gas (3% O_2, 4% CO_2, 93% N_2) for 1 minute and placed in an incubator at 37°C in 3% CO_2, 97% N_2. A medium change took place every 24 hours. The parasitaemia was observed microscopically by staining a smear of PRBC with 20% Giemsa staining solution and its requirement in this experiment was 1% with a 1% hematocrit.

3.6.3.2 Sample preparation

The sample solution was prepared with methanol: water (1:9) at a concentration of 2 mg/mL. A solvent control was included in each analysis to ensure that the antimalarial activity was because of the plant extract alone.

3.6.3.3 Assay procedure

The suspensions were dispensed in 96 well flat-bottomed microtitre plates in the following order: row 1 – blank (100 µl 1% hematocrit + 100 µl complete medium), row 2 – positive control (100 µl 1% parasite suspension + complete medium), row 3 to 12 - plant samples (conc. 100 µg/mL serially diluted two-fold in complete medium up to 0.195 µg/mL + 100 µl of 1% parasite suspension in each well). Chloroquine

diphosphate was used as the drug control and was serially diluted two-fold from 100 ng/mL to 0.195 ng/mL. Each well had a final volume of 200 µl. The microtiter plates were covered with a lid and placed in a chamber that was flushed with gas (3% O_2, 4% CO_2, and 93% N_2) for approximately 5 minutes and placed in an incubator for 48 hours at 37°C. After incubation, the samples were resuspended and 15 µl of the suspensions were transferred to 100 µl of Malstat (solution of 400 µl Triton 100, 4 g of L-lactate, 1.32 g tris-buffer and 22 mg APAD in 200 mL of mH_2O). To this was added 15 µl of NBT (solution consists of 160 mg NBT and 8 mg phenazine ethosulphate in 100 mL of mH_2O). Malstat was used to disrupt the red blood cell membranes and so expose the parasites to NBT. Air bubbles were removed with a hair dryer and the plates placed in a dark cupboard to develop. Once a colour change from yellow to purple was observed the absorbance of each well was read by using a microplate reader at 620 nm. The readings were plotted using GraphPad Prism v.2.01 and a non-linear dose response curve fitting analysis performed to determine the IC_{50}-values.

3.6.4 MTT cytotoxicity determination

The mammalian cell line, Chinese hamster ovarian (CHO) cells, was obtained from S. Schwager from the Department of Medical Biochemistry, University of Cape Town, South Africa. This method is a rapid colorimetric method for the determination of cellular growth and chemosensitivity (Mosmann, 1983) and uses methylthiazoyltetrazolium chloride (MTT) salt. The tetrazolium ring is cleaved in active mitochondria therefore only viable cells are able to reduce the yellow water-soluble coloured MTT to water-insoluble purple coloured formazan (Siewerts *et al.*, 1995).

3.6.4.1 Sample preparation

Plant samples of a concentration of 2 mg/mL were prepared in MeOH: H_2O (1:9), a solvent concentration that is not harmful to the Chinese hamster ovarian (CHO) mammalian cell line. Emetine dihydrochloride was used as the control drug.

3.6.4.2 Assay procedure

The cells were cultured in complete medium (45% Dulbecco's modified Eagle's medium, DMEM, 45% nutrient mixture F-12 HAM – Sigma ,HAMS and 10% Foetal Calf Serum, FCS). DMEM was prepared by stirring 13.53 g/L DMEM and 3.7 g/L $NaHCO_3$ in H_2O for 30 minutes, adjusting the pH to 7.1 and adding 0.05 g of the antibiotic gentamicin. DMEM was filtered using a 0.45 µm filter and filter sterilized using a 0.22 µm filter. The HAMS medium was prepared by dissolving 10.7 g/L of HAMS and 1.176 g/L NaHCO3 in water, adjusting the pH to 7.1, filtering the mixture and filter sterilizing it using a 0.22 µm filter. Both media were stored at 4°C. The foetal calf serum was heat inactivated by placing it in a 56°C water bath for 30 minutes after which it was stored at -20°C. Each sample was tested in triplicate. The initial

concentration of 100 µg/mL was dispensed into a 96 well microtiter plate and serially diluted in complete medium with 10-fold dilutions to give 4 concentrations with the lowest being 0.1 µg/mL. The cells were sub-cultured to obtain a suitable cell suspension and a small aliquot was mixed with crystal violet (1:1) to stain to enable counting. A concentration of 10^5 cells per mL was required. Row H was used as a blank and contained only complete medium. Row G to A contained 100 µl of complete medium and 100 µl of the cell suspension at concentration of 10^5 cells/mL. The final volume in each well was 200 µl. The plate was covered with a lid and placed in an incubator at 37°C for 24 hours with (5% CO_2, 95% N_2). After 24 hours the plant samples were added and the medium carefully aspirated from each well. Row G was used as a positive control and contained cells and medium only. The plant samples were added to Row F and A at different concentrations and the covered plate allowed to incubate at 37°C for 48 hours in 5% CO_2, 95% N_2. After the incubation period, 25 µl of the sterile MTT (5 mg/mL in PBS) was added to each well and the plates incubated for 4 hours at 37°C. The plates were then centrifuged at 2050 rpm for 10 minutes and the supernatant carefully aspirated from the well. The formazan crystal that remained in the wells was dissolved in 100 µl of DMSO and the plate gently shaken on the microtitre plate shaker for 5 minutes. The wells in column 1 was used to zero the reading of the spectrophotometer and the absorbance of the crystals was measured at 540 nm on a microtitre plate reader (Cambridge Technologies). The cell viability was calculated in each well using the formula:

$$\% \text{ Cell Viability} = \frac{A_{\lambda 540} \text{ test well (cells + drug)}}{A_{\lambda 540} \text{ cell control well (cells + no drug)}} \times 100$$

3.6.5 The radiometric method

The radiometric method used was described by S.H. Siddiqi in the Product and Procedure Manual of 1995 of the Bactec 460 TB System. The main component of this method is the 7H12 Middlebrook TB medium that contains the ^{14}C-labeled substrate (palmitic acid) as a source carbon. As the bacterium grows, it consumes the palmitic acid, which results in the release of $^{14}CO_2$ into the atmosphere of the sealed vial. The BACTEC-460 instrument (Johnston Laboratories, Towson MD) records the amount of $^{14}CO_2$ and processes it as a growth index (GI) on a scale of 0 – 999 (Lall and Meyer, 1999).

A sensitive *Mycobacterium tuberculosis* H37Rv ATCC strain was used in this investigation and was supplied by the Microbiology Laboratory of the National Health Laboratory Services, which is based at Groote Schuur Hospital, where it is routinely used to determine the bacterium's susceptibility to the first-line drugs rifampicin, isoniazid, and occasionally ethambutol. The bacterium was grown on Löwenstein-Jensen slants or in Bacto Middlebrook 7H9 liquid medium. A multi-drug resistant clinical isolate of *M. tuberculosis* that was resistant to rifampicin, isoniazid and ethambutol was also supplied by the same laboratory.

The sensitive *M. tuberculosis* H37Rv strain was inoculated into a BACTEC-vial containing 4 mL 7H12 Middlebrook TB medium. When the growth index of the bacterium vial reached values of 300 to 500, the broth culture was considered ready to be introduced into the test and control vials, which each contained 4 mL 7H12 Middlebrook TB medium. The test vials contained 0.1 mL of the broth culture and plant extract while the two positive control vials or drug controls contained 0.1 mL of the broth culture and 0.1 µg/mL isoniazid and 2.0 µg/mL rifampicin each. In addition, two negative controls were used, one with only 0.1 mL of broth culture and another prepared by adding 0.1 mL of a 1:100 dilution of the broth culture. The dilution was prepared in a special diluting fluid. For the plant extracts or purified fractions, a 5 mg/mL solution in 12.5% methanol or ethanol in Bacto Middlebrook 7H9 broth was prepared The plant sample test vials received 100 µL of each 5 mg/mL plant extract resulting in a 40-fold dilution. Each sample vial therefore contained 500 µg of extract or a 125 µg/mL sample solution. All the vials were incubated at 37°C and the growth index (GI) determined daily at the same time over a period of 4 to 12 days until the GI of the 1:100 control vial reached 30 or more. The sample vials with GI-values below or equal to that of the 1:100 control vials on the final day of the assay were considered active against *M. tuberculosis* at a concentration of 12 µg/mL. The percent inhibition was calculated as 1 – (growth index of test sample/growth index of control) x 100 = % as done by König *et al.* (2000).

3.7 Instrumental analysis

3.7.1 Nuclear Magnetic Spectroscopy

^{1}H and ^{13}C NMR spectra were run on a Varian UNITY-Inova Spectrometer using $CDCl_3$ or CD_2Cl_2 at 600 MHz and 150 MHz, respectively. The spectra were further processed using MestrRe-C Beta version 3.7.1 obtained from mestrec.com.

3.7.2 Mass Spectrometry

The GC-MS analyses were recorded at 70 eV using a Carlo Erba GC 6000 Vega Series equipped with a 40 m X 0.3 mm special performance capillary column (Lexus, PS089-OH) silanol-terminated (95%)-methyl-(5%)-phenylpolisiloxane copolymer stationary phase and He as the carrier gas. Analyses were performed using the following conditions: initial temperature, 130 °C; rate of increase 3 °C /min; final temperature, 270 °C, hold 10 minutes. The LC-MS spectra obtained for the initial identification of 2-methoxyethyl methacrylate were obtained in electron impact (EI) mode using a direct probe in an AMD 604 (AMD Intectra GmbH) mass spectrometer. The rest of the LC-MS/MS analysis was performed on an Applied Biosystems Sciex API-4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) using atmospheric pressure chemical ionization (APCI) for ion detection in an acidic chemical environment

(0.1% formic acid : acetonitrile 50%). The samples were infused at a flowrate of 10 µl/min. APCI was conducted in the positive mode with nitrogen as the nebulizer and an ion spray voltage of 5500.

3.7.3 High-Performance Liquid Chromatography

The initial chromatography in which 2-methoxyethyl methacrylate was isolated was done using a Shimadzu Liquid Chromatograph LC-10AS with an auto-injector, communications bus module, and Shimadzu diodearray detector SPD-M10A. Semi-preparative reverse phase HPLC was performed using a semi-preparative Higgins Analytical HAISIL 100 C_{18} column, 250 x 10 mm P/N HS-2510-M185, injection volume of 100 µL with a flow rate of 3 mL/min over a run time of 40 minutes and solvent gradient of 20% - 100% acetonitrile in water. Fractions were collected every 2 minutes using a fraction collector. This was repeated many times until sufficient sample material was available for each fraction for the bioassays. Analytical HPLC was performed using an octadecyl silica column (Supelco Discovery® C18 column, 15 cm x 4.6 mm, 5 µm). Separations were accomplished at room temperature with a solvent gradient of 20% - 100% acetonitrile in water in 30-minute long runs with a flow rate of 1 ml/min. In the second attempt to isolate the antimycobacterial compound(s) from *Polysiphonia virgata*, preparative separation was achieved using a Spectra-Physics IsoChrom LC HPLC equipped with a rheodyne injector, a Waters R401 Differential Refactometer, a Rikadenki chart recorder and a Whatman Partisil 10 column. The mobile phase used in this purification was hexane (85%) in ethyl acetate at a flow rate of 4 mL/min. Before injecting the sample into the column the extract was washed through a small silica column prepared by putting slurry of silica 60 in 80% hexane in ethyl acetate into a plugged Pasteur pipette. The portion that eluted from the column was further purified using the Spectra-Physics IsoChrom LC HPLC.

Chapter 4

ANTI-TUBERCULOSIS ACTIVITY OF *GELIDIUM PRISTOIDES*, *PLOCAMIUM CORALLORHIZA*, AND *POLYSIPHONIA VIRGATA* AND THE ANTI-PLASMODIAL ACTIVITY OF A FEW OF THE ALGAL EXTRACTS

4.1. Introduction

The algae that were investigated here have not been screened for anti-tuberculosis activity previously. In an antimicrobial screen of seventeen red algae collected in the Cape Peninsula, which was done at the University of the Western Cape by Cameron *et al.* (2001), it was shown that the crude extracts of *Plocamium corallorhiza* and *Gelidium pristoides* inhibited the growth of *Mycobacterium smegmatis*, a rapidly growing non-pathogenic mycobacterium (Howard and Byrd, 2000). In addition, the crude extract of *P. corallorhiza* also inhibited the growth of the fungus *Candida albicans*, while the crude extracts of *G. pristoides* and *P. virgata* inhibited the growth of the bacterium *Staphylococcus aureus.* At fractionation, the fractions of *G. pristoides* and *P. virgata* inhibited the growth of *S. aureus*, while the fractions of *P. corallorhiza* inhibited the growth of both *S. aureus* and *M. smegmatis*. Consequently, these findings served as strong justification for the selection of these species for this investigation. *G. pristoides* and *P. corallorhiza* were selected for their inhibitory activity against *M. smegmatis* and *P. virgata* was selected for its inhibition of *S. aureus* and its potential antimycobacterial activity.

Initially, the crude dichloromethane extracts of each alga showed no anti-tuberculosis activity, but when further purified using the Chromatotron, the anti-tuberculosis activity became evident in some of the fractions of each alga. However, as the purification continued, incorporating preparative layer chromatography, many of the individual compounds that constituted the purified fractions were reduced to 1 mg amounts or less, making complete structure elucidation with accessible instrumentation impossible. This chapter reports on the anti-tuberculosis activities of the purified fractions observed as the extracts were purified using the Chromatotron and preparative layer chromatography. Anti-tuberculosis activity was established as previously mentioned by selecting the samples with final growth index values of below or equal to the growth index of the 1:100 control. In addition, samples with GI-values above that of the 1:100 controls that appeared low relative to the maximum GI-values obtained per assay were also selected for further purification, bearing in mind that the active compound(s) could be in low concentration and

therefore give high growth indexes. The antiplasmodial activity of the initial crude extracts and purified fractions was also determined, as well as the cytoxicity of selected extracts.

4.2. Results

To confirm the findings of the project done by Cameron *et al.* (2001), the agar-overlay bioautography method was used to determine the inhibitory activity of the crude dichloromethane (DCM): methanol (MeOH) (2:1) algal extracts (for yields see Table 4.1) against *S. aureus* (Table 4.2).

Table 4.1. A record of the seaweed samples collected and the yields of obtained.

Seaweed	Sample no.	Wet mass (g)	Extract dry mass (g)	% yield
Gelidium pristoides	101.1	1000.42	27.89	2.8
Plocamium corallorhiza	102.1	1015.72	18.17	1.8
Polysiphonia virgata	103.1	495.32	8.08	1.6

Table 4.2 Rf-values obtained for the crude extracts in the agar-overlay bioautography method against *Staphylococcus aureus*.

SAMPLE NO.	ZONE	SAMPLE ZONE (MM)	FRONT (MM)	ZONE SIZE (MM)	RF-VALUE
***Gelidium pristoides* (101.1)**	1	49	165	10	0.30
		45	163.5	10	0.28
		44	165	10	0.27
	2	31	165	1	0.19
		28	163.5	2	0.17
		26	165	2	0.16
***Plocamium corallorhiza* (102.1)**	1	125	166	12	0.75
		124	166	10	0.75
		125	166	5	0.75
	2	46	166	8	0.28
		44	166	8	0.27
		50	166	9	0.30
	3	25	166	2	0.15
		20	166	1	0.12
		25	166	3	0.15
	4	17	166	2	0.10
		15	166	2	0.09
		17	166	2	0.10
***Polysiphonia virgata* (103.1)**	1	45	158	4	0.28
	top most zone	44	158	4	0.28
		40	158	4	0.25
	2	0	158	32	0.00
	At origin	0	158	35	0.00
		0	158	33	0.00

The plates had inhibition zones for *G. pristoides* (Figure 4.1) and *P. virgata* with the same Rf-values as well as new inhibition zones. The extract of *P. corallorhiza* showed zones of inhibition that were not present in the crude extracts of the abovementioned project.

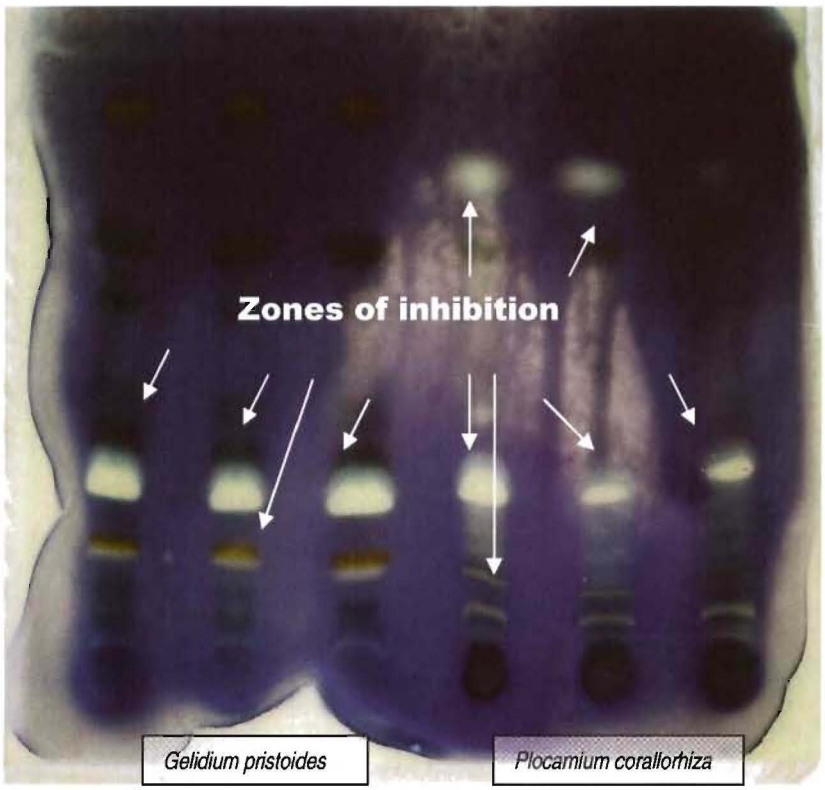


Figure 4.1. The inhibitions zones obtained against *Staphylococcus aureus* for the crude extracts of *Gelidium pristoides* and *Plocamium corallorhiza* in the overlay bioautography method.

Before the extracts were introduced into the BACTEC-460 system, attempts were made to test the extracts against *Mycobacterium aurum*, a bench top safe fast-growing mycobacterium, but problems occurred with culturing the organism. The DCM: MeOH (2:1) extracts and some of the fractions obtained using preparative layer chromatography were tested in a parasite lactate dehydrogenase assay against the sensitive D10 strain of *Plasmodium falciparum* (Figure 4.2 and 4.3).

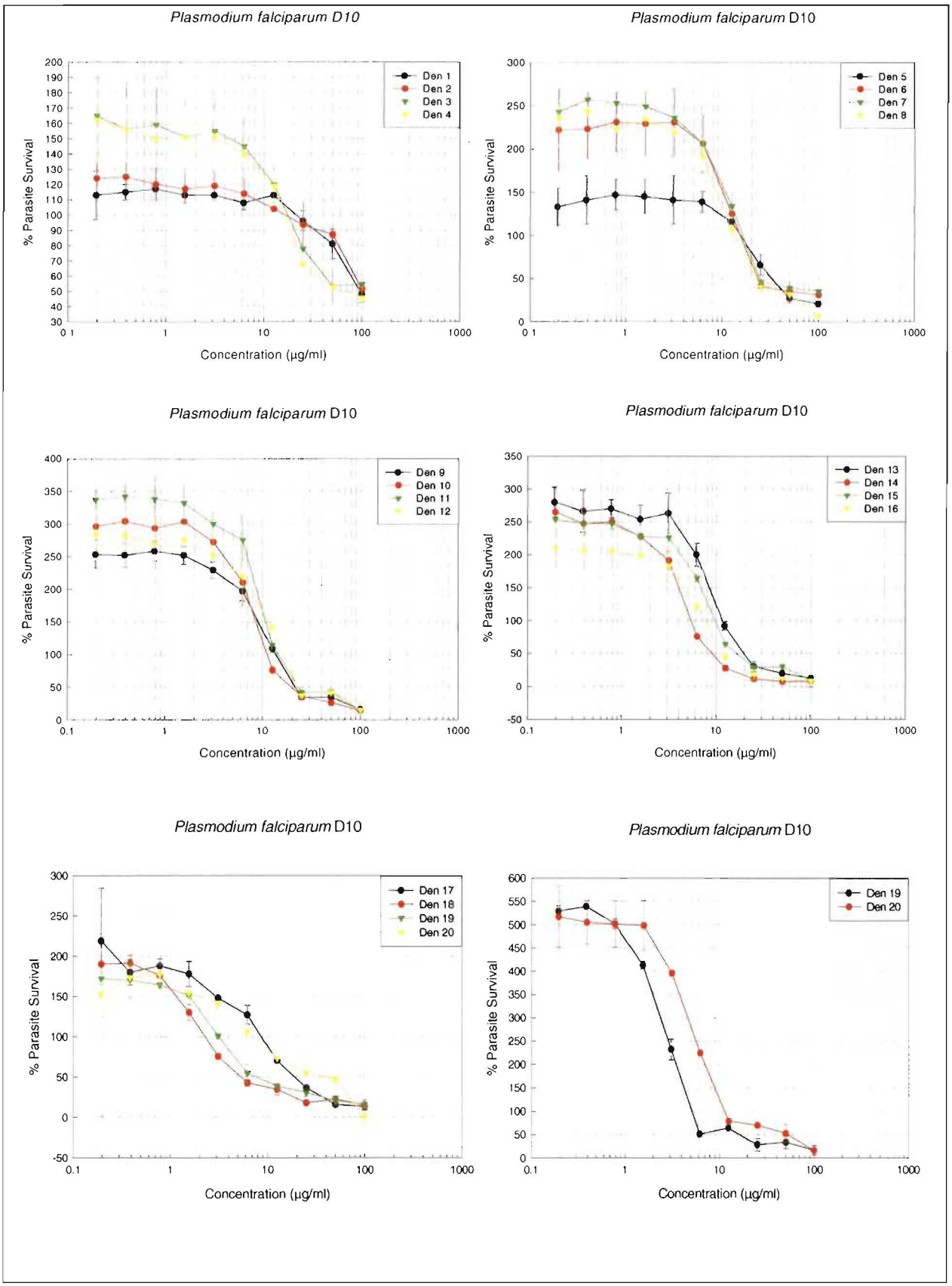


Figure 4.2. Dose-response curves obtained in the Parasite lactate dehydrogenase assay for *Plasmodium falciparum* sensitivity. Den 1 to Den 20 refers to fraction number 1 to fraction number 20 of the extract of *Plocamium corallorhiza* obtained using semi-preparative HPLC.

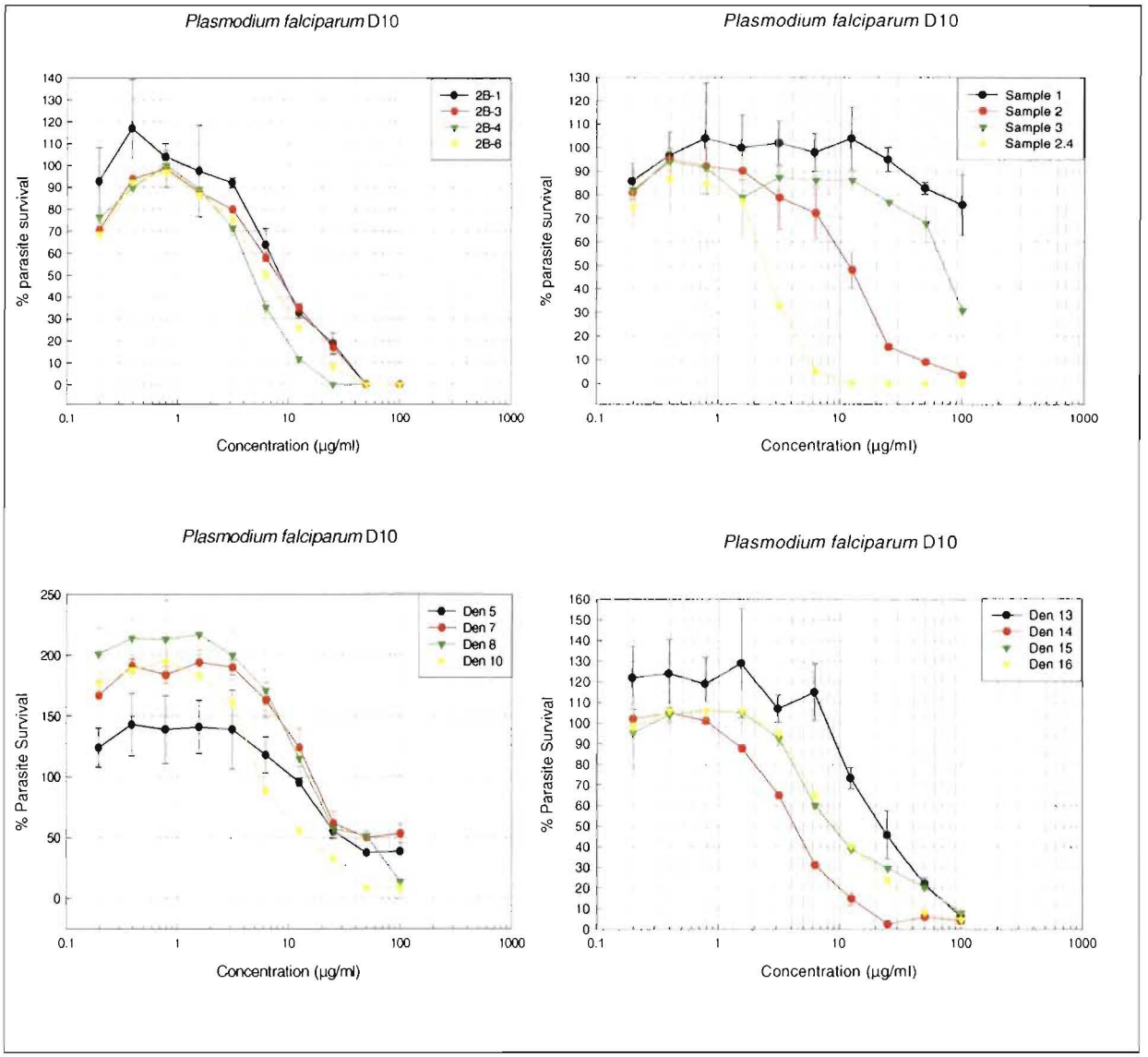


Figure 4.3. Dose-response curves obtained in the Parasite lactate dehydrogenase assay for *Plasmodium falciparum* sensitivity. 2B-samples refers to fractions of sample 102 (*Plocamium corallorhiza*) and Den-samples to the samples that were retested.

In this screen, the crude extract of *P. corallorhiza* inhibited the growth of *P. falciparum* with an MIC-value of 12.5 µg/mL (Table 4.3). Of the purified fractions, four fractions had antiplasmodial activity with MIC-values below 10 µg/mL. Although the crude extract of *G. pristoides* had a MIC-value of more than 100 µg/mL, three of its fractions inhibited the growth of *P. falciparum* with MIC-values below 20 µg/mL. The *P. virgata* crude extract had a MIC-value of 70 µg/mL and was not further investigated. Of the crude extracts that were tested in the parasite lactate dehydrogenase assay for *Plasmodium falciparum* sensitivity, that of *P. corallorhiza* and *P. virgata* showed the most potential with IC_{50}-values of 12.5 µg/mL and 70.0 µg/mL, respectively. The crude extract of *G. pristoides* had an IC_{50}-value of more than 100 µg/mL while the purified fractions had significant IC_{50}-values of 12.0 to 37.5 µg/mL demonstrating the importance of

Table 4.3. IC_{50}-values of the DCM:MeOH (2:1) crude extracts and their preparative layer chromatography purified fractions in the parasite lactate dehydrogenase assay against D10 strain of *Plasmodium falciparum*.

SAMPLE NO.	IC_{50}-values in µg/mL
101.1	>100
B1	37.5
B2	17.0
B3	12.0
B4	19.0
B5	22.5
B6	>100
102.1	12.5
B1	8.9
B3	8.0
B4	4.7, 2.4
B6	6.4
103.1	70.0

B in the sample no. refers to band: B1 being the least polar band and B6 the most polar band on the thin-layer chromatography plate.

fractionation when working with plant extracts. The fractions of *P. corallorhiza* were even more inhibitory to the growth of *P. falciparum* with IC_{50}-values of 2.4 to 8.9 µg/mL. For this reason, *P. corallorhiza* was selected for further purification on the HPLC and the 20 fractions so obtained were tested for antiplasmodial activity. Fraction 14 and 18 gave the best IC_{50}-values of 4.2 and 5.2 µg/mL, respectively (Table 4.4).

Table 4.4 IC_{50}-values of the HPLC purified DCM:MeOH (2:1) extract that were tested in the parasite lactate dehydrogenase assay against D10 strain of *Plasmodium falciparum*.

Fractions of Sample 102.1	IC_{50}-values in µg/mL	Repeats – IC_{50}-values (µg/mL)
1	95	Not tested
2	100	Not tested
3	100	Not tested
4	66	Not tested
5	34	30
6	24	Not tested
7	24	50
8	23	50
9	22	Not tested
10	20	16
11	24	Not tested
12	24	Not tested
13	20	12
14	9	4.2
15	18	9.0
16	12	9.6
17	Not reliable	19.0
18	Not reliable	5.2
19	Not reliable	8.0
20	50	40.0

Other fractions also showed potential with IC_{50}-values below 10 µg/mL. These results were consistent with the results obtained using preparative layer chromatography. The data of this assessment is reported in Appendix 2. When the same fractions were tested in the Bactec-460 method, two fractions, namely, 18 and 19 significantly inhibited the growth of *M. tuberculosis* (Table 4.5). These were further purified, but insufficient sample masses made structure analysis of the isolated compounds unobtainable. At this stage, the Chromatotron was introduced as an alternative method for purification.

Table 4.5 Growth-indexes of the 20 fractions of crude collected on the HPLC over. The assay ran over the period 24 October 2002 to 4 November 2002.

Sample no.	24/10	25/10	26/10	27/10	28/10	29/10	30/10	31/10	01/11	02/11	03/11	04/11
1:100 control	2	1	0	0	0	1	2	7	10	15	23	30
INH	10	7	4	4	3	2	3	2	2	2	4	3
RIF	6	3	1	0	0	0	0	0	0	0	0	0
Negative control	12	22	45	86	145	229	307	335	363	446	565	521
1	13	23	46	82	131	210	296	317	354	444	570	560
2	9	21	54	118	216	355	473	458	500	635	689	527
3	13	21	44	73	123	180	246	276	294	402	481	535
4	15	31	52	90	138	191	261	296	331	446	431	559
5	13	24	46	80	130	204	287	365	376	501	604	517
6	9	16	34	61	98	150	177	208	261	383	487	437
7	10	17	35	64	102	158	218	246	276	330	494	528
8	12	21	36	61	92	162	233	290	348	424	481	490
9	9	16	29	51	87	142	207	260	307	409	522	486
10	12	17	29	46	63	91	120	152	187	231	331	348
11	9	18	35	59	97	152	209	240	289	376	433	432
12	12	21	41	75	132	212	297	322	355	462	545	497
13	11	22	40	71	111	174	245	259	315	423	495	469
14	7	8	13	21	33	53	76	114	157	212	275	302
15	11	18	32	59	78	121	171	180	241	314	343	393
16	12	20	31	47	72	109	142	185	221	267	310	343
17	9	11	14	16	23	38	50	76	119	172	243	290
18	7	5	6	8	10	10	13	15	17	18	17	12
19	5	5	7	7	7	10	12	11	13	15	15	18
20	11	19	32	55	79	116	166	187	212	260	305	333

Sample numbers 1 to 20 refers to the purified fractions of *Plocamium corallorhiza* collected from the semi-preparative HPLC column. INH = isoniazid; RIF = rifampicin. Negative control = bacterium vial without a plant extract or control. The rows marked in red indicate the significantly antimycobacterial fractions.

When the crude extracts were fractionated using the Chromatotron, the first fractions were tested in the BACTEC-460 system against *M. tuberculosis*, but no inhibition of the mycobacterium was observed (Table 4.6). However, on further fractionation of the dichloromethane portion, all three algae had purified fractions with anti-tuberculosis activity (Table 4.7, 4.8, and 4.10). The water-soluble portion of all three crude extracts showed no inhibition of *M. tuberculosis* (sample 101, 102, and 103 in Table 4.12) and was not cytotoxic against the Chinese hamster ovarian cells in the MTT cytotoxicity determination with IC_{50}-values of above 100 µg/mL (Figure 4.4).

Table 4.6 Growth-indexes of the first fractions obtained after purification of the crude extracts using the Chromatotron.

Spec	08/08/03	09/08/03	10/08/03	11/08/03	12/08/03	13/08/03	14/08/03
1:100 control	4	4	6	12	18	29	45
Negative control	104	200	305	395	474	598	709
MeOH	111	231	357	494	654	810	867
12	53	90	149	227	308	393	483
13	57	125	207	331	471	651	798
14	54	127	231	372	484	656	828
15	55	140	282	423	452	354	298
16	42	130	298	473	619	725	855
21	77	182	309	453	564	712	885
22	96	203	321	462	695	878	999
23	91	198	285	414	584	778	939
24	99	220	351	451	730	894	999
25	121	261	397	534	747	913	898
26	111	238	374	498	641	798	869
31	96	220	351	512	640	803	880
32	111	230	341	477	599	768	869
33	130	276	422	578	738	759	919
34	126	271	398	564	114	798	803
35	84	194	315	434	614	810	869
38	104	218	326	434	604	779	875
39	84	196	350	502	706	884	964
112	80	187	312	470	653	864	860
114	69	149	248	350	529	726	852
115	102	222	353	503	653	833	941
116	124	272	432	559	778	957	999
117	70	136	203	267	319	408	427

Sample numbers starting with 1, 2 and 3 refers to the fractions of *Gelidium pristoides*, *Plocamium corallorhiza* and *Polysiphonia virgata*, respectively.INH = isoniazid; RIF = rifampicin. Negative control = bacterium vial without a plant extract or control. MeOH = methanol control.

Table 4.7 Growth-indexes of the purified fractions of the algae investigated over the period 6 September to 17 September 2003.

Sample no.	06/09	07/09	08/09	09/09	10/09	11/09	12/09	13/09	14/09	15/09	16/09	17/09
1:100 control	0	0	0	0	1	0	0	5	7	14	19	34
INH	5	4	0	1	0	1	1	1	0	1	0	1
RIF	2	0	0	0	0	0	0	0	0	0	0	0
ETHAM	9	9	8	5	4	3	4	2	2	2	2	3
Negative Control	10	14	32	52	91	167	252	369	462	542	575	578
1111	8	13	29	50	95	172	265	436	433	477	449	351
1112	4	7	17	35	72	126	212	313	375	448	516	480
112	5	10	23	43	80	161	295	411	489	655	672	628
114	10	12	19	25	31	54	70	110	141	155	213	296
115	8	12	24	38	69	80	92	104	123	198	189	228
116	7	10	21	38	no result	119	165	262	363	376	506	686
117	15	32	68	108	169	263	370	577	743	950	940	784
1131	17	34	68	102	137	210	264	390	505	472	441	430
1132	12	26	58	84	138	228	304	441	516	547	564	571
1133	5	12	30	50	79	146	213	356	484	604	649	585
1134	11	22	48	84	142	237	345	536	637	687	604	543
1135	15	29	66	107	175	298	409	547	566	499	366	347
11321	8	13	28	39	61	97	135	203	284	358	453	504
11322	8	12	26	44	70	116	178	no result	391	525	543	472
11323	6	10	18	24	30	43	58	289	105	155	210	237
11324	14	28	52	84	134	243	342	526	607	653	588	485
11325	10	15	25	39	52	110	149	250	363	523	656	739
11326	10	25	53	88	152	256	342	507	598	680	662	550
11327	17	33	57	89	136	209	268	402	517	622	581	582
141(1)	9	17	35	62	195	172	249	363	437	487	524	449
142(1)	4	6	15	33	64	131	208	348	506	678	747	796
143(1)	8	12	31	62	124	237	397	no result	999	999	935	590
144(1)	8	19	44	77	125	207	340	456	557	660	637	543
145(1)	9	18	43	74	137	247	373	543	645	630	661	608
141(2)	9	17	33	52	79	117	189	236	306	380	430	478
142(2)	4	5	12	30	70	149	244	421	635	743	888	866
143(2)	4	9	27	67	142	283	463	786	955	981	873	655
144(2)	7	14	30	58	99	167	260	326	406	484	502	552
145(2)	5	7	18	41	77	159	251	408	525	599	684	610
151	6	7	23	41	80	152	236	386	572	604	662	639
152	7	11	26	48	90	167	241	364	480	597	691	668
153	3	3	12	27	47	95	162	280	412	487	562	623
154	4	7	21	45	90	180	281	410	497	482	664	595
221	2	3	0	3	1	4	7	5	9	1	15	20
222	4	3	3	3	3	4	3	5	5	5	6	9

Sample numbers starting with 1 and 2 refers to the fractions of *Gelidium pristoides and Plocamium corallorhiza* respectively. INH = isoniazid; RIF = rifampicin; ETHAM = ethambutol; Negative control = bacterium vial without a plant extract or control. The rows marked in blue indicated relative moderate activity, while those in red indicate significant antimycobacterial activity.

Table 4.8 Growth-indexes of the purified fractions investigated over the period 11 September to 22 September 2003.

Sample no.	11/09	12/09	13/09	14/09	15/09	16/09	17/09	18/09	19/09	20/09	21/09	22/09
1:100 control	0	0	0	3	186	999	999	Contaminated				
INH	12	1	3	3	1	0	2	3	Not read	Not read	Not read	Not read
RIF	8	Not read	0	0	0	0	0	0	Not read	Not read	Not read	Not read
Negative control	10	5	31	75	142	241	323	391	420	430	403	469
223	7	0	8	19	35	64	133	225	327	431	577	644
161	3	0	0	2	6	14	23	52	93	153	252	366
162	9	4	31	91	172	306	491	675	740	756	890	582
163	8	3	25	60	132	247	474	590	611	570	600	476
164	7	4	28	77	171	350	669	909	939	780	680	543
165	7	3	20	60	137	269	487	651	687	684	716	584
166	7	3	23	68	139	281	527	738	690	738	657	495
167	6	2	17	51	114	250	517	771	870	884	645	445
16	7	3	24	67	132	261	481	no reading	523	472	469	454
311	10	2	5	12	23	54	117	888	386	571	666	680
312	9	1	5	8	10	10	13	16	15	17	19	22
313	7	1	1	3	3	6	8	18	20	43	86	167
314	7	1	1	3	2	3	5	13	23	52	116	242
315	8	2	2	5	4	7	13	27	57	120	221	358
316	9	2	9	17	24	38	70	120	183	324	270	407
317	8	4	30	71	160	273	447	577	530	481	410	408
381	8	2	11	33	70	135	250	411	454	481	484	442
382	10	4	22	53	112	196	309	378	461	519	524	575
383	10	3	19	47	104	188	293	444	470	559	574	497
384	6	2	10	29	73	162	338	537	497	570	607	557
385	8	3	30	80	164	295	479	676	632	665	639	573
36	6	0	10	30	70	156	285	474	538	690	773	780
37	8	4	25	69	137	256	358	469	582	737	656	586

Sample numbers starting with 1, 2 and 3 refers to the fractions of *Gelidium pristoides*, *Plocamium corallorhiza* and *Polysiphonia virgata*, respectively. INH = isoniazid; RIF = rifampicin; Negative control = bacterium vial without a plant extract or control. The rows marked in blue indicated relative moderate activity, while those in red indicate significant antimycobacterial activity.

Table 4.9 Anti-tuberculosis activities of the pure compounds, methoxyethyl methacrylate and hydroxyethyl methacrylate investigated over the period 27 April to 3 May 2004..

Sample no.	01/10	02/10	03/10	04/10	05/10	06/10	07/10	08/10
1:100 control	2	-	0	0	5	14	28	50
INH	33	-	32	29	26	23	22	18
RIF	15	-	9	6	5	3	2	1
Negative control	82	-	293	446	593	720	826	708
Solv EtOH	64	-	313	520	519	940	909	798
Solv MeOH	81	-	387	611	877	988	965	751
211	26	-	64	93	151	217	294	328
212	16	-	30	44	58	77	111	123
231	41	-	154	245	372	422	513	559
232	35	-	80	102	127	145	175	203
233	34	-	121	207	353	491	624	665
234	28	-	143	285	488	668	750	691
235	38	-	210	424	739	999	999	955
261	40	-	122	194	291	360	421	456
262	36	-	184	301	458	548	646	693
263	40	-	204	350	577	862	999	999
264	39	-	232	417	681	916	999	998
265	55	-	247	392	585	688	803	746
266	46	-	251	422	599	733	863	894
267	39	-	222	365	554	756	920	944

Sample numbers 2 refers to the fractions of *Plocamium corallorhiza*. INH = isoniazid; RIF = rifampicin; Negative control = bacterium vial without a plant extract or control. (-) indicates that no reading was taken. The rows marked in blue indicated relative moderate activity. Solv EtOH = ethanol control; Solv MeOH = methanol control.

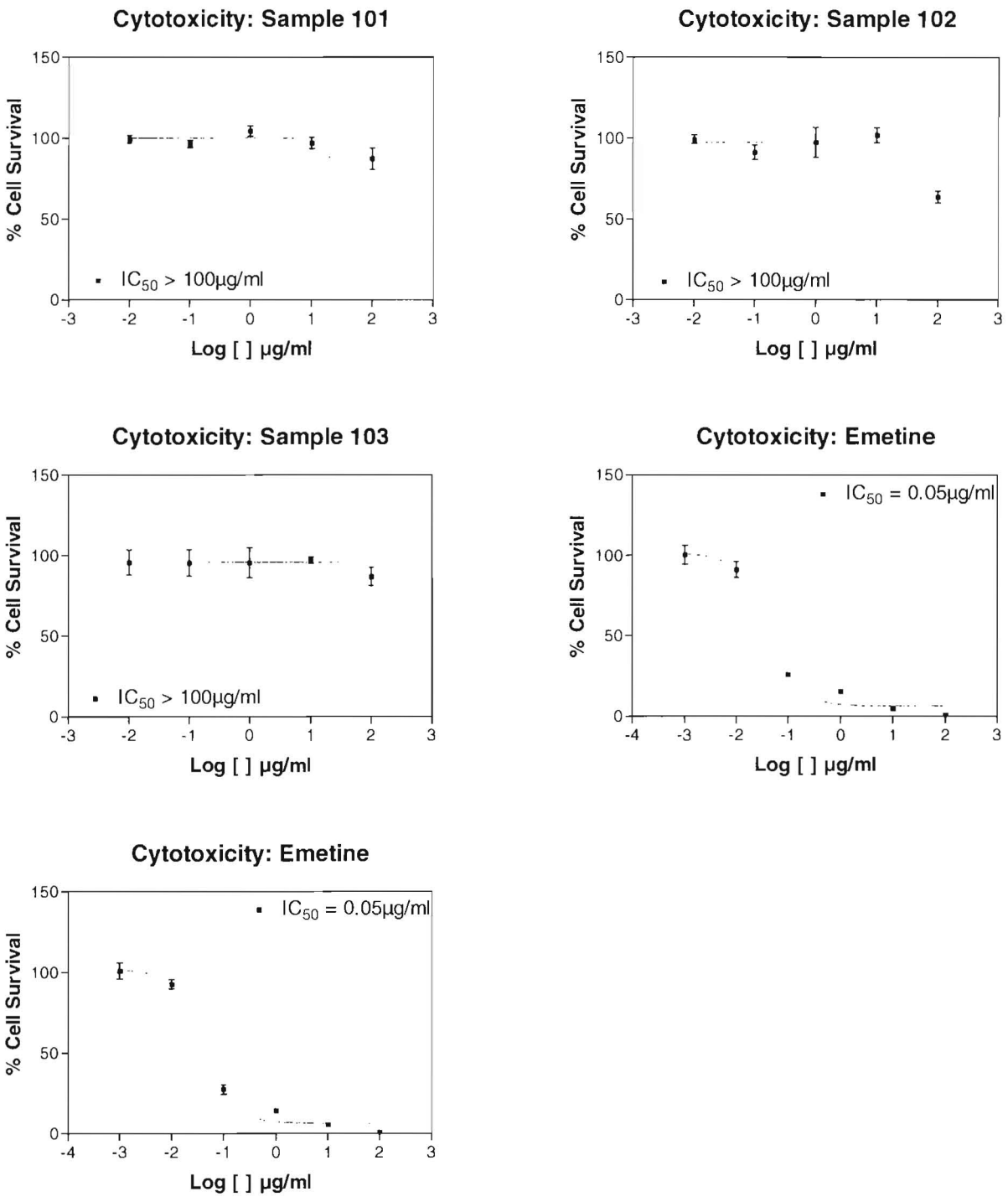


Figure 4.4. The percentage cell survival recorded for the water portions of Sample 101 (*G.pristoides*), 102 (*P. corallorhiza*) and 103 (*P. virgata*).

4.2.1 Anti-tuberculosis activity of purified fractions of *Gelidium pristoides*

The purified fractions numbered 122 and 123 inhibited the growth of *M. tuberculosis* by 91.7 and 79.2%, respectively, at a concentration of 12.5 µg/mL (Table 4.10 and Table 4.15). Other fractions that were also selected included fractions numbered 161 (Table 4.8) and 124 (Table 4.10). Both showed inhibition relative to the completely non-active fractions.

Table 4.10 Growth-indexes of the purified fractions investigated over the period 7 November to 23 November 2003.

Sample no.	07/11	08/11	09/11	10/11	11/11	12/11	13/11	14/11	15/11	16/11	17/11	18/11	19/11	20/11	21/11	22/11	23/11
1:100 control	1	0	0	0	0	0	1	1	3	3	5	0	1	2	9	21	48
INH	13	10	6	3	2	1	2	1	1	2	4	4	7	9	13	19	29
RIF	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
121	7	7	8	9	10	60	807	999	451	208	485	403	207	189	284	483	507
122	4	3	2	3	1	2	1	1	2	2	2	2	3	3	3	1	4
123	1	1	1	0	0	1	1	2	1	0	3	1	3	4	4	6	10
124	3	3	3	3	4	7	13	20	28	18	24	19	27	39	55	86	144
125	5	4	6	11	209	244	248	283	456	949	835	351	126	60	36	26	21
131	6	11	17	26	46	70	131	215	322	418	728	655	598	530	427	338	285
132	2	0	3	31	71	96	111	115	145	203	376	516	664	839	855	768	609
133	7	10	31	38	76	139	273	465	604	718	973	806	656	537	415	261	183
241	6	6	6	9	10	14	23	33	48	66	131	184	265	399	461	522	631
242	6	6	9	15	27	43	77	132	191	268	496	531	506	542	584	545	506
243	11	13	29	54	102	179	336	549	710	814	979	789	589	451	334	215	144
244	10	15	32	57	102	176	335	535	691	755	795	583	425	339	258	181	139
332	3	2	2	3	3	5	6	8	9	10	12	7	7	6	6	7	8
333	3	3	2	0	2	2	2	1	1	1	3	0	2	0	0	0	0
334	13	23	44	72	120	172	284	407	447	493	580	562	533	523	456	343	277
341	11	21	39	61	94	137	236	356	472	512	671	661	593	562	501	362	270
344	5	9	12	17	23	32	41	50	69	80	122	128	155	182	220	259	304
345	7	10	15	23	29	41	76	122	197	275	524	644	593	583	537	427	391
346	20	30	52	76	118	183	303	483	611	627	689	539	428	349	287	212	170

Sample numbers starting with 1, 2 and 3 refers to the fractions of *Gelidium pristoides*, *Plocamium corallorhiza* and *Polysiphonia virgata*, respectively. INH = isoniazid; RIF = rifampicin. The rows marked in blue indicated relative moderate activity, while those in red indicate significant antimycobacterial activity.

Table 4.11 Growth-indexes of the purified fractions investigated over the period 12 November to 2003 November 2003.

Sample no.	12/11	13/11	14/11	15/11	16/11	17/11	18/11	19/11	20/11
1:100 control	1	0	0	0	1	7	11	19	30
Negative control	29	54	91	154	238	472	538	570	589
EtOH	20	47	96	185	312	663	749	833	826
134	11	26	62	135	252	415	555	677	713
135	10	16	35	75	152	382	527	712	773
321	5	2	3	3	3	6	5	6	9
322	4	1	1	2	0	0	1	1	0
323	5	2	1	1	0	1	0	1	0
351	8	14	26	45	75	189	250	437	588
352	14	30	49	76	113	241	316	501	627
353	17	37	80	171	299	634	634	665	675
354	13	29	56	109	164	323	325	418	486
355	15	34	78	158	285	617	724	733	722
356	16	38	85	175	299	565	581	622	611
391	12	25	55	116	202	331	399	519	588
392	12	31	73	143	226	471	615	712	696
393	17	38	80	157	262	572	700	740	720
394	16	38	84	173	301	587	575	573	564

Sample numbers starting with 1 and 3 refers to the fractions of *Gelidium pristoides* and *Polysiphonia virgata*, respectively. Negative control = bacterium vial without a plant extract or control; EtOH = ethanol solvent control. The rows marked in red indicate significant antimycobacterial activity.

4.2.2 Anti-tuberculosis activity of purified fractions of *Plocamium corallorhiza*

This alga was purified using semi-preparative HPLC and the Chromatotron. Of the 20 fractions repeatedly collected from the semi-preparative HPLC column, only two fractions inhibited the growth of *M. tuberculosis* in the BACTEC-460 method (Table 4.5). This was a time consuming procedure with small fraction quantities as a reward. Preparative layer chromatography has the same drawback for large quantities of crude material. This served as a strong motivation for the purchase of the Chromatotron. Fractions 212 and 232 were only weakly active (Figure 4.9). Fractions 221 and 222 obtained from the Chromatotron inhibited the growth of *M. tuberculosis* by 41.2 and 73.5%, respectively, at a concentration of 12.5 µg/mL (Table 4.7 and Table 4.15). These fractions were combined as they were collected as successive bands from the Chromatotron and purified further.

Table 4.12 Growth-indexes of the purified fractions investigated over the period 2 December to 11 December 2003.

Sample no	02/12	03/12	04/12	05/12	06/12	07/12	08/12	09/12	10/12	11/12
1:100 control	2	1	3	2	3	6	9	14	24	37
Negative control	10	25	45	89	177	343	469	580	684	713
MeOH	16	31	61	115	206	373	483	446	480	523
EtOH	11	23	50	105	208	373	515	554	607	591
101	5	12	23	58	132	280	421	520	644	665
102	7	10	20	53	120	245	403	541	748	795
103	5	6	11	24	58	135	249	429	535	705
251	4	5	9	21	54	135	255	378	425	569
252	5	10	14	21	43	93	153	240	304	409
253	8	15	30	66	180	290	412	504	613	658
254	13	26	49	94	170	339	473	582	637	724
255	9	15	29	61	129	239	439	547	581	677
256	11	21	35	71	142	291	415	518	608	666
386	8	19	41	89	177	328	457	551	688	666
1134	insufficient to test									
1173	10	17	32	68	130	232	333	457	548	670

Sample numbers starting with 1, 2 and 3 refers to the fractions of *Gelidium pristoides*, *Plocamium corallorhiza* and *Polysiphonia virgata*, respectively. Negative control = bacterium vial without a plant extract or control. MeOH = methanol control, EtOH = ethanol control.

4.2.3 Anti-tuberculosis activity of the purified fractions of *Polysiphonia virgata*

The purified fractions 312 (the red marked sample in Table 4.8), 321,322, 323 (samples marked in red in Table 4.11), 332 and 333 (red marked samples in Table 4.10) showed *M. tuberculosis* inhibition of 26.7%, 70.0%, 100.0%, 100.0%, 83.3% and 100.0%, respectively (Table 4.15). Of these fractions, 312 was further purified while samples 321, 322, 323, 332 and 333 were not further purified due to low sample masses. Other fractions selected for further purification included fractions no. 313, 314 and 315 with more sample material and weak anti-tuberculosis activity (Table 4.8). Each fraction was purified until either the sample size became too small or the chromatography, using thin-layer chromatography and HPLC, indicated that a pure compound was obtained.

As the bioactive fractions were further purified, the sample masses in many cases became too small for further testing. To ensure a complete account of bioactivity, some of the purified fractions were combined and tested against *M. tuberculosis* (Tables 4.13 and 4.14). The active fraction 312 separated into many fractions with small sample masses. When those fractions were recombined fraction 31271 – 3128 inhibited the growth of *M. tuberculosis* (marked in red in Table 4.14) with a MIC-value of 12.5 µg/mL. Fraction 314 that showed moderate activity when compared with fraction 312, on further purification produced two active fractions, namely 3148A and 3147 that significantly inhibited the growth of *M. tuberculosis* at a MIC-value of 12.5 µg/mL (Table 4.14).

Table 4.13 Growth-indexes of the purified fractions investigated over the period 19 May to 26 May 2004.

Sample no.	19/05	20/05	21/05	22/05	23/05	24/05	25/05	26/05
1:100 control	1	0	0	2	5	13	24	55
Negative control	36	81	146	266	428	5548	594	640
INH	21	18	12	10	13	11	10	13
RIF	10	4	3	1	0	0	0	0
1611-1616 (12.5 µg/mL)	10	19	46	115	235	384	452	569
1611-1616 (6.25 µg/mL)	17	39	89	195	363	467	502	610
1611-1616 (3.125 µg/mL)	19	43	102	205	381	485	569	645
1617-16110 (12.5 µg/mL)	20	45	98	188	339	324	430	507
1617-16110 (6.25 µg/mL)	20	47	98	211	330	410	405	439
1617-16110 (3.125 µg/mL)	19	46	98	201	316	383	448	502
222221-4 (12.5 µg/mL)	19	37	76	165	282	369	423	535
222221-4 (6.25 µg/mL)	22	49	94	202	308	384	470	553
222221-4 (3.125 µg/mL)	18	43	86	182	271	365	426	526
312331-4 (12.5 µg/mL)	16	36	72	157	239	338	390	490
312331-4 (6.25 µg/mL)	16	37	83	173	291	387	463	530
312331-4 (3.125 µg/mL)	18	42	89	206	340	465	566	651
312335-12 (12.5 µg/mL)	19	46	107	169	148	194	277	314
312335-12 (6.25 µg/mL)	19	46	159	761	780	892	918	687
312335-12 (3.125 µg/mL)	21	53	109	206	357	435	465	543
3131-3137 (12.5 µg/mL)	14	28	44	75	120	160	217	325
3131-3137 (6.25 µg/mL)	17	39	76	146	256	265	429	584
3131-3137 (3.125 µg/mL)	19	42	84	164	278	347	419	441
3138A-12 (12.5 µg/mL)	20	49	103	222	266	315	663	941
3138A-12 (6.25 µg/mL)	25	58	119	233	374	464	555	624
3138A-12 (3.125 µg/mL)	25	62	122	228	363	437	522	557
31381-7 (12.5 µg/mL)	16	14	26	57	80	75	65	67
31381-7 (6.25 µg/mL)	23	57	118	269	429	533	607	643
31381-7C(3.125 µg/mL)	18	43	90	197	317	394	478	506

Sample numbers starting with 1, 2 and 3 refers to the fractions of *Gelidium pristoides*, *Plocamium corallorhiza* and *Polysiphonia virgata*, respectively. INH = isoniazid; RIF = rifampicin; Negative control = bacterium vial without a plant extract or control. The rows marked in blue indicated relative moderate activity.

Table 4.14 Growth-indexes of purified fractions measured over the period 27 May 2004 to 6 June 2004.

Sample no.	27/05	28/05	29/05	30/05	31/05	01/06	02/06	03/06	04/06	05/06	06/06
1:100 control	0	0	0	0	1	4	6	11	19	37	72
Negative control	48	105	170	256	456	446	545	603	680	550	556
31271-3128 (12.5 µg/mL)	24	32	30	27	24	22	19	20	18	22	13
31271-3128 (6.25 µg/mL)	25	49	73	93	150	168	242	301	429	552	807
31271-3128 (3.125 µg/mL)	26	14	64	150	231	254	299	348	413	453	647
3141-46 (12.5 µg/mL)	19	45	104	181	334	431	583	715	896	683	593
3141-46 (6.25 µg/mL)	21	50	122	203	393	489	731	708	527	404	441
3141-46 (3.125 µg/mL)	20	53	104	176	333	392	525	535	597	547	553
3148A (12.5 µg/mL)	27	40	40	40	41	30	33	33	28	28	32
3148A (6.25 µg/mL)	36	80	129	174	269	339	415	421	508	562	574
3148A (3.125 µg/mL)	35	89	157	252	413	480	581	603	608	465	401
3147 (12.5 µg/mL)	29	31	27	23	18	19	19	16	14	15	10
3147 (6.25 µg/mL)	44	72	99	109	141	143	180	225	294	407	648
3147 (3.125 µg/mL)	34	82	137	204	328	397	540	568	660	568	547
3151A-3154A (12.5 µg/mL)	28	70	145	232	394	519	650	658	722	724	694
3151A-3154A (6.25 µg/mL)	29	76	186	296	497	631	758	757	734	627	440
3151A-3154A (3.125 µg/mL)	48	104	188	279	431	478	628	611	667	598	506
31389-31813 (12.5 µg/mL)	31	74	140	92	315	737	985	999	808	433	628
31389-31813 (6.25 µg/mL)	26	65	139	207	360	476	637	603	651	576	506
31389-31813 (3.125 µg/mL)	32	73	159	228	395	473	610	602	642	559	484
3121-9 (12.5 µg/mL)	30	65	123	165	245	263	310	415	523	619	740
3121-9 (6.25 µg/mL)	21	53	96	153	245	291	369	507	613	639	659
3121-9 (3.125 µg/mL)	30	76	194	319	488	539	613	735	749	629	545
2222231-34 (12.5 µg/mL)	13	28	43	53	76	94	123	175	250	345	522
2222231-34 (6.25 µg/mL)	29	63	103	148	208	236	304	393	523	638	733
2222231-34 (3.125 µg/mL)	37	90	153	237	354	434	531	614	648	550	462

Sample numbers starting with 1, 2 and 3 refers to the fractions of *Gelidium pristoides*, *Plocamium corallorhiza* and *Polysiphonia virgata*, respectively. Negative control = bacterium vial without a plant extract or control. The rows marked in red indicate significant antimycobacterial activity.

Table 4.15 The percentage growth inhibition of *Mycobacterium tuberculosis* of the samples found active in this investigation using the BACTEC-460 method.

Sample number	Growth index of sample	Growth index of control	% Inhibition
31381-7 (12.5 µg/mL)	67	55	-21.8
122	4	48	91.7
123	10	48	79.2
221	20	34	41.2
222	9	34	73.5
312	22	Contaminated, est. at 30	26.7
321	9	30	70.0
322	0	30	100.0
323	0	30	100.0
332	8	48	83.3
333	0	48	100.0
31271-3128 (12.5 µg/mL)	13	72	81.9
3148A (12.5 µg/mL)	32	72	55.5
3147 (12.5 µg/mL)	10	72	86.1

Sample numbers starting with 1, 2 and 3 refers to the fractions of *Gelidium pristoides*, *Plocamium corallorhiza* and *Polysiphonia virgata*, respectively.

4.3. Discussion

Finding the same zones of inhibition against *S. aureus* in the agar-overlay bioautography method as were reported earlier by Cameron *et al.* (2001) suggested that the species might be in more or less the same phytochemical state, which initially served as motivation for their inclusion in this investigation. An increase in the number of zones of inhibition could indicate that the antimicrobial compounds of the algal extracts were present in much greater concentrations or greater number. This might be because these samples were taken in Glen Cairn, part of a bay namely, False Bay as opposed to Kommetjie (the collection site of the previous study) where the shore was exposed. This difference in the environment might have an impact on the composition of the secondary metabolites. For future investigations, it is also important to note the time of year that these seaweed samples were collected as seasonal variations could greatly influence the results as noted by Pratt *et al.* (1951) and Hornsey and Hide (1976).

Of the three algae screened against *Plasmodium falciparum*, the purified fractions of *P. corallorhiza* showed the greatest inhibition with IC_{50}-values of 2.4 µg/mL to 8.9 µg/mL. *G. pristoides* also showed potential as a source of antiplasmodial compounds, particularly after the fractionation of the dichloromethane extract. This underlines the importance of fractionation as often studies only screen the crude extracts and do not have fractionation as part of the standard screening procedure. Although many more extracts can be screened if the crude extract serves as the main indicator, many potential bioactive compounds could be overlooked. Antiplasmodial activity could potentially serve as an indicator for anti-tuberculosis activity. In 2000, König *et al.* investigated the anti-tuberculosis activity of 39 marine derived natural products and used a concentration range reaching values beyond 128 µg/mL (König *et al.*, 2000). Amongst other more active compounds the compounds isolated from the red algae *Laurencia obtusa*, *L. flexilis*, *L. rigida* and *Plocamium cartilagineum* possessed significant antimycobacterial activity ranging from MIC-values of 16 to 64 µg/mL. The sponge-derived compounds that were selected because of their antiplasmodial activity in a previous investigation were the most active antimycobacterial compounds. In addition, in recent years, antimalarial drugs have been tested against mycobacteria and anti-tuberculosis drugs investigated for the treatment of malaria. Bermudez *et al.* (1999) investigated the *in vitro* and *in vivo* activity of Mefloquine against *Mycobacterium avium* complex (MAC) and found that *in vitro* it was active at MICs of 8 to 16 µg/mL. *In vitro* it showed potential against MAC, particularly when combined with ethambutol, a first-line anti-tuberculosis drug. Isoniazid, another first-line anti-tuberculosis drug, was investigated for its ability to inhibit malarial transmission in the mosquito gut and found to affect the mosquito by an unknown mechanism (Arai *et al.*, 2004). These two examples show complementary action between antimalarial and anti-tuberculosis drugs. This might suggest that *in vitro* antimalarial activity could

serve as an indicator of antimycobacterial activity. This gives increased value to the readings obtained for the crude and purified fractions that were screened for antiplasmodial activity in this investigation.

Two fractions of the extract of *P. corallorhiza* moderately inhibited the growth of *M. tuberculosis*. These fractions were further purified, but the purified fractions that were tested did not show inhibition of *M. tuberculosis*. This might suggest that there is synergy between the compounds that make up the fraction and that it might be better to use the active fraction as opposed to a single isolate. 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). They suggested that interfering compounds could be masking the bioactive compounds. Therefore, it is safe to say that *P. corallorhiza* has anti-tuberculosis compounds as shown by the bioactive fractions and that further investigation is required into the aspect of synergy.

Of the three seaweeds screened in this investigation, the dichloromethane extract of *Polysiphonia virgata* had the most active fractions with the greatest inhibition. With purification, the antimycobacterial activity reduced slightly (Table 4.15), but remained significant. Where the fractions followed each other in succession off the circular centrifugal thin-layer chromatography plate, as was the case with fractions 321, 322 and 323, it could be that one compound could be spread out over these three bands. Tovar and Ballantine (2000) observed that when one antimicrobial zone on a thin-layer chromatography plate was further investigated on HPLC, many antimicrobial compounds were found. This suggests that the fractions of *P. virgata* hold many anti-tuberculosis compounds. One of the compounds was isolated and its elucidation is discussed in Chapter 5.

Two fractions of *G. pristoides* significantly inhibited the growth of *M. tuberculosis* at 12.5 µg/mL indicating that this commercial seaweed has potential as a source of antimycobacterial compounds. On further purification of the most polar fraction of the DCM extract, fraction no. 161, no inhibition was observed against *M. tuberculosis*.

When the fractions were collected from the Chromatotron, the numbers were allocated as the bands moved off the circular silica plates with increasing polarity with band no. 1 being the least polar and the higher band numbers the more polar. From the results, it is evident that the active bands were found around the first and second bands, which were the less polar portion of the dichloromethane extract.

The antimycobacterial activity of the active fractions compared very well with that of the drug controls isoniazid and rifampicin, since the inhibition was assessed at only one concentration and the actual MIC-values were not determined. In other investigations, inhibitory concentrations of up to 200 µg/mL were considered as significantly antimycobacterial (Okunade *et al.*, 2004). In a review of antimycobacterial natural products, Copp (2003) considered compounds having MIC-values of ≤ 64 µg/mL or ≥ 75% growth inhibition at 12.5 µg/mL or less concentration as significantly antimycobacterial. It is possible that some active fractions were not detected since only one low sample concentration was used. Antimycobacterial compounds could possibly have been in too low concentrations to inhibit the growth of *M. tuberculosis*.

The concentration of the sample plays a crucial role in this analysis, as one is limited in the organic solvent volume that can be introduced into the 12B-vials. In establishing which solvents and at what volumes they are permissible, acetonitrile, methanol and ethanol had no inhibitory effect on *M. tuberculosis* at a concentration of 12.5%. Therefore, of the 200 microliters normally submitted for testing, only 25 microliters could consist of the organic solvent. The rest, 175 microliters was either distilled water or growth medium. In this case, the growth medium 7H9 was used to ensure that if the sample was not completely soluble it would then form a suspension. With water, the less polar samples would not form a suspension, but rather an insoluble mass at the bottom of the eppendorf and poor solubility could possibly have resulted in false negatives. Another aspect of concentration is the question of how much of an extract or compound dissolves in the organic solvent selected. The correct sample mass weighed out for a high concentration to determine the minimum inhibition of a less polar sample with the small amount of solvent volume does not ensure that the entire expected sample makes it to the sample test vial. Thus, it appears that one could have more confidence in the assay results with smaller sample concentrations that work well for pure compounds, but could potentially give false negatives for crude extracts or purified fractions. Another point of concern is the testing of unstable compounds in an assay that could take 4 to 12 days to run to completion. One hopes that the values obtained had no relation with the break down products of the original marine plant derived compounds. Here other assays that use fast-growing mycobacteria have an advantage, although the results from those determinations could not give conclusive indications as to the anti-tuberculosis activity of the extracts against the sensitive or resistant strains of *M. tuberculosis*.

The non-cytotoxic water portions of the algal dichloromethane extracts are worth further investigation, particularly for *in-vivo* experiments. As these extracts were not purified, a complete assessment of their *in vitro* anti-tuberculosis activity cannot be given and should be considered in future investigations.

The samples tested against *M. tuberculosis* in the BACTEC-460 method was not tested in triplicate and there is no minimum inhibition concentration (MIC-value) reported for most of them. The cost involved with this assay could not bear the demands for such a large sample amount. However, the samples were screened with reliable controls and contamination could immediately be detected.

4.4. Conclusion

Of the three algae investigated for anti-tuberculosis activity, *Polysiphonia virgata* showed the greatest anti-tuberculosis activity. As an epiphyte on the commercial kelp *Ecklonia maxima*, it is a resource with economic potential. Instead of being separated as waste from *E. maxima*, which is exported and used in abalone farming (Anderson *et al.*, 2003), it could be processed for pharmaceutical use. The results obtained in this screen clearly showed that these algal extracts have anti-tuberculosis compounds and that seaweeds should be included in drug development programs. In addition, the antiplasmodial activities together with the anti-staphylococcal activities, obtained are equally promising and encourage investigations into many more bioactivities that could lead to the development of new drugs for the treatment of so many neglected and untreatable diseases.

Chapter 5

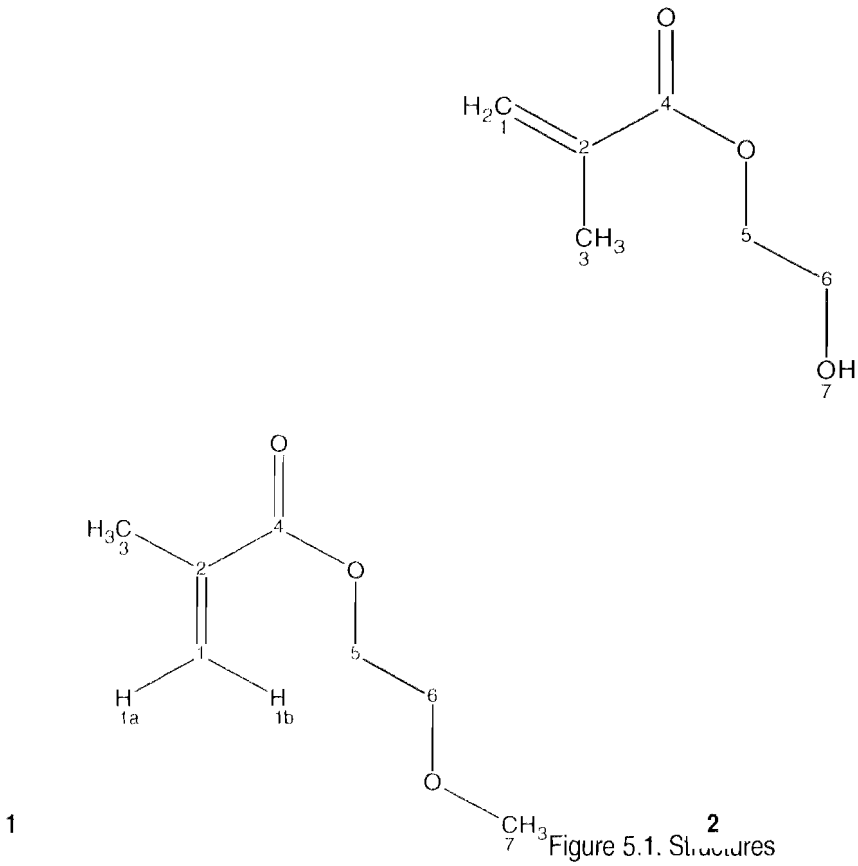
ISOLATION OF ANTI-TUBERCULOSIS COMPOUNDS FROM *POLYSIPHONIA VIRGATA*

5.1. Introduction

The fractions that inhibited the growth of *Mycobacterium tuberculosis* were purified repeatedly using the Chromatotron and preparative layer chromatography. Many new fractions were obtained after each purification step resulting in pure compounds of approximately 2 mg in mass. The compounds that appeared pure with analytical thin-layer chromatography were analysed using HPLC. Those that produced single peaks and that showed the potential of purity with a mass of approximately 1 to 2 mg (Table 5.3) were submitted for NMR analysis at the Chemistry Department of the University of Stellenbosch. The samples amounted to a total of eleven. From the NMR analysis, only four compounds presented spectra that supported their purity while the spectra of the other samples showed the presence of impurities. However, the spectra of all four pure compounds were identical suggesting them to be the same compound. These pure compounds were all isolated from the alga *Polysiphonia virgata*. The number one in the sample numbers indicate that they all initially came off the Chromatotron in the very first non-polar band. When that band was further chromatographed on the Chromatotron, it presented seven bands of which bands 313882, 3148A, 3155A, and 3156 were in four distinctly different bands and came from sub-bands within those bands. Significantly, the sample masses of the pure compounds were a strong determinant for selection because besides structure determination the antimycobacterial activity had to be evaluated. As a result, the impure compounds were not purified further because of their small sample masses. When the NMR spectra were analysed, the structures that were extracted were that of methoxyethyl methacrylate for all four compounds. A closely related derivative, 2-hydroxyethyl-2-methacrylate (HEMA) was available at the Polymer Institute of Stellenbosch University. Its NMR ^{1}H-spectrum was obtained to verify the characterization of MEMA and see whether its antimycobacterial activity was comparable with that of MEMA.

5.2 Results

5.2.1 Structure Elucidation



1 2

Figure 5.1. Structures of 2-methoxyethyl-2-methacrylate (**1**) and 2-hydroxyethyl-methacrylate (**2**).

The Electron Impact Mass Spectrum (EIMS) of (1) gave a molecular ion at m/z 144, with prominent fragment ions at m/z 113 (base peak), 69 and 41. EIMS m/z (rel. int.) 144 [M^+] (3), 113 [M^+-OCH_3] (100), 86 (11), 69 [$C_4H_5O^+$] (83), 45 (8), 41 [$C_3H_5^+$] (42). This is in agreement with the calculated molecular weight of 144.168 for the formula $C_7H_{12}O_3$. Two double quartets at δ 5.6 and 6.1, respectively long-range coupled in the COSY spectrum (Appendix 3) to a CH_3-group, were assigned to the non-equivalent olefinic protons H-1a and H-1b. In turn, the CH_3-group resonating as a double-doublet at δ 3.9 was identified from its long-range coupling to H-1a and H-1b. Two triplets at δ 3.7 and δ 4.2 were assigned to the two methylene groups at 5 and 6. Finally, a singlet at δ 3.6 was attributed to the methoxy methyl at position seven. The DEPT spectrum identified three methylene and two methyl groups. From the HSQC (Appendix 3) spectrum, the resonances of all the protonated carbons could be assigned. In the HMBC spectrum, the position of the carbonyl group at four was identified from 3-bond couplings from 3 CH_3 to C-4 and from H-

1a and H-1b to C-4. Further correlations, which confirmed the structure, were from 2 H-5 to C-4 and 3 CH_3 to C-1 and C-2. Full ^1H and ^{13}C assignments are given in Table 5.1.

Table 5.1. ^1H NMR (δ/ppm, multiplicity, J/Hz at 600 MHz and ^{13}C NMR (δ/ppm, multiplicity) at 150 MHz and ^1H – ^1H COSY and HMBC data in CD_2Cl_2 for compound 3155A.

Position	δ1H	HSQC	COSY ^1H-^1H	HMBC
1a	6.1 dq (1.5, 1.2)	125.5 t	H-1b, 3 CH3	C-2, C-3, C-4
1b	5.6 dq (1.5, 1.2)	125.5 t	H-1a, 3 CH3	C-3
2		136.8 s		
3	1.9 dd (1.2, 0.9)	18.4 q	1a, 1b	C-1, C-2, C-4
4		167.4 s		
5	4.2 t (4.8)	64.2 t	H-6	C-4, C-6
6	3.7 t (4.8)	69.4 t	H-5	C-5, C-7
7	3.6 s	70.9 q		C-6

The ^{1}HMR spectrum of 2-hydroxyethyl-2-methacrylate showed the expected protons at δ 6.1, 5.6, 4.3, 3.9 and 1.9 that fits the assignments of the ethyl methacrylate portion of the molecule, but was without the CH_3 protons of the methoxy group at δ 3.6 (Table 5.2).

Table 5.2 ^1H-NMR assignments for compound 2 (J in Hz in parentheses)

H	δ $CDCl_3$
1a	6.1 dq (1.5, 1.2)
1b	5.6 dq (1.5, 1.2)
3	1.9 dd (1.2, 0.9)
5	4.3 t (4.8)
6	3.9 t (4.8)

Table 5.3 Purified samples submitted for NMR analysis.

Sample no.	Sa mass (mg)	Experiments	Purity	Structure
1611	1.00	^1H	Impure	-
1617	2.00	^1H	Impure	-
3138A	0.96	^1H	Impure	-
3141	1.60	^1H	Impure	-
3148A	0.98	^1H	Pure	MEMA
3155A	1.8	^1H, ^{13}C, DEPT, COSY, GHSQC, GHMQC	Pure	MEMA
3156	1.04	^1H, ^{13}C	Pure	MEMA
16110	2.18	^1H	Impure	-
222224	1.05	^1H	Impure	-
313882	2.02	^1H, ^{13}C	Pure	MEMA
2223221	2.14	^1H	Impure	-

Methoxyethyl Methacrylate

2–Methoxyethyl-2-methacrylate (MEMA) is a known compound, also known as ethylene glycol methyl ether methacrylate. As a synthetic monomer, it plays a very important role as a copolymer for example

with styrene (Stejskal and Kratochvil, 1978; Elliot *et al.*, 2003). It is commonly used to prepare polymeric materials in the pharmaceutical industry (Peppas *et al.*, 2000). Its polymers have shown excellent blood compatibility (Tanaka *et al.*, 2002) which is an important consideration for blood contacting devices such as catheters, dialyzers and blood containers (Zou *et al.*, 2002). In addition, MEMA has been added to antifouling paint mixtures as a copolymer to increase the control of the release rates of the biocide cuprous oxide (Yonehara *et al.*, 2001). To determine its anti-tuberculosis activity, it was purchased from Sigma-Aldrich Co. Related monomers are easy to get hold of, as was the case with 2-hydroxyethyl-2-methacrylate (HEMA) which was obtained from the University of Stellenbosch. It plays an important role in the preparation of soft contact lense polymers (Nicolson and Vogt, 2001).

5.2.2 Anti-tuberculosis activity of MEMA and HEMA

MEMA inhibited the growth of *M. tuberculosis* by 66.7% at a MIC-value of 100 µg/mL (Table 5.4 and Table 5.6) when it was tested in the Bactec-460 method. At 50 µg/mL, it showed slight inhibition of the growth of *M. tuberculosis* (Table 5.4). HEMA showed greater inhibition of *M. tuberculosis* of 76.7% at a MIC-value of 100 µg/mL (Table 5.6). It too showed slight inhibition of the growth of *M. tuberculosis* at a concentration of 50 µg/mL (Table 5.4). When it was tested for cytotoxicity against Chinese hamster ovarian cells, it did not show significant cytotoxicity with IC_{50}-values above 100 µg/mL (Figure 5.2). MEMA also showed moderate inhibition of the multi-drug resistant strain if *M. tuberculosis* (Table 5.5)

Table 5.4 Anti-tuberculosis activities against *Mycobacterium tuberculosis* H37Rv of the pure compounds, methoxyethyl methacrylate and hydroxyethyl methacrylate investigated over the period 27 April to 3 May 2004.

Sample	27/04	28/04	29/04	30/04	01/05	02/05	03/05
1:100 control	3	0	2	2	7	15	30
Negative control	45	133	231	410	619	842	918
INH	23	24	17	15	16	17	15
RIF	16	17	11	10	8	5	5
MEMA 100 µg/mL	13	10	7	8	7	8	10
MEMA 50 µg/mL	16	16	16	20	27	33	43
MEMA 25 µg/mL	23	41	59	85	132	210	356
MEMA 12.5 µg/mL	26	69	124	213	347	533	750
MEMA 6.25 µg/mL	27	85	166	297	461	658	841
MEMA 3.125 µg/mL	26	83	170	305	482	631	741
MEMA 1.5625 µg/mL	27	92	177	324	540	631	747
HEMA 100 µg/mL	13	9	7	6	7	7	7
HEMA 50 µg/mL	21	29	33	34	41	49	57
HEMA 25 µg/mL	22	42	95	177	330	557	872
HEMA 12.5 µg/mL	21	51	112	220	419	678	908
HEMA 6.25 µg/mL	21	61	133	249	460	701	849
HEMA 3.125 µg/mL	27	87	190	334	529	739	844
HEMA 1.5625 µg/mL	22	74	159	276	478	671	750

Table 5.5. Anti-tuberculosis activity of MEMA against a multi-drug resistant clinical strain of *Mycobacterium tuberculosis*.

Sample (Conc. in µg/mL)	23-Mar-06	24-Mar-06	25-Mar-06	26-Mar-06	27-Mar-06
Negative control	162	346	570	852	999
1:100	2	1	4	10	49
Rifampicin	85	136	164	193	234
Solvent control	114	174	240	325	443
MEMA (125)	53	69	83	115	143
MEMA (62)	88	149	257	442	705
MEMA (31)	87	163	315	520	784
MEMA (15)	112	210	390	633	879
MEMA (7.8)	119	241	393	632	909

Table 5.6 The percentage growth inhibition of *Mycobacterium tuberculosis* by MEMA and HEMA in the BACTEC-460 method.

Sample no.	Growth index of sa	Growth index of control	% Inhibition
MEMA 100 µg/mL	10	30	66.7
MEMA 50 µg/mL	43	30	-43.3
HEMA 100µg/mL	7	30	76.7
HEMA 50 µg/mL	57	30	-90

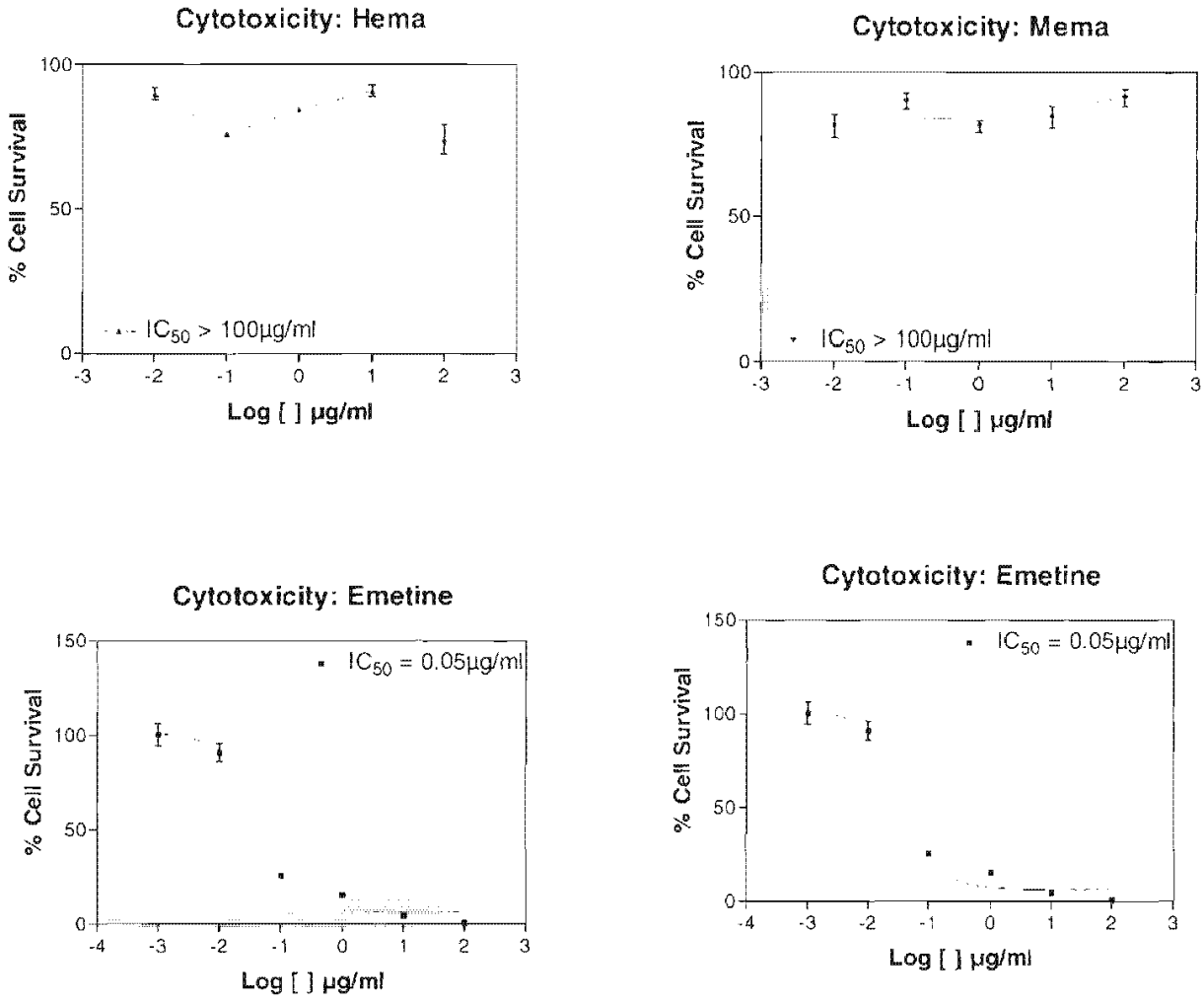


Figure 5.2. The percentage cell survival recorded for the MEMA and HEMA against CHO-cells in the cytotoxicity determination.

5.3 Discussion

The results clearly show the isolation of 5.84 mg of MEMA from *P. virgata* (0.072% of crude extract and 0.00118% of the whole alga). The methoxy group places the compound under the isolations of methoxylated compounds from marine organisms that are common as shown by the many publications on such natural products. For example, malyngamides were reportedly isolated from a marine cyanophyte *Lyngbya majuscula* (Cardellinall *et al.*, 1978; Mynderse and Moore, 1978; Loui and Moore, 1979; Todd and Gerwick, 1995; Wu *et al.*, 1997; Mesguiche *et al.*, 1999) and methoxylated fatty acids from the red seaweed *Schizymenia dubyi* (Barnathan *et al.*, 1998). The methoxylated fatty acids included 9-methoxypentadecanoic, 9-methoxyheptadecanoic, 13-methoxyheneicosanoic and 15-methoxytricosanoic acids. 7(S)-methoxytetradec-4(E)-enoic acids showed activity against *Staphylococcus aureus* and *Bacillus subtilus*. Many α-methoxylated fatty acids were identified in the phospholipids of marine sponges (Carballeira, 2002) (Figure 5.3).

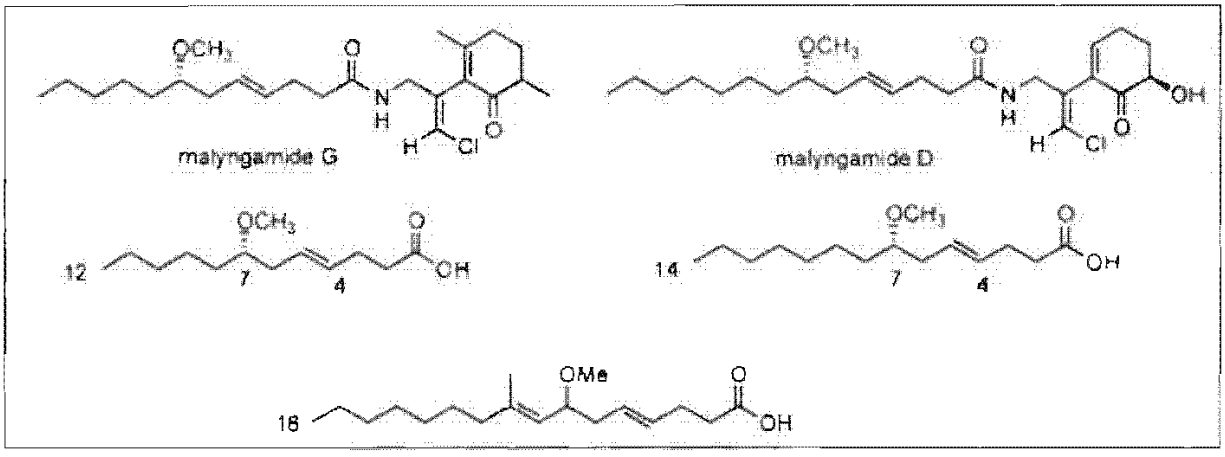


Figure 5.3. Two malyngamids and three methoxylated fatty acids, namely, (-)-7-methoxydodec-4(*E*)-enoic acid, 7(*S*)-methoxytetradec-4(*E*)-enoic acid and 7-methoxy-9-methylhexadeca4(*E*),8(*E*)-dienoic acid were isolated from *Lyngbya majuscula* (Carballeira, 2002).

However, this was the first report of the isolation of the commercially available MEMA from an alga and specifically from *P. virgata*. Concerning commercially available compounds, a red alga of the genus Asparagopsis produced small quantities of carbon tetrachloride, bromoform and halogenated acetones of which monobromoacetone is a major component of tear gas (Josephson, 1997). The isolation of methyl ethers from red algae is also common, particularly from other species of Polysiphonia, for example, *Polysiphonia lanosa* where the methyl, ethyl and n-propyl ethers of lanosol where reported to have cytotoxic activity against human colon adenocarcinoma (Shoeib *et al.*, 2004).

Earlier in the description of MEMA, reference was made to its current uses of which its role as monomer in antifouling paints suggests the possibility it being an environmental pollutant. Algae are known to accumulate environmental pollutants and are often used as pollution indicators along with mussels (Moy and Walday, 1996). In their investigation, Moy and Walday (1996) looked at bioaccumulation of the radiolabelled hydrophobic toxicants, namely, polychlorinated biphenyl (^{14}C-PCB$_{77}$) and a polycyclic aromatic hydrocarbon, benzo[a]pyrene (^{14}C-BaP) and found both mussels and algae rapidly absorbed the contaminants. They noted that with the algae, the pollutant concentration was determined on the whole plant and a distinction between absorption into the plant and adsorption on to the surface of the seaweed was not made. Similarly, in this investigation no provision was made for compounds that might be adsorbed to the mucus surface of the seaweed. Moy and Walday (1996) also noted that the algal polysaccharides that make up the outer cell walls "act as a sponge which adsorbs cations and lipophilic compounds from the environment". Therefore, the isolation of MEMA from *P. virgata* will have to be further investigated, particularly clarifying whether it was an authentic plant compound or adsorbed onto the surface of the alga or absorbed into the alga as an environmental pollutant.

Another observation made by Moy and Walday (1996) was the variation in the time it took for the seaweeds and mussels to release the contaminant and that possibly a pool of contaminants could be stored over a period. The concentration of BaP reduced over a period of one and two months once the pollutant was removed. This was mainly due to metabolic degradation. The concentration of PCB however did not show this reduction and the pollutant was retained in the organisms for much longer. In this investigation, 5.84 mg (Table 5.3) from 8.08 g (Table 4.1) of extracted material (0.07%) was present at the time of collection and this could have accumulated over time or have been the result of a single event.

In scientific literature, much is said about the harmful environmental impact of organic tin-based antifouling paints on marine organisms (Huggett *et al.*, 1992; Michel and Averty, 1999) not targeted by antifouling agents applied to underwater marine structures (Yamamoto *et al.*, 1997;Yebra *et al.*, 2004). After attempting different solutions, tributyltin self-polishing copolymer paints (TBT-SPC) in which MEMA is a key monomer are currently the most successful tool in dealing with biofouling on ships and other underwater structures. However, tributyltin continues to be harmful to the environment and new tin-free biocides are under investigation, such as copper-based self-polishing copolymer technology. In the process, little has been done to account for the monomers, of which methacrylates are the most important, leaching from the copolymer paints during seawater exposure. Since these algae were collected in Sweet Water, south of Hout Bay harbour there were not many possible sources for MEMA as an ingredient in

antifouling paints of underwater structures. Hout Bay harbour is a small harbour with vessels ranging from fishing boats to yachts.

In the process of searching for new environmental safe antifouling compounds, natural compounds are considered a good option. Sessile marine organisms have their own armamentarium of compounds to prevent them from being fouled by other marine species. In recent years, a few antifouling compounds have been isolated from marine organisms for example; a diterpene-alkaloid was isolated from a marine sponge (Hattori *et al.*, 1997). Another possibility that could justify the absorption of MEMA into the algal thallus is its bioactivity that was demonstrated to some extent in this investigation. The alga could take the compound from the environment and use it in its own biodefense. This investigation is the first report of MEMA isolated from a marine plant and its impact on algae should be established in future investigations.

In a review compiled by Cantrell *et al.*, 2001, they reported on the antimycobacterial activity of plant terpenoids with MIC-values below 64 µg/mL most of which were determined using the BACTEC-460 system. They reported on the MIC-values of the current first-line anti-tuberculosis drugs to show how the terpenoids compare with today's chemotherapeutic agents. It is interesting to note that pyrazinamide had a MIC-value *in vitro* of 100 µg/mL while the other first-line drugs had MIC-values well below 10 µgl/mL. This is relevant to this study as the MIC-value of both MEMA and HEMA were determined to be 100 µg/mL and prompts an investigation into its *in vivo* activity, particularly the *in vivo* activity of MEMA against a drug-resistant strain of *M. tuberculosis*.

Chapter 6

ISOLATION OF 2-METHOXYETHYL-2-METHACRYLATE FROM *POLYSIPHONIA VIRGATA*

6.1 Introduction

The isolation of (2-methoxyethyl-2-methacrylate (MEMA) or ethylene glycol methyl ether methacrylate from *Polysiphonia virgata* (0.072% of the crude extract and 0.0012% of the whole alga) raised a few questions, which will be addressed in this chapter. These included whether MEMA was isolated as an authentic plant compound, or an artifact or environmental pollutant, was it absorbed into the cells of the seaweed or adsorbed onto the algal mucus surface. To clarify the latter the fresh alga was soaked in various organic solvents such as dichloromethane, ethanol and methanol to remove compounds that might be adsorbed to the algal surface. The resultant solutions were submitted for LC-MS/MS analysis and detection set for a [M+1] ion at m/z 145. The sample was infused into an Applied Biosystems Sciex API-4000 mass spectrometer at atmospheric pressure using chemical ionization for ion production.

In the previous chapter, the isolation of MEMA was not guided by bioactivity alone. Initially, the fractions were tested for antimycobacterial activity, but with increased purity, the sample size reduced significantly. Pure compounds in sufficient amounts for characterization were selected for identification and subsequently tested for anti-tuberculosis activity. Thus, MEMA could not be said to be the major anti-tuberculosis compound in the seaweed, but rather a compound present in sufficient amounts for structure determination. The fact that MEMA is a known monomer immediately presented the likelihood that it could be an environmental pollutant that bioaccumulated in the seaweed. Marine vessels and underwater structures coated with antifouling paints in which MEMA was probably a monomer might have been the sources from which it leached. However, the seaweed sample from which it was isolated was collected at Sweet Water, which is at an exposed area on the Atlantic Ocean of the Cape Peninsula, south from Hout Bay. Hout Bay has a small harbour, which is home to fishing boats and a few yachts. Alternatively, MEMA could have come from a chemical spill in the ocean in the area. To get answers to these possibilities 5 litres of seawater were collected at the sites where the seaweeds were collected and screened for the presence of MEMA using a LC-MS.

Another very important possibility is that the presence of the methacrylate could be ascribed to enzymatic biosynthesis within the thallus of the alga or by bacteria associated with the alga. This, however, could not be determined in this investigation. Instead, a closer look at published literature was taken and will be discussed later in this chapter.

Environmental events that could influence the quality of algae include upwelling in southern Benguela, which is in season from September to March. During this season the water temperatures of newly upwelled water is 14° to 16°C (Verheye *et al.*, 1992) as opposed to the normal 10° to 12°C. Upwelling brings about an increase in mesozooplankton (Verheye *et al.*, 1992) and so grazing of the algae in the effected area. To determine whether there was an increase in mezozooplankton in the Sweet Water area chlorophyll-a concentration images using an ocean colour sensor was obtained from the Department of Oceanography, University of Cape Town.

6.2 Results

The mass spectra of the solutions obtained from dipping 200 g of freshly collected *P. virgata* in 500 mL of first ethanol, followed by dichloromethane and finally methanol did not show the presence of the characteristic (M+1) molecular ion of MEMA at m/z 145. The solvent partitioning procedure in which the seawater was partitioned between water and chloroform to extract the organic compounds from the seawater samples yielded 3.4 mg and 6.3 mg for the samples taken in Simonstown harbour at the East Gate and Boathouse, respectively. The Sweet Water extract amounted to 8.6 mg and the seawater extract obtained from Glen Cairn to 2.4 mg. However, MEMA was not detected in the mass spectra of these chloroform extracts of the seawater samples.

The chlorophyll-a concentration images obtained from the Oceanography Department showed cloud cover on the day we collected the seaweed (Figure 6.1). As a result, we could not derive any information from the ocean colour sensor images, which contain chlorophyll-a concentration data.

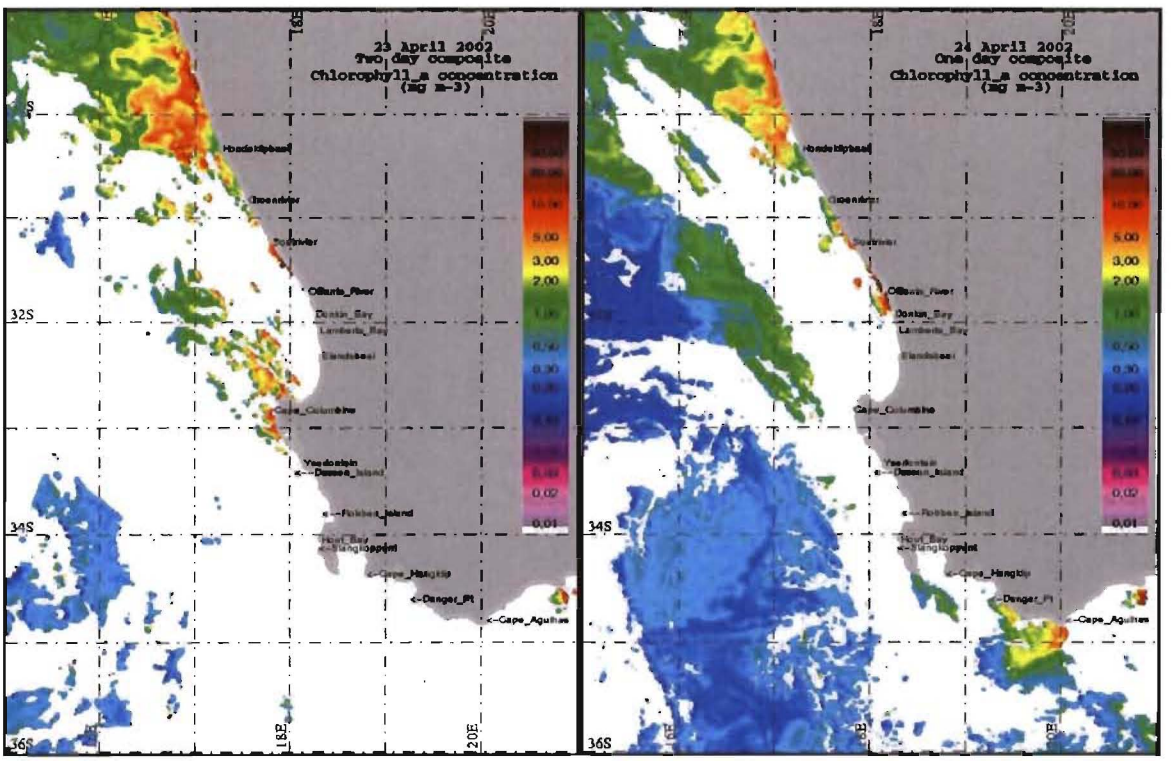


Figure 6.1 Chlorophyll-a concentration images for 23 April to 24 April 2002 obtained from the Department of Oceanography, University of Cape Town.

6.3 Discussion

The absence of MEMA from the organic solvents into which the samples were dipped would suggest that it was not adsorbed onto the algal surface. Its absence from the collected seawater samples at the sites of collection would suggest that it is not present in the marine environment as a pollutant. The LC-MS/MS method used for its detection was sufficiently sensitive in that it registers ions present in the solvent in nanogram quantities. The 5 liters of water samples seem to be an adequate volume of seawater for the evaluation of organic compounds at coastal sites. This was demonstrated in an investigation done by Gshwend *et al* .(1982) when they looked at volatile organic cornpounds in 4 liters of seawater samples and were able to detect alkanes, aldehydes, dimethyl polysulfides, and many more compounds.

It could be that MEMA is the product of biosynthesis, by either the seaweed or microorganisms associated with the alga. A possible precursor of MEMA, acrylic acid, is frequently mentioned in environmental marine publications in the context of the by-product of dimethylsulfoniopropionate (DMSP) degradation. DMSP is a tertiary sulphonium compound produced by marine algae and plant halophytes (Yoch, 2002). Degrading seaweeds release a considerable amount of acrylic acid, as shown in Figure 6.2 (Yoch, 2002).

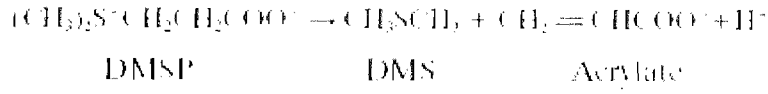
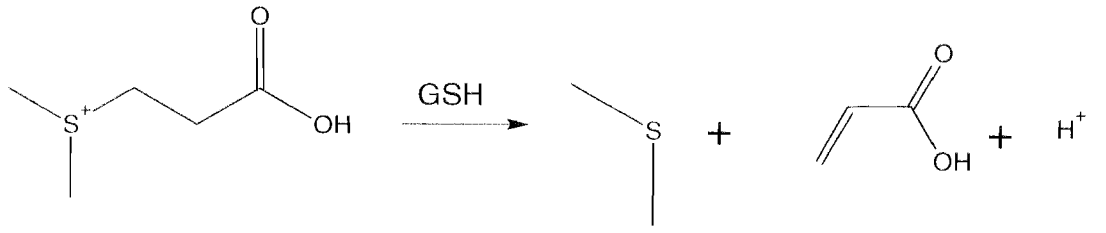


Figure 6.2 Degradation of DMSP to DMS and an acrylate by the enzyme DMSP lyase (Yoch, 2002).

In 1935, Haas (1935) observed that the seaweed *Polysiphonia fastigata* evolved dimethyl sulfide shortly after collection. In 1956, Cantoni and Anderson (1956) found a dimethylpropiothetin bromide (DMPT) cleaving enzyme in the extracts of *Polysiphonia lanosa* and they suggested the catalytic reaction to be:

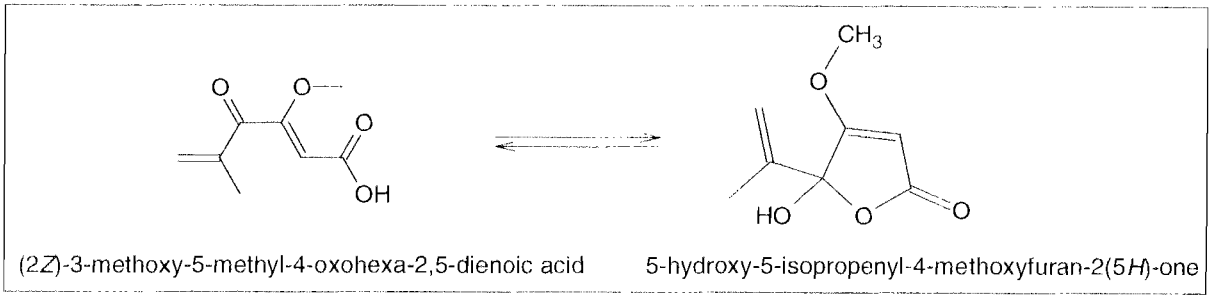


(2-carboxyethyl)(dimethyl)sulfonium dimethyl sulfide acrylic acid hydrogen(1+)

In fact, acrylic acid was identified as the major component of the products formed during the enzymatic cleavage and the substrate, DMPT, was reported to be present in as high a concentration as 0.04 M. Steinke *et al.* (1998) found that more than one dimethylsulfoniopropionate lyase isozyme could be associated with a particular organism when they investigated the phytoplankton *Emiliania* huxleyi. In another investigation, Watanabe *et al.* (1977) obtained an ethylene-synthesizing enzyme from the red alga *Porphyra tenera*. The enzyme was acrylate decarboxylase and formed ethylene from an acrylate detected in its presence. More recently, Van Alstyne *et al.* (2001) completed this picture by describing the activated defenses of temperate marine macroalgae. *Polysiphonia hendryi* was one of the species investigated and the only red alga to show detectable amounts of DMSP which was considered to be present as a primary chemical defense. In addition, significant DMSP lyase activity was reported for *P. hendryi*. The release of DMS was associated with damage to the algal tissues due to herbivory or crushing and the acrylic acid served as a feeding deterrent not because of toxicity, but rather because of its unpleasant taste.

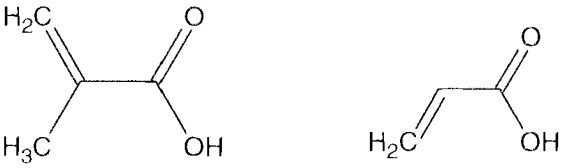
Glombitza isolated acrylic acid as an antimicrobial compound from *Polysiphonia urceolata* in its free and bound form together with dimethyl-β-propiothetin its precursor (Glombitza, 1970a; Glombitza, 1970b). Glombitza was not the first to report the antibiotic potential of acrylic acid and its derivatives. Walton examined the antimicrobial potential of β-aroyl acrylic acids and their derivatives in 1957 (Walton, 1957a; Walton, 1957b). He justified the investigation by comparing their structure to that of the antibiotic penicillic acid and synthesized alkyl 4-oxo-2-alkenoates (Figure 6.3). Other investigators followed in searching for new antibiotics from acrylic acid derivatives. Iwasaki *et al.* (1977) synthesized novel acrylamides and

found N-n-propyl-cis-β-n-butylsulfinylacrylamide to be highly herbicidal against *Digitaria adiscendens* and *Amaranthus ascendens.*



(2*Z*)-3-methoxy-5-methyl-4-oxohexa-2,5-dienoic acid 5-hydroxy-5-isopropenyl-4-methoxyfuran-2(5*H*)-one

Figure 6.3. Structures were taken from Walton (1957a)



methacrylic acid acrylic acid

Figure 6.4 Structures of methacrylic and acrylic acid.

The importance of acrylic acid lies in its close structural association with methacrylic acid (Figure 6.4) with the only difference in the structure being the methyl group. The biosynthesis of a methacrylate such as 2-methoxyethyl methacrylate (Figure 6.5) seems probable when compared to the formation of an acrylate as shown in Figure 6.1 with the addition of a methoxyethyl and methyl group.

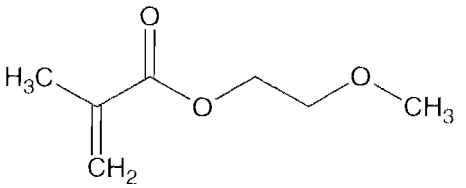


Figure 6.5 Structure of 2-methoxyethyl methacrylate.

Alternatively, microorganisms associated with the alga could have synthesized the methacrylate. From published literature, it appears as if *Polysiphonia* is host to various marine microorganisms. The fungus *Geniculosporium sp.* was isolated from an unidentified species of Polysiphonia and under controlled conditions produced botryane metabolites (Krohn *et al.*, 2005). Another fungus, *Apiospora montagnei* isolated from *P. violacea* presented a diterpene, three acids and a methyl ester of 9-hydroxyhexylitaconic acid (Klemke *et al.*, 2004).

In a culture broth of a photosynthetic bacterium, Smith *et al.* (1994) found that the bacterium photoassimilated 3–mercapto-2-methylpropionate as a source of sulphur and that a methacrylate accumulated in the medium. Ansede *et al.* (1999) investigated the metabolism of acrylate to β-hydroxypropionate in a salt marsh sediment bacterium *Alcaligenes faecalis* and its association with DMSP degradation. DMSP degraded outside the bacterial cell resulting in extracellular accumulation of the acrylate. The acrylate was was further metabolized to β-hydroxypropionate. The overall reaction was described as follows:

$(CH_3)_2S^+ CH_2CH_2CO \longrightarrow (CH_3)_22S + CH_2{=}CHCO_2^- + H^+ \longrightarrow HOCH_2CH_2CO_2^- \longrightarrow CO_2 + X$ (X represents an unknown metabolite).

The role of the acrylate and its analogs (acylamide and methacrylate) appeared to induce DMSP lyase activity. Thus, it appears that the biosynthesis of methacrylates by the alga and/or microorganisms is highly possible and more likely the reason for the presence of 2-methoxyethyl methacrylate in *Polysiphonia virgata.*

Chapter 7

ISOLATION OF ANTIMYCOBACTERIAL COMPOUNDS FROM *POLYSIPHONIA VIRGATA:* RE-INVESTIGATION

7.1 Introduction

The isolation of MEMA from *Polysiphonia virgata* necessitated a re-investigation into its bioactive compounds to determine whether other compounds were responsible for the primary antimycobacterial activity. In the re-investigation, bioassay-guided fractionation of the dichloromethane extract of the dried alga led to the isolation of a mixture of fatty acids and hydrocarbons according to the 1HMR spectra of the bioactive components. Further identification of the fatty acids and hydrocarbons was achieved by gas chromatography mass spectrometry (GC-MS) analysis. The sample was first methylated to form methyl esters of the fatty acids since direct analysis in gas chromatography (GC) is made complicated by the tendency of lipids samples to be highly polar, less volatile and to form hydrogen bonds (Brondz, 2002). Antimycobacterial activity of the purified fractions and mixture of fatty acids and hydrocarbons was assessed using *Mycobacterium smegmatis* since *Mycobacterium tuberculosis* could not be used in the intial stages of this investigation. However, *Mycobacterium smegmatis* is a non-pathogenic bacterium and good indicator of possible anti-tuberculosis activity as demonstrated by a recent investigation in which the initial bioactivity against *M. smegmatis* assisted the development of a new drug against tuberculosis by Andries *et al.* (2005). The antimycobacterial activity of the identified fatty acids were determined by testing commercially available standards against *M. smegmatis* using direct bioautography and *M. tuberculosis* H37Rv and a multi-drug resistant isolate of *M. tuberculosis* in the Bactec-460 radiometric method.

7.2 Results

The fractionation of the crude extract using flash chromatography resulted in 45 fractions (Appendix 1: Table A2) of which many were active against *M. aurum* (Table 7.1 and Figure 7.1) and *M. smegmatis* (Table 7.2).

Table 7.1. Rf-values obtained for the fractions in the direct bioautography method against *Mycobacterium aurum* on 29 August to 1 September 2005.

Sample no.	Solvent front (mm)	Zone distance (mm)	Zone size (mm)	Rf-value
1	89	85	5	0.96
2	89	88	3	0.99
3	89	85	6	0.96
	89	25	2	0.28
4	89	80	7	0.90
	89	25	6	0.28
5	89	25	10	0.28
6	89	25	20	0.28
7	89	24	14	0.27
8	89	no zone		
9	89	no zone		
10	83	82	2	0.99
11	83	22	7	0.27
12	83	23	10	0.28
13	83	22	10	0.27
14	83	23	10	0.28
15	83	20	10	0.24
16	83	20	10	0.24
17	83	20	7	0.24
18	83	no zone		
19	88	28	30	0.32
20	88	24	25	0.27
21	88	18	25	0.20
22	88	19	28	0.22
23	88	19	28	0.22
24	88	20	33	0.23
25	88	20	32	0.23
26	88	22	39	0.25
27	88	23	35	0.26
28	84	20	36	0.24
29	84	20	35	0.24
30	84	18	32	0.21
31	84	18	30	0.21
32	84	15	25	0.18
33	84	15	30	0.18
34	84	15	28	0.18
35	84	15	30	0.18
36	84	17	20	0.20
37	89	no zone		
38	89	10	22	0.11
39	89	10	21	0.11
40	89	10	20	0.11
41	89	10	30	0.11
42	89	10	3	0.11
43	89	no zone		
44	89	no zone		
45	89	no zone		
Ciprofloxacin			11	
Ciprofloxacin			11	

Table 7.2. Rf-values obtained for the fractions in the direct bioautography method against *Mycobacterium smegmatis* on 29 to 30 August 2005.

Sample no.	Solvent front (mm)	Zone distance (mm)	Zone size (mm)	Rf-value
1	88	no zone		
2	88	no zone		
3	88	no zone		
4	88	20	7	0.23
5	88	22	14	0.25
6	88	25	19	0.28
7	88	26	37	0.30
8	88	no zone		
9	88	no zone		
10	88	no zone		
11	88	no zone		
12	88	no zone		
13	88	no zone		
14	88	no zone		
15	88	no zone		
16	88	no zone		
17	88	no zone		
18	88	no zone		
19	90	30	30	0.33
20	90	25	30	0.28
21	90	20	30	0.22
22	90	20	30	0.22
23	90	20	30	0.22
24	90	20	30	0.22
25	90	22	30	0.24
26	90	20	30	0.22
27	90	15	30	0.17
28	86	18	37	0.21
29	86	18	33	0.21
30	86	15	32	0.17
31	86	15	30	0.17
32	86	15	30	0.17
33	86	15	24	0.17
34	86	17	32	0.20
35	86	12	22	0.14
36	86	12	16	0.14
37	90	no zone		
38	90	18	33	0.20
39	90	15	30	0.17
40	90	14	28	0.16
41	90	16	36	0.18
42	90	13	27	0.14
43	90	no zone		
44	90	no zone		
45	90	no zone		
Ciprofloxacin			10	
Ciprofloxacin			10	

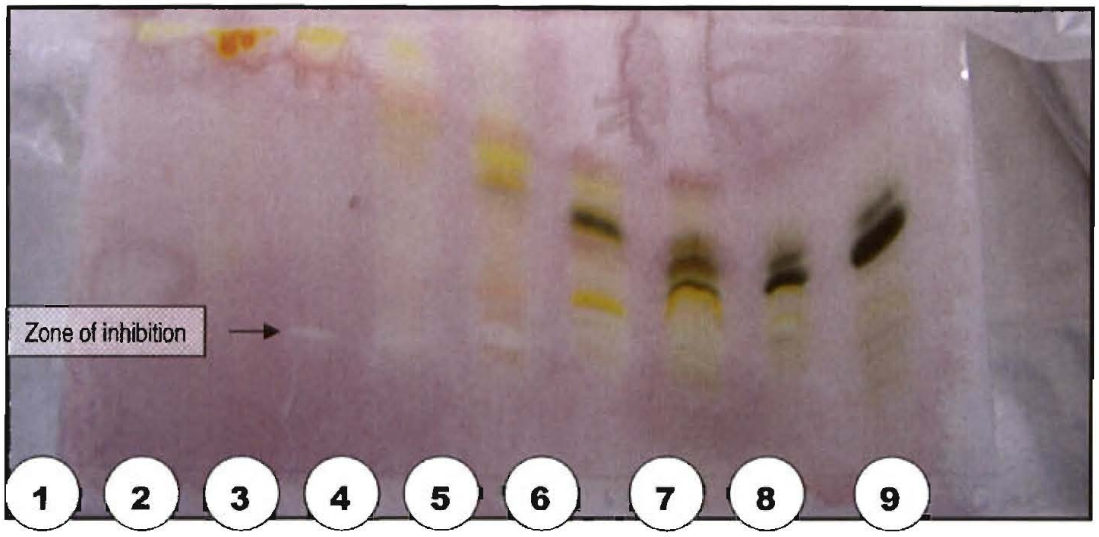


Figure 7.1 The inhibition zones obtained against *Mycobacterium aurum* in the direct bioautography method. Numbers 1 to 9 indicate the fraction numbers as assigned in Table 7.2.

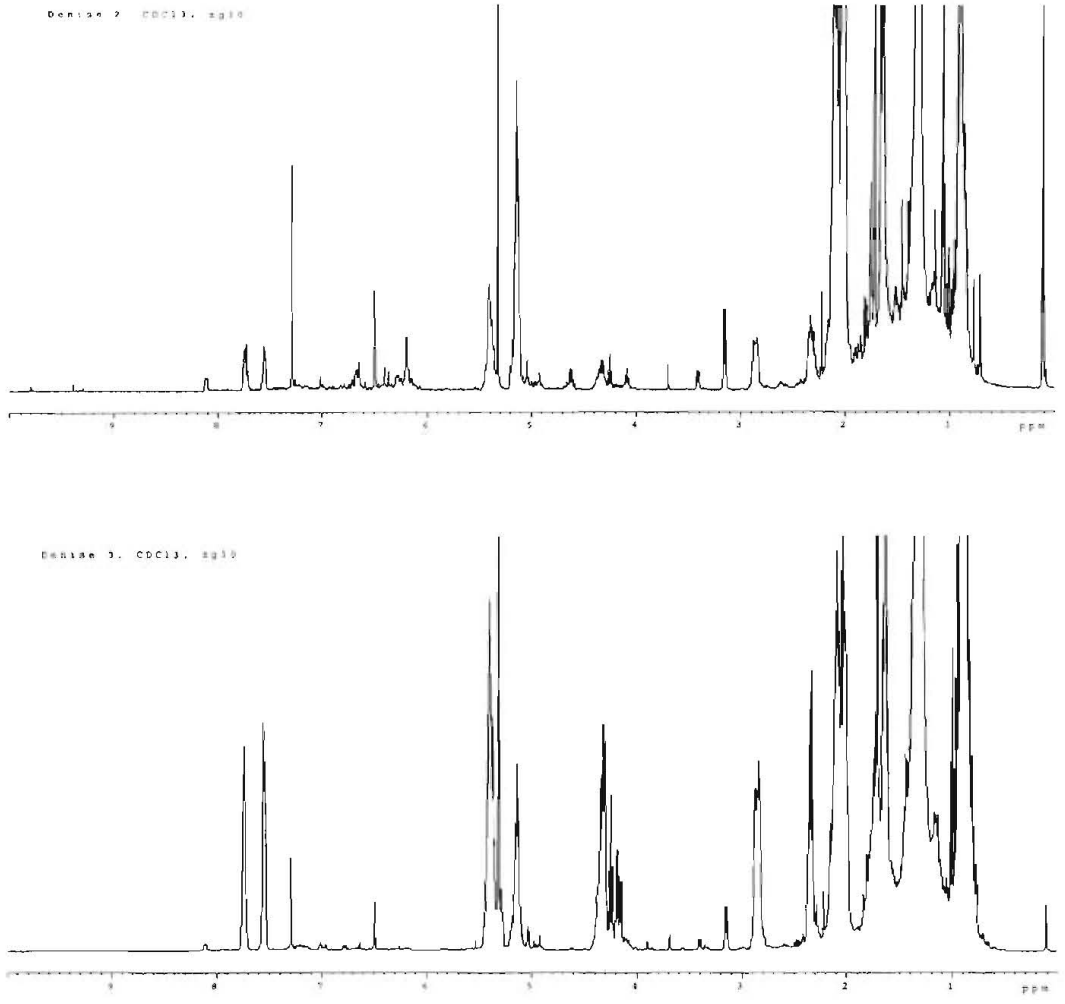


Figure 7.2 1HNMR of Fraction 2 (above) and 3 (below) obtained using flash chromatography.

Fraction 2 and 3 were combined since their tlc-profile and 1H NMR spectra (Figure 7.2) were the same and the resultant samples further purified using the Chromatotron. Fractions 17 to 30 were also combined for the same reason. Fractions 5 to 10 were kept separately and further purified (Appendix 1: Table A1.3) using preparative HPLC on a Spectra-Physics IsoChrom LC HPLC with a mobile phase of hexane (85%) in ethyl acetate at a flow rate of 4 mL/min. The purified samples were screened for activity against *M. smegmatis* using the direct bioautography method and the bioactive zones reported in Table 7.3.

Table 7.3. Inhibition zones obtained of the undeveloped purified fractions in direct bioautography against *Mycobacterium smegmatis*.

Sample no.	Mass (mg)	Zone size (mm)
6/1	1.9	5
6/2	0.6	5
6/3	0.9	5
6/6	2.5	5
6/9	0.7	20
7/1	2.6	6
7/2	0.8	5
1730/1Wash		6
R1730/2	16.7	6
R1730/3	2.6	6
R1730/4	13.2	5
R1730/5	4.6	6
R1730/6	5.3	6
R1730/7	3.8	6
R1730/8	14.5	5
6/665	1.4	5
6/52	0.6	5
1730/1	34.9	6
1730/2	26.3	8
1730/3	4.9	15
1730/4	4.7	15
1730/5	1.7	14
1730/6	2.6	13
1730/7	1.7	6
1730/8	1.7	6
1730/9	10.0	6
2&3/13	10.9	6
2&3/14	4.9	6

Sample 6/9 with a small sample size of 0.7 mg was the most active against *M. smegmatis* with an inhibition zone size of 20 mm in diameter. The next active samples were those of 1730/3 to 1730/6 with inhibition zones of about 14 mm in diameter. All samples were further purified using thin-layer chromatography and fractions with about 1 mg in mass were tested against *M. smegmatis* (Table 7.4). Sample 1730/1 produced only one bioactive fraction namely 1730/11 compared to sample 1730/2 that produced 2 bioactive fractions and 1730/3 three bioactive fractions. Of sample 2&3, fractions 131 and 142 appeared to hold the bioactive compounds.

Table 7.4 Inhibition zones obtained of purified samples against *M. smegmatis*.

Sample no.	Mass (mg)	Zone size (mm)
6/91	4.8	9
1730/11	26	7
1730/21	17	8
1730/27	16.2	8
1730/31	6.8	10
1730/32	9.4	9
1730/37	9.4	8
1730/41	5.8	8
1730/54	2.8	9
2&3/131	9.5	10
2&3/142	6.2	9
R/31	6.2	10
Ciprofloxacin		12
Ciprofloxacin		12

The active samples were submitted for 1HNMR analysis after which samples 1730/31,1730/27, R/31, 2&3/142, 1730/31, 1730/32, 1730/41, 1730/54, 6/9, and 7/11 were combined and numbered C1730(49.4 mg) because their 1H NMR spectra were the same (Figure 7.3). Samples R/69, R611, R/55, 1730/55, and 1730/66 produced the same 1HNMR spectrum and were also combined (Figure 7.4) into sample number 1730/11 (20.5 mg).

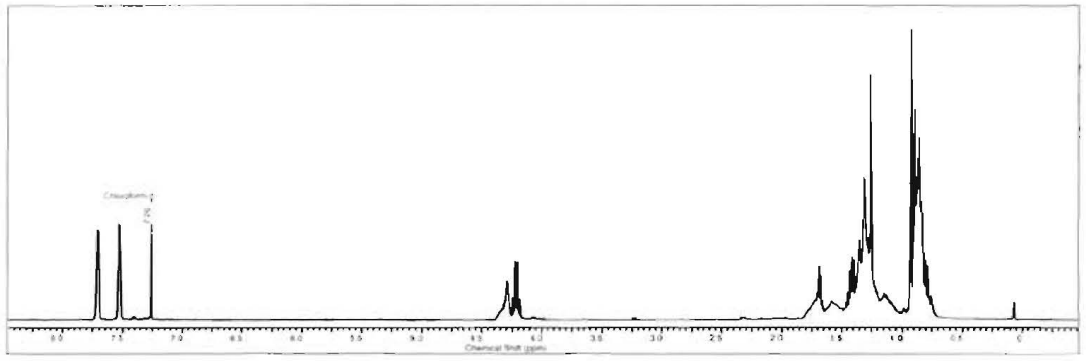


Figure 7.3 1HNMR spectrum of sample 1730/27.

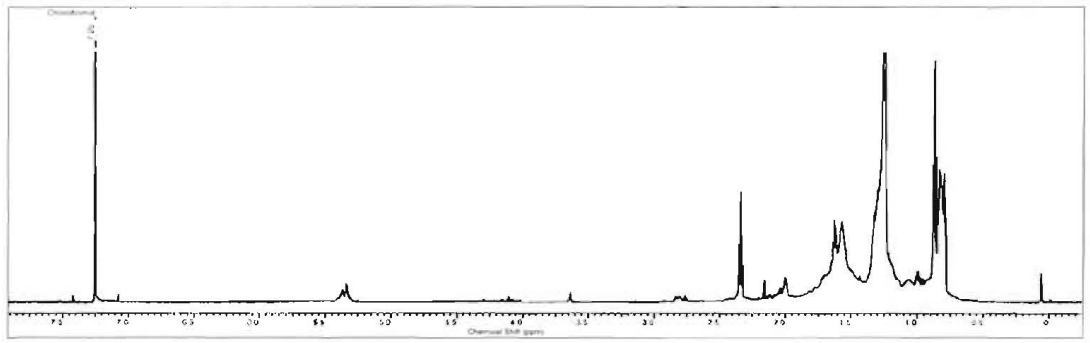


Figure 7.4. 1HNMR spectrum of sample R/69

The combined samples were tested against *M. smegmatis* and the minimum inhibition concentration (MIC) determined to be 15.62 µg (zone size 4 = mm) and 7.4 µg (zone size = 4 mm) for sample C1730 and

1730/11, respectively which compared well with Ciprofloxacin that showed a 12 mm diameter zone at an amount of 12.5 micrograms on the thin-layer chromatography plate (Table 7.5).

Table 7.5. The zones of inhibition of sample C1730 and 1730/11 obtained against *M. smegmatis.*

Sample no. (sample mass on tlc-plate)	Zone size (mm)
C1730 (500)	9
C1730 (250)	7
C1730 (125)	8
C1730 (62.5)	7
C1730 (31.25)	4
C1730 (15.62)	4
C1730 (7.8)	0
1730/11 (1000)	10
1730/11 (500)	10
1730/11 (250)	10
1730/11 (125)	7
1730/11 (62.5)	6
1730/11 (31.25)	6
1730/11 (15.62)	5
1730/11 (7.8)	4
Ciprofloxacin (12.5 µg)	12
Ciprofloxacin (12.5 µg)	12

It was noted that the 1HNMR peaks around 7.5 ppm were likely to be due to phthalates that were present as contaminants. However, the signals more upfield were suspected to be because of fatty acids and/or hydrocarbons. Therefore, the two samples were methylated and submitted for GC-MS analysis. After methylation, the samples weighed 10.0 mg (C1730E) and 6.4 mg (1730/11E). Hydrocarbons, phthalates and importantly methyl esters were tentatively identified (Figure 7.5, Figure 7.6 and Table 7.6, Table 7.7, Table 7.8) confirming the presence of fatty acids (Table 7.9). The detailed ion chromatograms of the tentitative identification are in Appendix 6. All the methyl esters (Table 7.8) in the mixture were identified by comparing the retention times of GC peaks with those of the standard compounds under the same chromatographic conditions using commercially available standards purchased from Sigma Aldrich Co.(Appendix 6).

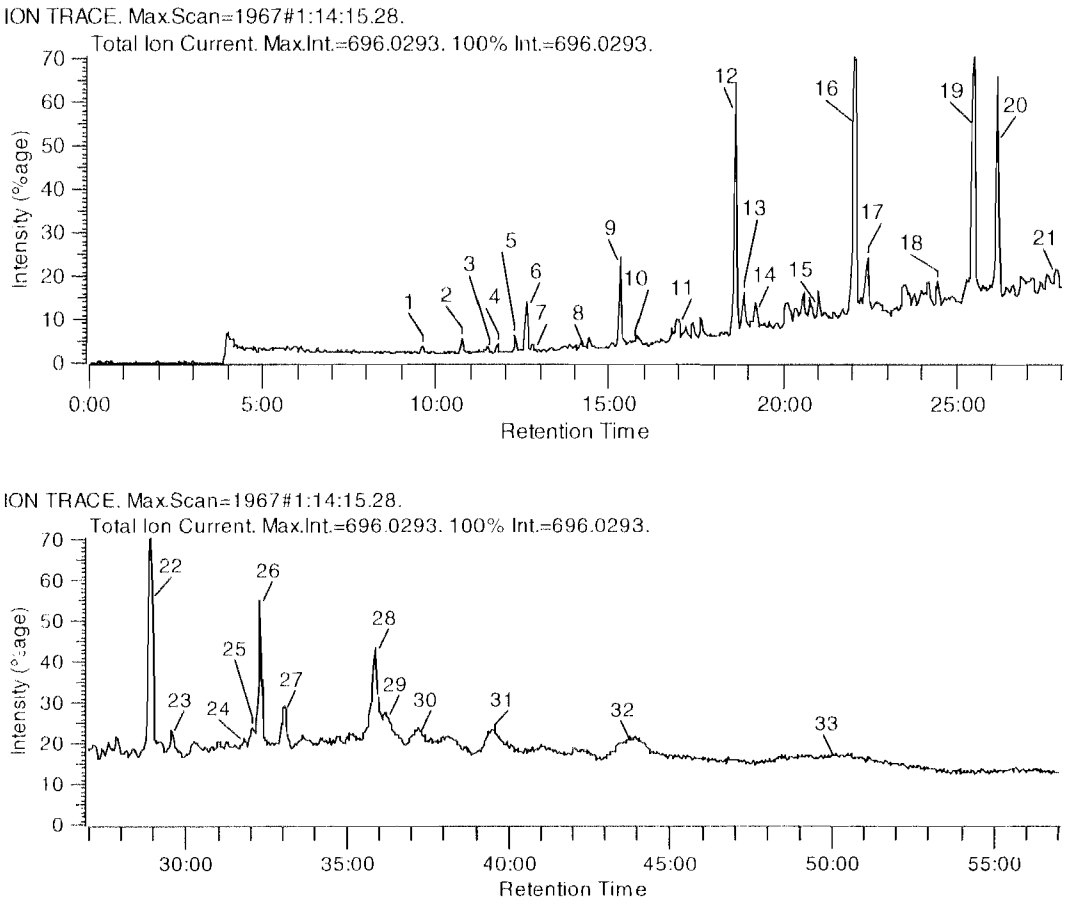


Figure 7.5 GC-MS total ion chromatogram of sample 1730/11E

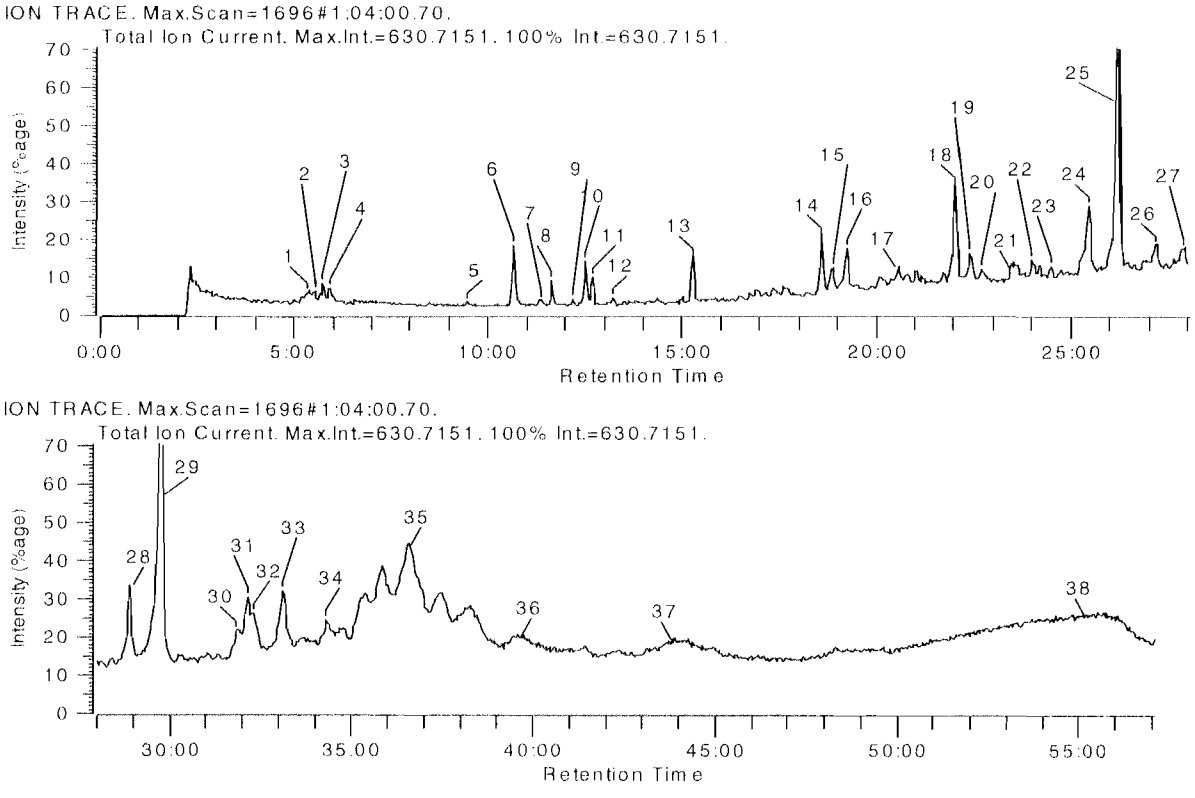


Figure 7.6 GC-MS total ion chromatogram of sample C1730E.

Table 7.6. The tentative identities of the components of samples 1730/11E.

ION NO. of 173011E	COMPOUND
1	Aliphatic hydrocarbon
2	Dimethyl phthalate
3	Unknown compound
4	Unsaturated cyclic hydrocarbon
5	Pentadecane
6	Butylated hydroxytoluene
7	Methyl dodecanoate
8	Aliphatic hydrocarbon
9	Hexadecane
10	Diphenylamine
11	Aliphatic hydrocarbon
12	Heptadecane
13	Aliphatic hydrocarbon
14	Methyl tetradecanoate
15	Aliphatic hydrocarbon
16	Octadecane
17	Aliphatic hydrocarbon
18	Aliphatic hydrocarbon
19	Nonadecane
20	Methyl hexadecanoate
21	Aliphatic hydrocarbon
22	Icosane
23	Phthalate
24	Methyl (Z,Z)-9,12-octadecadienoate
25	Methyl Z-9-octadecenoate
26	Henicosane
27	Methyl octadecanoate
28	Docosane
29	Phthalate
30	Aliphatic hydrocarbon
31	Tricosane
32	Tetracosane
33	Phthalate

Table 7.7. The tentative identities of the components of samples C1730E

ION NO. of C1730E	COMPOUND
1	Aliphatic alcohol
2	Unsaturated aliphatic hydrocarbon
3	Aliphatic alcohol
4	Unsaturated aliphatic hydrocarbon
5	Aliphatic hydrocarbon
6	Dimethyl Phthalate
7	Unknown compound
8	Unsaturated cyclic hydrocarbon
9	Aliphatic hydrocarbon
10	Butylated hydroxytoluene
11	Methyl dodecanoate
12	Unknown compound
13	Phthalate
14	Heptadecane
15	Phthalate
16	Methyl tetradecanoate
17	Aliphatic hydrocarbon
18	Octadecane
19	Aliphatic hydrocarbon

ION NO. of C1730E	COMPOUND
20	Methyl pentadecanoate
21	Aliphatic hydrocarbon
22	Phthalate
23	Aliphatic hydrocarbon
24	Nonadecane
25	Methyl hexadecanoate
26	Phthalate
27	Aliphatic hydrocarbon
28	Icosane
29	Phthalate
30	Methyl(Z,Z)-9,12-octadecadienoate
31	Methyl Z-9-octadecenoate
32	Aliphatic hydrocarbon
33	Octadecanoate
34	Phthalate
35	Phthalate
36	Aliphatic hydrocarbon
37	Aliphatic hydrocarbon
38	Phthalate

Table 7.8 Methyl Ester and related fatty acids observed in sample 1730/11E and C1730E.

SAMPLE NO (ION. NO)	METHYL ESTERS OF FATTY ACIDS	FATTY ACIDS
1730/11E (7)	Methyl dodecanoate	Dodecanoic acid
1730/11E (14)	Methyl tetradecanoate	Tetradecanoic acid
1730/11E (20)	Methyl hexadecanoate	Hexadecanoic acid
1730/11E (24)	Methyl (Z,Z)-9,12-octadecadienoate	9,12-Octadecadienoic acid
1730/11E (25)	Methyl Z-9-octadecenoate	9-Octadecenoic acid
1730/11E (27)	Methyl octadecanoate	Octadecanoic acid
C1730E (11)	Methyl dodecanoate	Dodecanoic acid
C1730E (16)	Methyl tetradecanoate	Tetradecanoic acid
C1730E (20)	Methyl pentadecanoate	Pentadecanoic acid
C1730E (25)	Methyl hexadecanoate	Hexadecanoic acid
C1730E (30)	Methyl(Z,Z)-9,12-octadecadienoate	9,12-Octadecadienoic acid
C1730E (31)	Methyl Z-9-octadecenoate	9-Octadecenoic acid
C1730E (33)	Octadecanoate	Octadecanoic acid

Table 7.9 Saturated and unsaturated fatty acids isolated from *P. virgata* and molecular formulas as obtained from The Merck Index 12th edition (1997).

Systematic name	Common name	Molecular formula
SATURATED FATTY ACIDS		
Dodecanoic acid	Lauric acid	$C_{12}H_{24}O_2$
Tetradecanoic acid	Myristic acid	$C_{14}H_{28}O_2$
Hexadecanoic acid	Palmitic acid	$C_{16}H_{32}O_2$
Octadecanoic acid	Stearic acid, Emersol	$C_{18}H_{36}O_2$
Pentadecanoic acid		$C_{15}H_{30}O2$
UNSATURATED FATTY ACIDS		
(Z,Z)-9,12-Octadecadienoic acid	Linoleic acid, 9,12-linoleic acid	$C_{15}H_{32}O_2$
(Z)-9-Octadecenoic acid	Oleic acid	$C_{18}H_{34}O_2$

Of the fatty acids identified in the GC-MS analysis only dodecanoic acid, stearic acid, palmitic acid, oleic acid, linoleic acid and myristic acid could be obtained commercially for further analysis. Pentadecanoic

acid had to be omitted from further analysis as it could not be obtained within the time of this investigation. Antimycobacterial activity of the fatty acids was assessed against *Mycobacterium tuberculosis* H37Rv and a clinical multidrug-resistant (MDR) strain of *M. tuberculosis* in the Bactec-460 radiometric method and *M. smegmatis* using direct bioautography. In the radiometric method, dodecanoic acid, myristic acid, linoleic acid and oleic acid significantly inhibited the growth of *M. tuberculosis.* Oleic acid was the most bioactive compound with an MIC-value equal to 25 µg/mL showing growth index values (GI-values) similar to rifampicin, which was used as the control drug (Table 7.10).

Table 7.10 Growth-indexes of the fatty acids in the Bactec-460 radiometric method using the H37Rv strain of *M. tuberculosis* obtained over the period 14 March 2006 to 20 March 2006.

Sample (Conc. in µg/mL))	14-Mar-06	15-Mar-06	16-Mar-06	17-Mar-06	18-Mar-06	19-Mar-06	20-Mar-06
Negative control	46	131	297	548	710	979	959
1:100 control	0	0	0	1	10	23	52
Solvent control	50	103	148	179	184	192	208
Rifampicin	19	11	5	2	1	0	0
Stearic acid (50)	33	64	113	151	179	220	258
Stearic acid (25)	29	80	183	353	559	825	999
Stearic acid (12.5)	36	126	326	664	913	999	999
Stearic acid (6.25)	39	135	293	560	626	868	949
Stearic acid (3.125)	62	214	399	660	778	877	810
Dodecanoic acid (50)	9	3	1	2	1	1	0
Dodecanoic acid 25)	15	25	42	68	87	128	183
Dodecanoic acid (12.5)	32	105	221	371	445	680	779
Dodecanoic acid (6.25)	41	140	251	451	520	617	613
Dodecanoic acid (3.125)	36	126	263	461	571	739	731
Myristic acid (50)	10	4	3	4	2	1	1
Myristic acid (25)	16	30	64	118	176	291	388
Myristic acid (12.5)	25	71	150	249	385	557	806
Myristic acid (6.25)	34	106	222	385	529	696	871
Myristic acid (3.125)	42	141	273	466	581	730	666
Linoleic acid (50)	8	1	0	0	0	0	0
Linoleic acid (25)	12	22	52	94	158	249	364
Linoleic acid (12.5)	17	42	92	177	248	349	508
Linoleic acid (6.25)	27	78	158	274	453	623	835
Linoleic acid (3.125)	22	74	158	278	402	561	799
Palmitic acid (50)	29	59	86	109	128	137	154
Palmitic acid (25)	33	104	217	354	591	937	999
Palmitic acid (12.5)	29	112	253	433	774	999	999
Palmitic acid (6.25)	39	147	289	431	635	971	977
Palmitic acid (3.125)	43	158	290	398	590	728	989
Oleic acid (50)	6	0	0	0	0	0	0
Oleic acid (25)	5	1	0	0	0	0	0
Oleic acid (12.5)	13	31	80	194	289	367	588
Oleic acid (6.25)	16	50	124	243	320	380	513
Oleic acid (3.125)	18	66	158	290	417	555	859
C1730 (125)	34	82	125	172	200	246	296
C1730 (62.5)	30	117	253	462	571	587	850
C1730 (31.25)	42	148	292	511	632	687	766
C1730 (15.625)	41	154	285	487	620	697	822
C1730 (7.8125)	42	155	298	531	641	713	804
C1730 (125)	26	74	133	205	261	295	408
C1730 (62.5)	39	125	244	343	377	356	560
C1730 (31.25)	36	130	289	447	537	602	742
C1730 (15.625)	38	149	306	467	562	654	827
C1730 (7.8125)	34	130	279	452	450	594	790

However, it showed only moderate inhibition at 50 µg/mL against MDR *M. tuberculosis* (Table 7.12). Dodecanoic acid had an MIC-value of 50 µg/mL with GI-values similar to rifampicin; while at a concentration of 25 µg/mL it could be considered moderately active against *M. tuberculosis* H37Rv. It inhibited the growth of the MDR-strain to the same extent and at the same concentrations. Myristic acid and linoleic acid also had MIC-values equal to 50 µg/mL with linoleic acid showing moderate inhibition at a concentration of 25 µg/mL against *M. tuberculosis* H37Rv. The MDR-strain was also moderately inhibited by myristic acid at 25 µg/mL, while linoleic acid showed inhibition at 50 µg/mL only. Stearic acid and palmitic acid was moderately active at 50 µg/mL against *M. tuberculosis* H37Rv, while myristic acid showed moderate inhibition of *M. tuberculosis* H37Rv at a MIC-value of 25 µg/mL. The fatty acid and hydrocarbon mixture was also tested against *M. tuberculosis* H37Rv and showed moderate inhibition at 125 µg/mL. Dodecanoic acid, linoleic acid and oleic acid inhibited the growth of *M. smegmatis* with MIC-values of 3.125 µg/mL, 1.56 µg/mL and 0.8 µg/mL respectively as opposed to stearic acid, myristic acid and palmitic acid that showed no inhibition of *M. smegmatis* (Table 7.11).

Table 7.11 Inhibition zones recorded for the fatty acids against *Mycobacterium smegmatis* using direct bioautography.

Fatty Acid (mass in µg)	Zone size (mm)
Stearic acid (50)	0
Stearic acid (25)	0
Stearic acid (12.5)	0
Stearic (6.25)	0
Dodecanoic acid (50)	13
Dodecanoic acid (25)	12
Dodecanoic acid (12.5)	11
Dodecanoic acid (6.25)	11
Dodecanoic acid (3.125)	9
Dodecanoic acid (1.5625)	0
Dodecanoic acid (0.8)	0
Dodecanoic acid (0.4)	0
Myristic acid (50)	0
Myristic acid (25)	0
Myristic acid (12.5)	0
Myristic acid (6.25)	0
Linoleic acid (50)	13
Linoleic acid (25)	13
Linoleic acid (12.5)	10
Linoleic acid (6.25)	9
Linoleic acid (3.125)	8
Linoleic acid (1.56)	5
Linoleic acid (0.8)	0
Linoleic acid (0.4)	0
Palmitic acid (50)	0
Palmitic acid (25)	0
Palmitic acid (12.5)	0
Palmitic acid (6.25)	0
Oleic acid (50)	13
Oleic acid (25)	13
Oleic acid (12.5)	13
Oleic acid (6.25)	9

Fatty Acid (mass in µg)	Zone size (mm)
Oleic acid (3.125)	9
Oleic acid (1.56)	7
Oleic acid (0.8)	5
Oleic acid (0.4)	0
Ciprofloxacin (12.5)	9
Ciprofloxacin (12.5)	9

Table 7.12. Growth-indexes of the fatty acids in the Bactec-460 radiometric method using a clinical multidrug-resistant strain of *M. tuberculosis* obtained over the period 23 March 2006 to 27 March 2006.

Sample (Conc. in µg/mL)	23-Mar-06	24-Mar-06	25-Mar-06	26-Mar-06	27-Mar-06
Negative control	162	346	570	852	999
1:100	2	1	4	10	49
Rifampicin	85	136	164	193	234
Solvent control	114	174	240	325	443
Oleic acid (50)	50	63	94	172	284
Oleic acid (25)	35	48	98	229	478
Oleic acid (12.5)	88	147	319	673	947
Oleic acid (6.25)	94	167	304	617	836
Oleic acid (3.125)	114	212	377	707	928
Dodecanoic acid (50)	34	22	15	15	12
Dodecanoic acid (25)	63	67	89	141	218
Dodecanoic acid (12.5)	124	215	376	657	749
Dodecanoic acid (6.25)	116	221	388	660	804
Dodecanoic acid (3.125)	116	208	336	591	782
Linoleic acid (50)	36	37	45	67	91
Linoleic acid (25)	56	70	123	244	435
Linoleic acid (12.5)	89	148	314	633	846
Linoleic acid (6.25)	108	190	386	666	807
Linoleic acid (3.125)	177	327	510	735	799
Myristic acid (50)	19	10	5	4	5
Myristic acid (25)	45	44	64	104	157
Myristic acid (12.5)	84	140	288	447	605
Myristic acid (6.25)	105	199	373	658	861
Myristic acid (3.125)	93	178	316	565	828

7.3 Discussion

Fatty acids are important components of terrestrial plant oils (Evans, 1996b) and the same is true for marine plants. Many investigations into marine animal and plant lipids have published (Dembitsky and Srebnik, 2002; Carballeira, 2002; Sanina *et al.*, 2004; Huang and Wang, 2004). The fatty acids identified in this investigation of *P. virgata* are common to red algae and specifically to the genus Polysiphonia. Johns *et al.* (1979) investigated the fatty acid composition of 10 seaweeds from Australian waters. *Polysiphonia pungens* was one of the species investigated and showed high levels of polyunsaturated fatty acid, such as, arachidonic acid. Palmitic acid was the main saturated fatty acid. Cis-vaccenic acid, *cis*-9-hexadecenoic or palmitoleic acid and myristic acid were minor components in the fatty acid mixture. Pettitt *et al.* (1989) investigated the lipids of red the red algae *Chondrus crispus* and *Polysiphonia lanosa*. The major phospholipids were phosphatidylcholine and phosphatidylglycerol with trace amounts of

phosphatidic acid and diphosphatidylglycerol. The major glycolipids were mono glycosyl- diacylglycerol, diglycosyldiacylglycerol and sulphoquinovosyldiacylglycerol. Galactose was the major component of both monoglycosyldiacylglycerol and diglycosyldiacylglycerol. The major fatty acids detected were palmitic, oleic, arachidonic and eicosapentaenoic acids. Huang and Wang (2004) investigated 16 species of seaweeds from China and found species, such as, *Porphyra haitanensis* and *Undaria pinnatifida* to have lipids totaling 168.3 and 124.1 mg/g dry weight, respectively. The lipids comprised of 9 fatty acids, 14 alkanes, 1,1´-bicyclopentyl and cholesta-3,5-diene and the fatty acids were divided into saturated and unsaturated fatty acids. The seaweeds were tested for antioxidant activity and it was established that the type of lipid was a vital consideration for this bioactivity and not the overall lipid content. An red alga with a lipophilic portion high in antioxidant activity, *Rhodomela confervoides*, had only 37.7% mg/g dry weight in lipid content, but the most unsaturated fatty acids (46% of lipophylic content), such as, 11-hexadecenoic acid and 5,8,11,14,17-eicosapentaenoic acid. *Plocamium telfairiae* was one of the algae with the lowest antioxidant activity and had the least amount of unsaturated fatty acids (7%). In another study, Vaskovsky *et al.* (1996) determined the polar lipid and fatty acid composition of 24 macroalgae from the Yellow Sea. They found glycolipids, such as, monogalactosyldiacylglycerol and phospholipids such as phatidylcholine present as the major polar lipid components. The red algae were rich in C_{20} polyunsaturated fatty acids mainly arachidonic and eicosapentaenoic acids. Rezanka and Dembitsky (2001) isolated polyhalogenated homosesquiterpenic acids from *Plocamium cartilagineum* from the Mediterranean. Halogenated fatty acids are very common in the marine environment and many have been isolated from algae and other marine organisms as shown in a review done by Dembitsky and Srebnik (2002). Many other lipids were isolated from other marine organisms as shown in Table 7.13.

Table 7.13. A few exarnples of fatty acids isolated from marine organisms.

Organism	Type	Examples of Fatty acids	Bioactivity	References
Amphimedon compressa	Sponge	2-methoxyhexadecanoic acid		Carballeira *et al.*, 1998a
Amphimedon viridis	Sponge	5,13-nonadecadienoic acid		Carballeira and Shalabi, 1994
Callyspongia fallax	Sponge	Methoxylated fatty acids		Carballeira and Pagan, 2001
Calyx podatypa	Sponge	9,13-dimethyltetradecanoic acid		Carballeira and Pagán, 2000
Chondria armata	Red alga	Domoic acid		Zaman *et al.*, 1997
Codium dwarkense	Green alga	Octadecadienoic acid		Dembitsky *et al.*, 2003
Eunicea succinea	Sponge	(5Z,9Z)-14-methyl-5,9-pentadecadienoic acid	Antimicrobial	Carballeira *et al.*, 1997
Eunicea succinea	Gorgonian	(5Z,9Z)-14-methyl-5,9-pentadecadienoic acid	Antimicrobial	Carballeira *et al.*, 1997
Gracilaria verrucosa	Red alga	Chlorinated C12 fatty acid metabolites		Shoeb and Jaspars, 2003
Holothuria mexicana	Sea cucumber	7-methyl-6-octadecenoic acid		Carballeira *et al.*, 1996
Hyalosiphonia caespitosa	Red alga	C20 polyunsaturated fatty acids		Vaskovsky *et al.*, 1996
Kappaphycus alvarezzi	Red alga	Oleic acid, linoleic acid, heptadecanoic acid	Antioxidant	Fayaz *et al.*, 2005
Oceanapia sp.	Sponge	C14 acetylenic fatty acid	Antimicrobial	Matsunaga *et al.*, 2000
Parietochloris incisa	Green alga	Arachidonic acid		Bigogno *et al.*, 2002
Peyssonnelia caulifera	Red alga	Ω3 fatty acids	Dna methyl-transferases inhibitors	McPhail *et al.*, 2004
Plocamium telfairiae	Red alga	Hexadecanoic acid	Antioxidant	Huang and Wang, 2004

Organism	Type	Examples of Fatty acids	Bioactivity	References
Ptilota filicina	Red alga	Conjugated triene-containing fatty acids		Wise *et al.*, 1994
Ptilota filicina J. Agardh	Red alga	Icosapentaenoic acids		Lopez and Gerwick, 1987
Schizymenia dubyi	Red alga	Methoxy fatty acids		Barnathan *et al.*, 1998
Sporothrix flocculosa	Fungus	9-heptadecenoic acid	Antibiotic	Benyagoub *et al.*, 1996
Sporothrix flocculosa	Fugus	4-methyl-7,11-heptadecadienoic acid	Antifungal, antimicrobial	Choudhury and Traquair, 1994
Stolonica sp.	Ascidian	3,6-epidioxy-7,10-tetrahydrofurano C26 unsaturated fatty acids	Cytotoxic	Davies-Coleman *et al.*, 2000
Synthetic Compound		3-methylnonadecanoic acid	Larvicidal	Hwang *et al.*, 1978
Synthetic Compound		(Z)-2-methoxy-5-hexadecenoic acid	Antimicrobial	Carballeira *et al.*, 1998b
Tanacetum balsamita	Plant	Hexanoic acid, octanoic acid	Antimicrobial	Kubo and Kubo, 1995
Trigonia fasciculata	Plant	Oleic acid		Mafezoli *et al.*, 2003

This was not the first isolation of bioactive fatty acids from a South African red alga. In an investigation done by Cameron *et al.* (2001) the bioactive compounds isolated from the alga *Plocamium corallorhiza* were partially characterized as long chain fatty acids. Shoeb and Jaspars (2003) investigated a South African red alga *Gracilaria verrucosa*, which was collected from Port Elizabeth and isolated the three fatty acid derived compounds shown in Figure 7.6. All three compounds were suggested to originate from dodec-2-enoic acid.

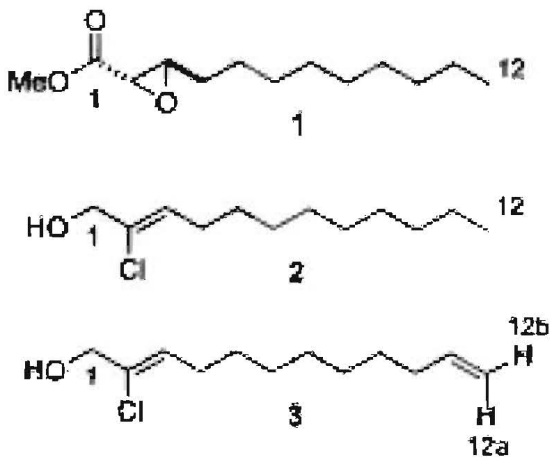


Figure 7.7. 3-nonyloxirane-2 carboxylic acid methyl ester (1), 2-chlorododec-2-en-1-ol (**2**) and 2-chlorododec-2,11-dien-1-ol (**3**) were isolated from *Gracilaria verrucosa* (Shoeb and Jaspars, 2003).

Hydrocarbons are also common in algae. In the above study, 14 hydrocarbons, such as, heptadecane, octadecane and triacontane were isolated (Haung and Wang, 2004). The microalga *Botryococcus braunii* produced a large amount of hydrocarbons such as *n*-alkadienes and trienes, triterpenoid botryococcenes and methylated squalenes as determined by Metzger and Largeau (2005). For this reason, edible algae have to be analyzed to determine the healthfulness of the product as was done in the case of the brown

alga *Himanthalia elongata* where it was found to have a low aliphatic hydrocarbon content of between 14.8 and 40.2 µg/g dry weights (Crespo and Yusty, 2004) which were considered safe. At the isolation of volatiles from the seagrass *Zostera marina* part of the major constituents were characterized pentadecane, heptadecane and nonadecane (Kawasaki *et al.*, 1998). *Sesquiterpene* hydrocarbons were isolated from a new sponge species *Cymbastela hooperi* (Wright and König, 1996). Giese *et al.* (1999) detected iodinated and brominated hydrocarbons in an investigation where 29 algal species were collected and the release of volatile iodinated hydrocarbons determined.

Many publications are available about fatty acids with antibiotic activities. Findlay and Patil (1984) isolated the fatty acids (6Z,9Z,12Z,15Z)-hexadecatetraenoic acid, (6Z,9Z,12Z,15Z)-octadecatetraenoic acid and (6Z,9Z,12Z)-hexadecatrienoic acid as the antibacterial compounds from the diatom *Navicula delognei*. It inhibited the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Proteus vulgaris*. Benyagoub *et al.* (1996) isolated 9-heptadecenoic acid and 6-methyl-9-heptadecenoic acid as the compounds responsible for the antibiotic activity of the fungus *Sporothrix flocculosa*. Ohta *et al.* (1993) investigated the antibiotic activities of 10 fatty acids and their methyl esters and found γ-linolenic acid (C18:3) to have the highest antibiotic activity. α-Linolenic acid along with eicosapentaenoic acid and docosahexaenoic acid also had strong antibiotic activity.

The antimycobacterial activity of long-chain fatty acids have been demonstrated by a few investigations. A potent antimycobacterial compound, 3-nitropropionic acid was isolated from endophytic fungi found on Thai medicinal plants with a minimum inhibition concentration of 3.3 µM (Chomcheon *et al.*, 2005). Antimycobacterial (MIC-value of 25 µg/mL) and antiplasmodial activity (IC_{50}-value of 7.2 µg/mL) was reported for scleropyric acid isolated from the twigs of *Scleropyrum wallichianum* (Suksamrarn *et al.*, 2005). Stavri *et al.* (2004) investigated the hexane extract of hops, namely *Humulus lupulus* for antimycobacterial compounds and found a fatty acid mixture to be the bioactive principle. Amongst the fatty acids were palmitic, stearic and oleic acid with small quantities of lignoceric, arachidic, behenic and linoleic acids. All saturated fatty acids were inactive against *Mycobacterium fortuitum*, while the unsaturated fatty acids, namely oleic and linoleic acids showed minimum inhibitory concentrations of between 4 and 16 µg/mL. Kondo and Kanai (1972) looked at the lethal effect of long-chain fatty acids on mycobacteria and found that out of 11 long-chain fatty acids, oleic, linoleic and myristic acid showed the greatest activity against *Mycobacterium tuberculosis* and *M. bovis*. Seidel and Taylor (2004) investigated the dried roots of *Pelargonium reniforme* and *P. sidoides* for antibacterial activity against *Mycobacterium aurum* and *M. smegmatis* and showed the active components to contain palmitic, oleic and linoleic acid as

the major bioactive components. Although the initial mixture of fatty acids and hydrocarbons were highly active against *M. smegmatis* in direct bioautograph, but weakly active against *M. tuberculosis* in the radiometric method, the antimycobacterial activity of the individual fatty acids, such as oleic acid and linoleic acid are consistent with the above investigations.

Chapter 8

DISCUSSION AND CONCLUSION

8.1 Discussion

The primary objective of establishing whether the algal extracts of *Plocamium corallorhiza*, *Gelidium pristoides,* and *Polysiphonia virgata* contain antimycobacterial compounds was achieved in this investigation. All three algae presented fractions with antimycobacterial activity against *Mycobacterium tuberculosis* in the Bactec-460 method with *P. virgata* showing the greatest number of bioactive fractions. The secondary objective of isolating the bioactive compounds from the algae was achieved for *P. virgata*, but not for *P. corallorhiza* and *Gelidium pristoides*. Initially, a moderately anti-tuberculosis compound, namely, 2-methoxyethyl methacrylate was isolated from *P. virgata*. A follow-up investigation revealed that instead of the methacrylate, long-chain fatty acids were the major antimycobacterial compounds in *P.virgata*.

A difference in the conditions at the time the two samples of *P. virgata* were collected seems to have influenced the phytochemical composition of the alga. In the first collection, *P. virgata* was obtained from the tidal pools as opposed to being cut from the stipes of the kelp *Ecklonia maxima* at the second collection. The tidal pools sample was removed from the kelp stipes for much longer allowing more time for degradation by-products, such as the methacrylate to be formed as discussed in Chapter 6. This is likely to be the reason why the methacrylate was not detected in the extract of the second collection. For future algae investigations it is recommended to ensure that the algae are cut from the holdfast or host plants to reduce the formation of degradation by-products. In addition, after cleaning the algae it should be dipped in boiling water to inactivate enzymes as was done in a study by Sanina *et al.*, 2004. Seasonal variations could also have influenced the results as noted by Pratt *et al.* (1951) and Hornsey and Hide (1976). The second sample of *P. virgata* was collected at the middle of winter in July 2004 while the first sample was collected in the middle of autumn in April 2002. If a future investigation is aimed at following up a particular aspect of this investigation, the time of year the samples are collected should also be taken into account.

Since MEMA is commercially available, a structure-activity relationship investigation should be done to improve the antimycobacterial activity. The diagram below indicates the regions in the structures of MEMA and HEMA, where simple chemical modifications could be used to generate a considerable number of new compounds.

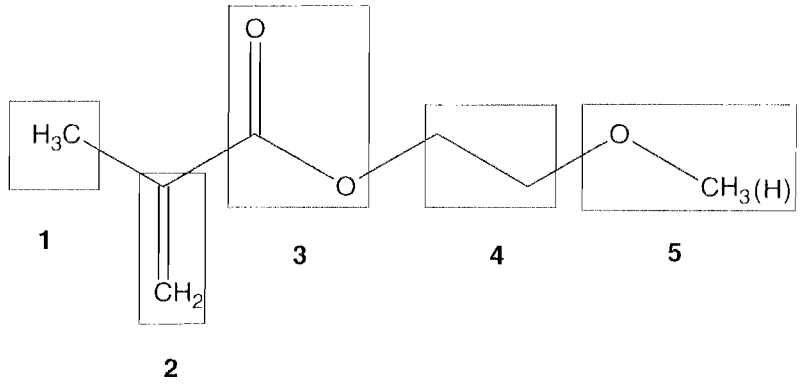


Figure 8.1 A diagram of the modifiable regions in the structures of MEMA and HEMA.

1. Replacement by H or a variety of alkyl groups.
2. Functionalisation of the double bond leading to diols, alcohols, epoxides, cyclopropanes, and aziridines.
3. Formation of new esters, amides, thioesters.
4. Variation of chain length to improve lipophilicity.
5. Formation of esters, sulphonic acid esters, ethers, amines, ureas and sulphonyl ureas.

Derivatives of MEMA have been patented and should be easy to obtain, and their syntheses do not have to be attempted (see Appendix 4). In addition, *in vivo* antimycobacterial activity of MEMA and its analogs should be investigated. MEMA and its analogs should also be tested against various mycobacteria and other pathogenic microorganisms. A difficulty that arises after finding a natural product with pharmaceutical potential is the production of significant quantities of the bioactive compound by straightforward extraction or by synthesis with the latter being the preferred root. Often the structures of marine compounds are complex and their syntheses involve such long procedures that industrial synthesis does not seem economically viable (Munro *et al.*, 1999). With MEMA, the simple compound has already been synthesized. A thorough investigation into its biological activities is recommended.

The extraction procedure used in this investigation with dichloromethane: methanol (2:1) proved highly effective. However, the initial purification technique of preparative layer chromatography (PLC) was very

time consuming. Since seaweeds contain a large number of secondary metabolites with each purified band separating into many individual bands, repeated preparative layer chromatography that involved scraping the bands from the glass plates and desorbing the compounds using various solvents took too long. The Chromatotron, which was introduced in the second year of this investigation, resolved this problem immediately. The ease of collecting the bands as they progressed off the centrifugal plates reduced the time of fractionation significantly. With purification, the number of bands did not reduce, but it was easier to collect and dry the resultant solutions. As with all chromatographic procedures, establishing the most effective mobile phase takes time. Another method that could have been used for purification is open-column chromatography. In the investigation of Cameron *et al.* (2001) which led to this investigation, PLC was used together with open-column chromatography. The bioactive compounds reported in that investigation was indicated by their positions on the TLC plates. As a result, TLC was the initial method of choice in this investigation. The Chromatotron used the same principles and it was the logic choice to build on previous findings.

The BACTEC-460 procedure gave a direct indication of anti-tuberculosis activity in the partially purified fractions. The direct assessment against a sensitive strain of *M. tuberculosis* ensured that possible negatives with *M. aurum* or *M. smegmatis* did not escape *M. tuberculosis* exposure. However, the re-investigation of *P. virgata* showed that *M. smegmatis* is an ideal candidate for large-scale investigations together with the direct bioautography method, which worked very well and eliminated the problems of the insolubility of plant compounds with agar-based assays. The bioactivity of the fractions against *M. smegmatis* was right on target as demonstrated by the subsequent inhibition of *M. tuberculosis* in the radiometric method. It would be of value to test the bioactive fractions of the algae against multi-drug resistant strains of *M. tuberculosis*, as there is an increasing demand for more effective drugs. In addition, it would be useful to test the bioactive fractions and isolated compounds in combination with existing first-line tuberculosis drugs and to evaluate their combined cytotoxicity.

The cytotoxicity analysis could prove useful as toxicity over the long treatment periods is currently problematic and could lead to the discontinuation of treatment. In addition, the bioactive fractions and isolates could also be introduced into an *in vivo* anti-tuberculosis model. The water portions of the algal extracts should also be tested in an *in vivo* model, as low cytotoxicity was observed *in vitro* against Chinese hamster ovarian cells. No inhibitory activity was found against *M. tuberculosis in vitro*, but *in vivo* testing is worth an investigation as the water portion is one of the main reasons for the existence of the current seaweed industry and a significant finding might add value to an argument for a local agar plant linked to a pharmaceutical industry.

The inhibition of the growth of *Plasmodium falciparum* by the purified fractions of *P. corallorhiza* with IC_{50}-values of 2.4 µg/mL to 8.9 µg/mL demonstrates the potential of this alga as a source of antimalarial compounds. A follow-up investigation into it antimalarial compounds is highly recommended. Such a study should include the algae used in this investigation with brown and green seaweeds.

The crude extracts of the three seaweeds did not inhibit the growth of *M. tuberculosis*, but the purified fractions showed inhibition. This is a valuable strategy in screening plant extracts as many investigations rely on the finding for the crude extracts and do not include fractionation unless the crude extract is bioactive. From the results of this investigation, it is clear that fractionation is a vital part of the experimental procedure and bioactivities can only be reported if the extract was fractionated.

This investigation was undertaken with the assumption that the antimicrobial activities of the three seaweeds, namely, *Gelidium pristoides*, *Plocamium corallorhiza* and *Polysiphonia virgata*, in the investigation by Cameron *et al.* (2001) would lead to anti-tuberculosis compounds. The assumption was confirmed by the significant inhibition of *Mycobacterium tuberculosis* by the algal purified fractions in the Bactec-460 radiometric method and the subsequent isolation of long-chain fatty acids as the major anti-tuberculosis compounds from *P. virgata*.

8.2 Conclusions

This investigation has demonstrated that seaweeds are potential sources of anti-tuberculosis compounds. As an untapped local resource for pharmaceutical agents, the number of investigations that could be launched to investigate their pharmaceutical potential is unlimited. In this study, only three algae were investigated. With South Africa's diverse marine plant resource, many red, brown, and green algae remain to be investigated and should form part of any drug development program.

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Appendix 1

SAMPLE MASSES

Table A1.1 Purified fractions and the sample masses obtained using the Chromatotron.

Sa no.	Sa mass (mg)	Sa no.	Sa mass (mg)	Sa no.	Sa mass (mg)
1111	55.84	21	120.81	1032	1663.51
11111	1.09	211	46.59		
11112	31.64	212	57.61	31	183.84
111121	1.24			311	42.33
111122	24.33	22	304.32		
111123	8.71			312	25,42
11113	29.84	23	205.94	3122	0.42
		231	3.49	3123	7.64
1112	27.07	232	142.03	3124	0.44
11121	0.94	233	20.9	3124	1.61
11122	11.5	234	10.88	3125	-2.62
111221	41.23	235	10.91	3126	-9.11
111222	7.58			3129	0.6
111223	3.87	24	68.46		
		241	1.73	(2531)31231	0.37
11123	5.68	242	27.32	(2533)31233	5.2
111231	-0.08	243	10.11	312331	2.12
111232	10.3	244	54.8	312332	0.05
111233	0.73			312333	0.75
		25	84.55	312334	0.84
112	68.09	251	0.95	312335	0.44
1121	3.65	252	44.75	312336	0.12
11221	21.84	253	7.73	312338	0.18
112211	125.68	254	1.21	312339	0.23
112212	20.46	255	7.75	3123310	0.37
112213	4.13	256	5.7	3123311	0.32
				3123311A	0.39
11223	3.87	26	153.12	3123312	0.61
112231	4.05	261	8.33	3123312	0.58
112232	2.04	262	3.63		
112233	0.68	263	4.14	31234	0.93
		264	10.64		
112221	0.41	265	10.24	(2535)31235	-15.46
112222	1.8	266	12.15		
112223	5.74	267	18.93	3127	4.04
1122231	0.01			31271	-1.51
1122232	2.94	221	49.06	31272	0.34
1122233	2.38	222	36.54	31272A	0.1
112224	2.18	223	126.59	31273	0.74
112225	2.63	222RET	21.66	31274	1.36
				3128	1.96
101 DCM	2546.29	2221	4.91		
11	423.65	22211	2.87	31210A	0.52
		22212	1.2	31210	0.51
113	133.95			3121	1.61
1131	6.07	2222	17.6	31211	-0.83
11311	0.39	22221	3.78	31212	0.58
11312	2.72	222211	0.48	31213	0.73
11313	1.1	222212			
		222213	1.12	313	12.52
1132	1.19	2222121	0.19	3131	0.47
11321	4.86	2222122	24.58	3132	0.50
11322	1.89	2222123	7.92	3133	1.15
11323	6.08			3134	0.46
11324	1.94	22222	12.3	3135	-3.02
11325	89.83	222221	0.46	3136	0.7
		222222	2.84	3137	1.57
11325111	2.51	222223	6.5	3138	4.41
11325112	28.68	222224	1.05	3138A	0.96
11325113	16.56			3139	0.13
113251131	0.54	2222231	0.23	31310	0.32
113251132	7.97	2222232	1.51	31311	0.3
1132511321	0.26	2222233	2.37	31312	1.53
1132511322	6				
1132511323	5.8	2222234	1.29	(2611)31381	0.15
1132511324	1.33	22222333	0.59	(2612)31382	0.76
113251133	3.74	2223	31.92	(2613)31383	-13.53
113251134	0.00	22231	0.93	(2614)31384	0.69
113251135		22232	28.16	(2615)31385	0.73
113251136	-1.22	222321	29.38	(2616)31386	0.4
113251137	0.43	2223211	0.06	(2617)31387	0.67
113251135	0.68	2223212	0.21	(2618)31388	3.08
113251138	3.28	2223213	Lost	313882	2.02

Sa no.	Sa mass (mg)	Sa no.	Sa mass (mg)	Sa no.	Sa mass (mg)
				313883	0.18
113252	21.92	222322	32.91	313884	0.15
113253	1.77	2223221	2.14	313885	0.32
		2223222	3.42	(2619)31389	0.64
11326	7.96	2223223	2.54	(26110)313810	0.65
11327	2.04	2223224	8.07	(26111)313811	0.75
1133	2.51	22232242	0.51	(26112)313812	0.33
1134	0.8	22232243	3.08	313813	0.56
1135	1.54				
		2223225	2.17	314	22.56
114	92.36	2223226	1.85	3141	1.6
		2223227	0.81	3141	2.34
115	39.07	2223228		31411A	0.37
		22232213	0.76	31411 (11)	0.8
116	19.48	22232214	0.26	31412	0.38
				31413	0.47
117	26.53	22232223	1.58	31413A	0.06
1171	0.44			31414	0.17
1172	0.1	22232231	0.62	31415	1.1
1173	0.94	22222333	0.59	31416	0.57
1174	0.05			3145	1.19
1175	0.06	22232253	1.31	3146	0.83
				3147	3.89
12	156.96	222323	2.46	3148	0.35
121	8.08			3148A	0.98
122	92.6	2224	9.49	3143(2)	0.71
123	26.46	22241	7.48	3143	-1.23
124	23.37	222411	0.5	3147	0.6
125	3.01	222412	5.25	31410	0.34
		222413	1.56	31410	-2.78
13	105.29			31411A	0.37
131	55.14	2225	0.42	315	9.61
132	35.18			3151A	0.55
133	23.73			3151	0.80
134	7.5			3152	0.55
135	21.03			3153	0.41
				3154	0.46
14	825.93			3154a	0.39
141	1.76			3154A	0.74
142	7.18			3155A	1.8
143	170.67			3155	1.01
144	129.93			3156	1.04
145	5.54			3156A	0.74
141*	3.43			3157	0.31
142*	38.65			3158	-3.93
143*	84.67			3159	-14.26
144*	239.66				
145*	32.98				
				316	7.43
15	640.95			317	325.81
151	65.82				
152	89.35			32	29.45
153	3.98			321	2.09
154	447.22			322	9.18
				323	10.38
16	28.78				
16	507.92			33	21.41
161	13.88			331	0.13
1611	1.00			332	5.81
1612	0.25			333	6.33
1613	0.07			334	2.31
1614	0.28				
1615	0.81			34	20.56
1616	0.23			341	9.59
1617	2.00			342	0.15
1618	0.43			343	1.07
1619	0.65			344	5.93
16110	2.18			345	2.04
1611A	0.64			346	5.88
1613A	0.34				
1615A	0.46				

Sa no.	Sa mass (mg)	Sa no.	Sa mass (mg)	Sa no.	Sa mass (mg)
				35	52.22
162	66.25			351	5.1
163	87.62			352	11.26
164	83.42			353	224.71
165	9.77			354	11.71
166	77.94			355	6.84
167	43.87			356	5.63
				36	4.5
				37	11.78
				38	212.46
				381	1.41
				382	70.21
				383	48.78
				384	7.69
				385	14.74
				386	30.87
				39	48.81
				391	14.24
				392	10.39
				393	15.66
				394	3.58

Table A1.2 Sample masses obtained using flash chromatography.

Sa no.	Solvent System	Fractions	Mass (mg)
1	1 - 6 = 100:0 Hex:EtOAc	1 - 12	**110.1**
2	7 - 10 = 95:5	13 -14	**55.7**
3	11 - 18 = 90:10	15 - 16	**125.2**
4	19 - 24 85:15	17	**22.7**
5	25 - 48 = 80:20	18 - 20	**22.7**
6	49 - 68 = 75:25	21 - 23	**37.8**
7		24 - 27	**45.5**
8		28	**28.5**
9		29	**25.7**
10		30 - 36	**347.3**
11		37 - 39	**44.7**
12		40 - 41	**14.8**
13		42 - 45	**39.9**
14		46 - 51	**54.9**
15		52 - 53	**24.6**
16		54 - 63	**75.5**
17	69 - 131 = 70:30	64 - 72	**52.7**
18		73	**7.1**
19		74 - 79	**36.8**
20		80 - 81	**17.8**
21		82 - 89	**35.9**
22		90 - 91	**9.2**
23		92 - 95	**15.9**
24		96 - 98	**11.6**
25		99 - 107	**34.5**
26		108 - 109	**7.6**
27		110 - 111	**13.1**
28		112 - 116	**29**
29		117 - 125	**32.1**
30		126 - 134	**30.1**
31	132 - 153 = 65:35	135 - 148	**60**
32		149 - 153	**17.6**
33	154 - 171 = 60:40	154 - 162	**44.4**
34		163 - 171	**38**
35	172 - 195 = 55:45	172 - 175	**20.6**
36		176 - 184	**38.6**
37		185	**4.4**
38	196 - 203 = 50:50	186 - 203	**72.1**
39	204 = 50:50	204	**25.7**
40	205 = 40:60	205	**67.2**
41	206 = 30:70	206	**17.8**
42	207 = 20:80	207	**71.5**
43	208 = 10:90	208	**335.4**
44	209 = 0:100	209	**258.6**
45	210 = EtOH	210	**1046**

Table A1.3 Masses of preparative HPLC purified fractions.

Sample no.	Sample mass (mg)
5/1	3.5
5/2	3
5/3	3.5
5/4	10.9
5/5	5
5/6	3.8
5/7	22.8
5/8	9.1
5/9	6.7
6 Hex	24.8
6 EtOAc	1.5
7 Hex	52.1
7 EtOAc	1.8
6/1	1.9
6/2	0.6
6/3	0.9
6/4	1.6
6/5	1.6
6/6	2.5
6/7	1.1
6/8	1.4
6/9	0.7
6/10	1
6/11	1
7/1	2.6
7/2	0.8
7/3	0.8
7/4	1.1
7/5	1.3
7/6	0.7
7/7	1.4
7/8	0.5
7/9	2.2
7/10	0.6
7/11	0.7
7/12	1.8
1730Hex	187.1
1730EtOAc	57.1
1730Crude	
1730/1	1.9
1730/2	16.7
1730/3	2.6
1730/4	13.2
1730/5	4.6
1730/6	5.3
1730/7	3.8
1730/8	14.5
6/664	0.7
6/61	0.1

Sample no.	Sample mass (mg)
6/62	0.2
6/63	0.0
6/64	0.2
6/65	0.4
6/66	
6/67	0.8
6/68	0.2
6/69	0.1
6/610	0.4
6/661	-0.3
6/662	0.2
6/663	0.1
6/664	
6/665	1.4
6/51	0.1
6/52	0.6
6/53	0.8
6/54	0.4
6/55	0.3
6/56	0.4
6/57	0.3
6/58	0.8
2&3Hex	152.8
2&3EtOAc	2.5
1730/1	34.9
1730/2	26.3
1730/3	4.9
1730/4	4.7
1730/5	1.7
1730/6	2.6
1730/7	1.7
1730/8	1.7
1730/9	10.0
1730/10	17.7
1730/11	8.7
1730/12	5.2
1730/13	2.9
2&3/1	3.6
2&3/2	1.3
2&3/3	0.9
2&3/4	1.1
2&3/5	0.4
2&3/6	13.2
2&3/7	16.6
2&3/8	14.3
2&3/9	59.5
2&3/10	24.7
2&3/11	6.3
2&3/12	10
2&3/13	10.9
2&3/14	4.9
1730/11	26
1730/21	17

Sample no.	Sample mass (mg)
1730/27	16.2
1730/31	6.8
1730/32	9.4
1730/37	9.4
1730/41	5.8
1730/54	2.8
2&3/131	9.5
2&3/142	6.2
6/91	4.8
R/31	6.2

Appendix 2

PARASITE LACTATE DEHYDROGENASE ASSAY

The data are were processed on 20020610 (15:25:14)

Row of wells A:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	8.51	29.7	70.2	80.8	112.	101.	94.6	89.3	94.6	84.0
Row of wells B:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	5.20	31.2	76.0	72.9	96.8	100	92.7	81.2	95.8	88.5
DC-1										
means:	6.85	30.5	73.1	76.8	104.	100.	93.6	85.3	95.2	86.2
stdev:	2.33	1.03	4.12	5.61	11.2	.752	1.39	5.73	.814	3.18
Row of wells C:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	8.91	24.7	52.4	81.1	100	87.1	87.1	98.0	88.1
Row of wells D:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	2.66	13.3	28	73.3	102.	118.	120	117.	137.	125.
DC-2										
means:	1.33	11.1	26.3	62.9	91.9	109.	103.	102.	117.	106.
stdev:	1.88	3.12	2.29	14.7	15.1	13.1	23.2	21.3	27.7	26.3
Row of wells E:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	4.85	9.70	17.4	47.5	74.7	81.5	75.7	71.8	86.4	88.3
Row of wells F:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	2.80	7.47	22.4	49.5	73.8	81.3	80.3	78.5	67.2	92.5
DC-3										
means:	3.82	8.59	19.9	48.5	74.2	81.4	78.0	75.1	76.8	90.4
stdev:	1.45	1.57	3.50	1.38	.654	.173	3.28	4.70	13.5	2.95
Row of wells G:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	4.08	13.2	30.6	67.3	80.6	81.6	90.8	84.6	85.7	93.8
Row of wells H:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	5.15	12.3	39.1	76.2	77.3	86.5	98.9	87.6	79.3	81.4
DC-4										
means:	4.61	12.8	34.8	71.8	78.9	84.1	94.8	86.1	82.5	87.6
stdev:	.758	.632	6.05	6.32	2.32	3.51	5.76	2.07	4.47	8.79

Row of wells A:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	14.1	22.3	42.3	81.1	97.6	92.9	97.6	107.	102.	97.6
Row of wells B:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	4.49	15.7	46.0	74.1	92.1	96.6	103.	88.7	98.8	100
DC-5										
means:	9.30	19.0	44.2	77.6	94.8	94.7	100.	97.9	100.	98.8
stdev:	6.80	4.68	2.62	4.96	3.89	2.60	4.04	12.9	2.45	1.66
Row of wells C:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	59.7	89.1	80.4	100	100	107.	104.	102.	97.8	103.
Row of wells D:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	46.8	90.4	76.5	102.	103.	108.	97.8	107.	94.6	107.
DC-6										
means:	53.2	89.7	78.5	101.	101.	108.	101.	104.	96.2	105.
stdev:	9.17	.915	2.71	1.50	2.25	.637	4.57	3.72	2.22	2.95
Row of wells E:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	5.82	11.6	29.1	69.9	86.4	83.4	85.4	96.1	97.0	99.0
Row of wells F:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	8.57	13.3	32.3	63.8	92.3	88.5	92.3	89.5	95.2	90.4
DC-7										
means:	7.19	12.4	30.7	66.8	89.3	86.0	88.9	92.8	96.1	94.7
stdev:	1.94	1.18	2.30	4.30	4.22	3.58	4.91	4.66	1.30	6.04

The data are being processed on 20020624 (14:42:44)

Row of wells A:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	0	22.2	34.5	69.1	93.8	112.	108.	133.	103.
Row of wells B:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	0	15.5	31.0	58.6	90.5	82.7	100	101.	81.8

2B-1

means:	0	0	18.8	32.8	63.8	92.1	97.5	104.	117.	92.8
stdev:	0	0	4.74	2.49	7.43	2.34	20.9	6.11	22.3	15.4
Row of wells C:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	0	14.7	36.4	58.1	80.6	89.1	98.4	94.5	71.3
Row of wells D:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	0	19.2	33.8	57.6	79.2	86.1	98.4	93.0	70

2B-3

means:	0	0	16.9	35.1	57.9	79.9	87.6	98.4	93.8	70.6
stdev:	0	0	3.18	1.82	.316	.982	2.11	8.43	1.05	.931
Row of wells E:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	0	0	11.1	37.3	69.0	88.8	102.	90.4	77.7
Row of wells F:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	0	0	12.4	33.3	73.6	89.1	99.2	89.1	74.4

2B-4

means:	0	0	0	11.7	35.3	71.3	89.0	100.	89.8	76.0
stdev:	0	0	0	.913	2.80	3.24	.182	2.23	.939	2.37
Row of wells G:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	0	5.55	23.0	51.5	75.3	84.9	95.2	84.9	63.4
Row of wells H:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	0	10.8	28.8	48.6	75.6	88.2	99.0	100	73.8

2B-6

means:	0	0	8.18	25.9	50.1	75.5	86.6	97.1	92.4	68.6
stdev:	0	0	3.71	4.11	2.07	.197	2.38	2.73	10.6	7.34

Row of wells A:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	84.8	81.0	98.7	113.	103.	108.	110.	121.	103.	91.1
Row of wells B:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	66.6	84.6	91.4	94.8	92.3	95.7	90.5	88.0	89.7	80.3
Sample 1										
means:	75.7	82.8	95.0	104.	98.0	102.	100.	104.	96.7	85.7
stdev:	12.8	2.54	5.14	13.4	8.12	9.28	13.8	23.6	9.93	7.63
Row of wells C:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	4.54	9.09	16.3	53.6	80	88.1	96.3	97.2	98.1	83.6
Row of wells D:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	2.41	8.87	14.5	42.7	64.5	69.3	83.8	87.0	91.9	78.2
Sample 2										
means:	3.48	8.98	15.4	48.1	72.2	78.7	90.1	92.1	95.0	80.9
stdev:	1.50	.155	1.30	7.70	10.9	13.3	8.83	7.19	4.41	3.82
Row of wells E:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	29.2	72.3	78.0	88.6	87.8	83.7	66.6	90.2	90.2	78.0
Row of wells F:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	32.5	63.4	75.6	83.7	84.5	91.0	91.0	92.6	97.5	86.1
Sample 3										
means:	30.8	67.8	76.8	86.1	86.1	87.3	78.8	91.4	93.9	82.1
stdev:	2.29	6.32	1.72	3.44	2.29	5.17	17.2	1.72	5.17	5.74
Row of wells G:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	0	0	0	1.83	33.9	76.1	80.7	77.9	69.7
Row of wells H:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	0	0	0	0	8.04	32.1	78.1	88.5	95.4	79.3
Sample 2.4										
means:	0	0	0	0	4.94	33.0	77.1	84.6	86.6	74.5
stdev:	0	0	0	0	4.39	1.24	1.42	5.49	12.3	6.77

DENISE'S FRACTIONS

Row of wells A:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	52.1	88.0	100.	112.	105.	113.	112.	117.	111.	102.
Row of wells B:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	44.1	74.1	91.6	113.	111.	113.	115	117.	119.	125

DEN 1

means:	48.1	81.1	96.2	113.	108.	113.	113.	117.	115.	113.
stdev:	5.63	9.80	6.49	.362	4.62	.241	1.54	.317	5.09	15.8
Row of wells C:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	51.2	85.7	83.1	103.	107.	113.	110.	113.	118.	115.
Row of wells D:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	50.9	88.6	103.	104.	120.	126.	123.	128.	133.	132.

DEN 2

means:	51.1	87.1	93.4	104.	114.	119.	117.	120.	125.	124.
stdev:	.224	2.09	14.5	.958	9.32	9.17	8.95	10.5	10.2	11.3
Row of wells E:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	55.3	60.6	75.5	111.	130.	140.	134.	142.	135.	147.
Row of wells F:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	53.9	46.0	80.2	128.	159.	171.	168.	176.	177.	182.

DEN 3

means:	54.6	53.3	77.8	120.	145.	155.	151.	159.	156.	165.
stdev:	.969	10.3	3.34	12.1	20.0	21.6	24.3	23.8	30.0	24.7
Row of wells G:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	50.6	51.9	71.4	125.	148.	163.	166.	161.	171.	181.
Row of wells H:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	40.8	53.7	63.4	112.	133.	138.	137.	140.	141.	143.

DEN 4

means:	45.7	52.8	67.4	119.	140.	151.	151.	150.	156.	162.
stdev:	6.92	1.28	5.64	9.24	10.4	17.6	20.2	14.2	20.8	27.4

The data are being processed on 20021021 (14:44:12)

Row of wells A:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	23.1	30.5	74.3	114.	130.	121.	131.	134.	121.	118.

Row of wells B:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	18.0	23.8	57.1	119.	147.	160.	159.	160	160.	148.
D5										
means:	20.6	27.1	65.7	116.	139.	141.	145.	147.	141.	133.
stdev:	3.56	4.78	12.1	3.53	12.0	27.9	19.5	17.8	27.9	21.4
Row of wells C:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	30.1	27.7	36.1	125.	183.	203.	203.	207.	200	189.
Row of wells D:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	31.7	42.8	46.0	125.	230.	258.	255.	255.	247.	255.
D6										
means:	30.9	35.2	41.0	125.	206.	231.	229.	231.	223.	222.
stdev:	1.14	10.7	6.99	6.76	33.2	38.9	36.7	34.1	33.6	46.9
Row of wells E:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	36.5	39.6	47.6	144.	212.	230.	247.	255.	263.	249.
Row of wells F:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	34.3	38.8	44.7	123.	200	243.	250.	252.	250.	237.
D7										
means:	35.4	39.2	46.1	134.	206.	236.	249.	253.	257.	243.
stdev:	1.54	.619	2.01	14.5	8.97	9.28	2.21	2.34	9.01	8.40
Row of wells G:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	7.04	29.5	35.2	102.	180.	215.	228.	221.	242.	225.
Row of wells H:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	6.15	33.8	44.6	112.	204.	223.	243.	226.	247.	247.
D8										
means:	6.59	31.7	39.9	107.	192.	219.	235.	223.	244.	236.
stdev:	.628	3.01	6.64	6.71	17.2	5.36	10.5	3.55	3.84	15.7
Row of wells A:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	17.1	40.7	36.8	105.	207.	238.	261.	269.	265.	268.
Row of wells B:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	14.4	28.9	31.3	114.	186.	220.	242.	248.	239.	238.

D9

means:	15.7	34.8	34.0	109.	197.	229.	252.	258.	252.	253.
stdev:	1.87	8.39	3.90	6.50	14.9	12.4	13.9	15.2	18.4	21.1
Row of wells C:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	13.3	22.6	32	72	185.	241.	276	265.	282.	265.
Row of wells D:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	14.7	29.5	39.3	80.3	236.	303.	331.	321.	326.	327.

D10

means:	14.0	26.0	35.6	76.1	210.	272.	303.	293.	304.	296.
stdev:	1.00	4.83	5.19	5.88	35.8	43.8	38.9	39.5	30.8	44.2
Row of wells E:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	21.0	45.6	47.3	119.	301.	314.	354.	361.	357.	347.
Row of wells F:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	9.67	40.3	37.0	109.	248.	287.	309.	316.	327.	327.

D11

means:	15.3	42.9	42.2	114.	275.	300.	332.	338.	342.	337.
stdev:	8.04	3.74	7.26	6.80	37.7	19.0	31.6	32.0	21.5	14.1
Row of wells G:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	15.6	43.7	39.0	153.	235.	278.	293.	298.	318.	312.
Row of wells H:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	14.2	38.9	35.0	132.	203.	227.	259.	242.	246.	255.

D12

means:	14.9	41.3	37.0	142.	219.	252.	276.	270.	282.	284.
stdev:	.947	3.38	2.82	14.6	22.6	35.9	24.0	39.3	50.9	40.0

Row of wells A:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	12.5	21.8	25	87.5	187.	242.	239.	260.	243.	264.
Row of wells B:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	12.5	17.8	35.7	96.4	212.	285.	269.	280.	289.	296.

D13										
means:	12.5	19.8	30.3	91.9	200	263.	254.	270.	266.	280.
stdev:	0	2.84	7.57	6.31	17.6	30.7	21.6	13.7	32.1	22.8
Row of wells C:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	8.06	4.83	9.67	30.6	75.8	201.	225.	250	235.	267.
Row of wells D:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	8.19	9.83	13.1	24.5	75.4	181.	229.	250.	259.	263.
D14										
means:	8.13	7.33	11.3	27.6	75.6	191.	227.	250.	247.	265.
stdev:	9.34	3.53	2.43	4.28	.280	13.8	2.61	.579	16.6	2.69
Row of wells E:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	9.37	32.8	31.2	62.5	168.	231.	240.	242.	248.	254.
Row of wells F:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	10.4	26.8	26.8	65.6	161.	222.	214.	253.	246.	253.
D15										
means:	9.91	29.8	29.0	64.0	164.	226.	227.	247.	247.	254.
stdev:	.758	4.20	3.10	2.24	5.34	6.26	18.1	8.16	1.53	.676
Row of wells G:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	6.94	11.1	23.6	48.6	134.	202.	208.	225	229.	237.
Row of wells H:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	8.98	13.4	13.4	40.4	108.	165.	191.	187.	186.	184.
D16										
means:	7.96	12.2	18.5	44.5	121.	183.	199.	206.	207.	210.
stdev:	1.44	1.67	7.16	5.77	18.1	26.5	12.2	26.4	30.1	37.6
Row of wells A:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	10.4	20.8	27.0	84.7	161.	192.	195.	202.	195.	215.
Row of wells B:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	9.52	15.0	34.9	90.4	183.	224.	229.	230.	233.	242.

D13										
means:	9.97	17.9	31.0	87.5	172.	208.	212.	216.	214.	229.
stdev:	.631	4.06	5.54	4.06	15.2	22.7	24.2	19.9	27.0	19.5
Row of wells C:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	4.44	1.48	7.40	28.1	76.2	170.	195.	208.	200	223.
Row of wells D:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	4.58	5.34	8.39	20.6	76.3	159.	196.	212.	217.	223.
D14										
means:	4.51	3.41	7.90	24.3	76.3	164.	196.	210.	208.	223.
stdev:	9.59	2.73	.699	5.32	2.79	7.65	.983	2.89	12.4	2.79
Row of wells E:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	6.10	28.2	31.2	64.8	159.	205.	212.	213.	216.	223.
Row of wells F:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	6.71	25.3	30.5	68.6	155.	200	193.	222.	221.	226.
D15										
means:	6.41	26.8	30.9	66.7	157.	202.	203.	218.	218.	224.
stdev:	.431	2.03	.495	2.66	2.52	3.77	13.9	6.11	3.96	1.73
Row of wells G:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	4.10	10.2	23.9	49.3	130.	182.	186.	197.	200.	210.
Row of wells H:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	6.74	10.6	16.8	43.8	108.	151.	168.	165.	168.	170.
D16										
means:	5.42	10.4	20.4	46.5	119.	167.	177.	181.	184.	190.
stdev:	1.86	.282	5.03	3.88	15.4	22.0	12.5	22.2	22.7	28.4
Row of wells A:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	8.23	10.5	15.2	32.9	105.	180	241.	243.	264.	270.
Row of wells B:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	12.3	13.8	23.0	43.0	153.	263.	327.	347.	356.	352.
D17										
means:	10.2	12.2	19.1	38.0	129.	221.	284.	295.	310.	311.
stdev:	2.87	2.30	5.50	7.16	33.9	58.7	61.1	73.6	65.2	57.7
Row of wells C:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	14	24	18	44	56	196	364	428	430	456

Row of wells D:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	17.0	31.7	21.9	53.6	56.0	285.	441.	519.	560.	568.
D18										
means:	15.5	27.8	19.9	48.8	56.0	240.	402.	473.	495.	512.
stdev:	2.17	5.44	2.79	6.82	6.89	63.1	54.7	64.7	92.6	79.4
Row of wells E:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	23.8	42.8	38.0	66.6	52.3	247.	407.	509.	535.	521.
Row of wells F:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	9.52	23.8	19.0	61.9	50	216.	419.	495.	542.	538.
D19										
means:	16.6	33.3	28.5	64.2	51.1	232.	413.	502.	539.	529.
stdev:	10.1	13.4	13.4	3.36	1.68	21.8	8.41	10.1	5.05	11.7
Row of wells G:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	17.0	65.8	73.1	78.0	226.	397.	536.	536.	539.	563.
Row of wells H:	100	50	25	12.5	6.25	3.12	1.56	.78	.39	.195
	14.5	39.5	66.6	79.1	222.	393.	460.	466.	472.	470.
D20										
means:	15.8	52.7	69.9	78.6	224.	395.	498.	501.	505.	517.
stdev:	1.76	18.5	4.59	.790	2.76	2.69	53.8	49.4	46.7	65.4

Appendix 3

HPLC, NMR AND MASS SPECTRA

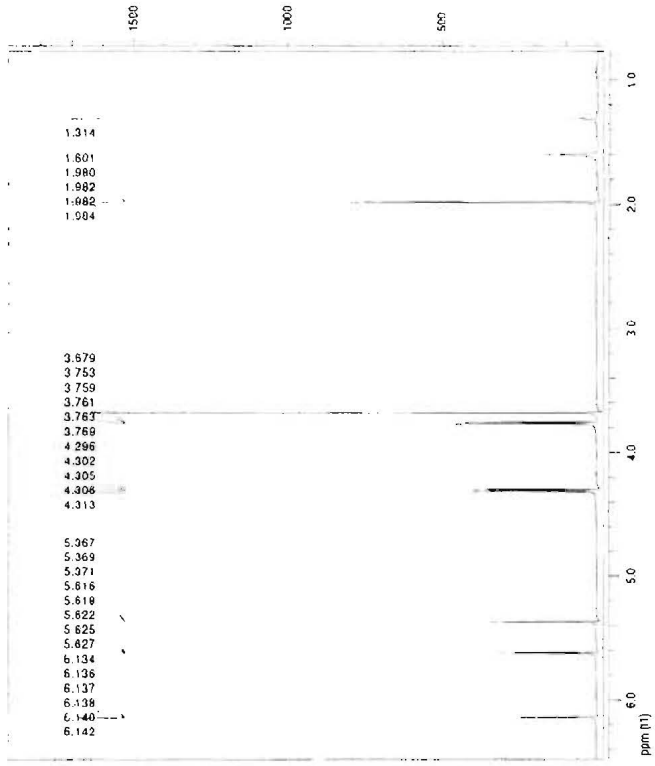


Figure A3.1. ^{1}H NMR spectrum of 3155A in CD_2Cl_2, 600 MHz.

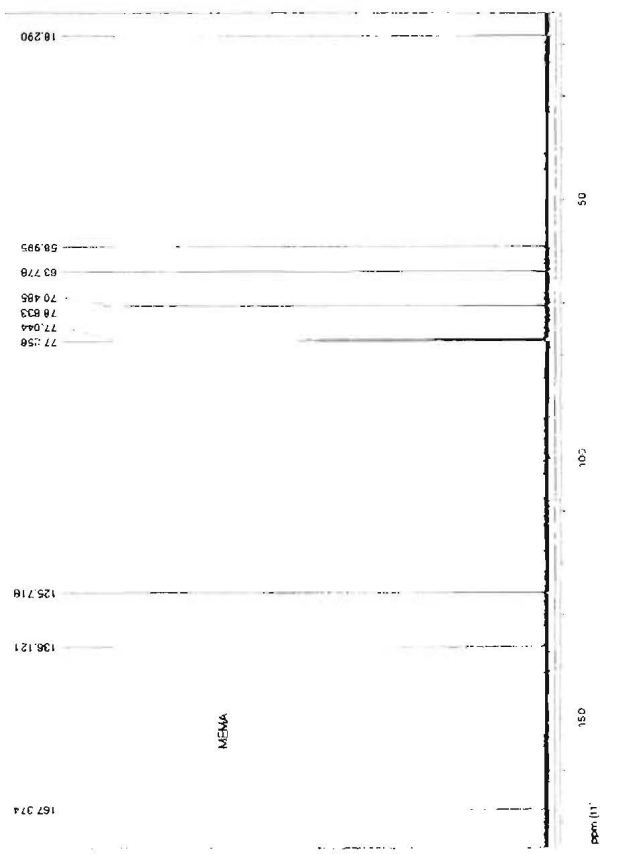


Figure A3.2. ^{13}C NMR spectrum of MEMA in CD_2Cl_2, 150 MHz.

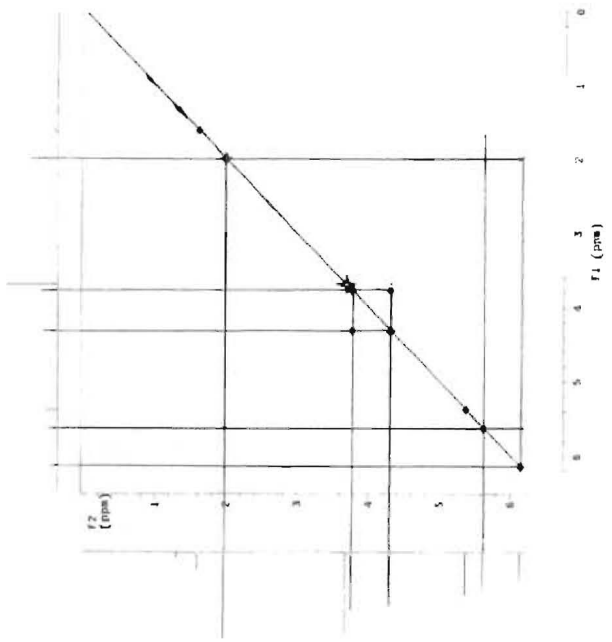


Figure A3.3. COSY spectrum of 3155A in CD_2Cl_2.

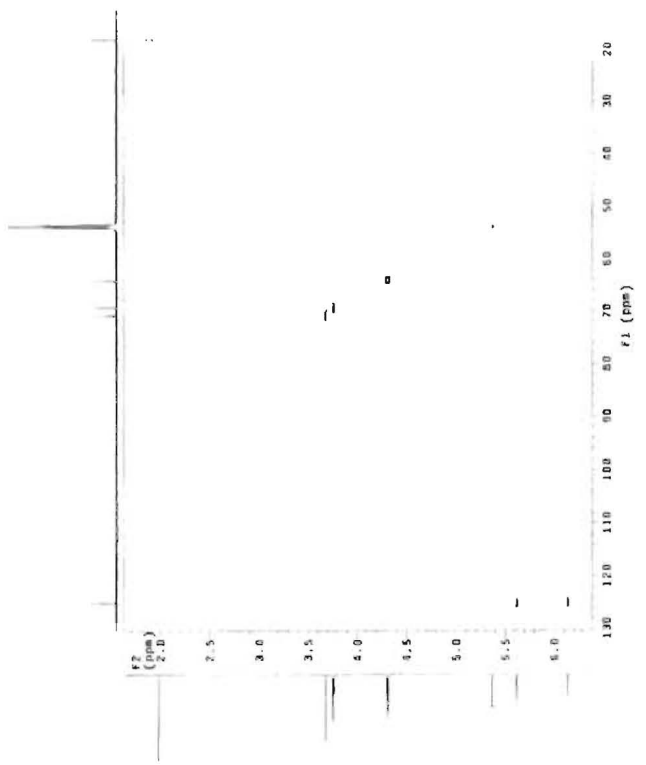


Figure A3.4 HSQC spectrum of 3155A in CD_2Cl_2.

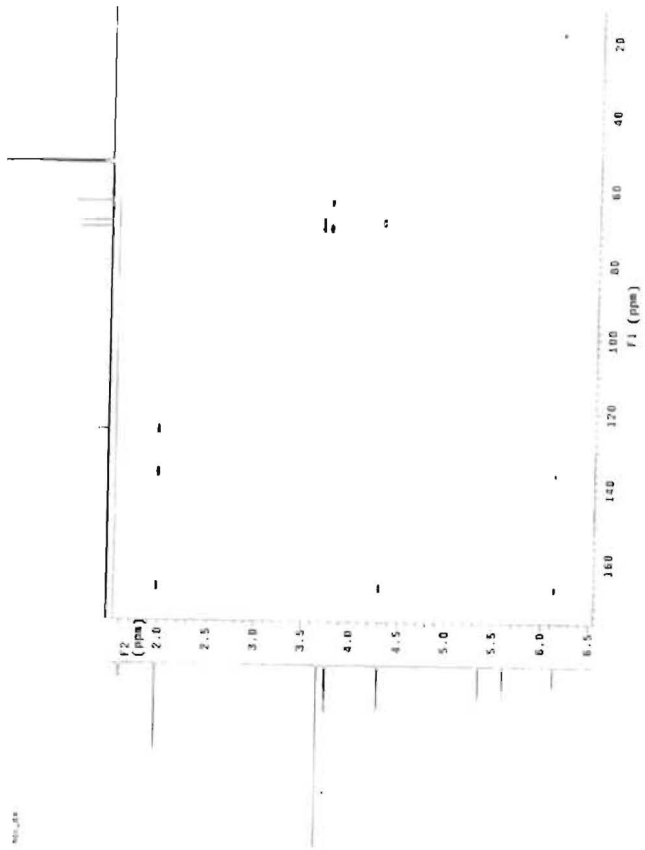


Figure A3.5. HMBC spectrum of 3155A in CD_2Cl_2.

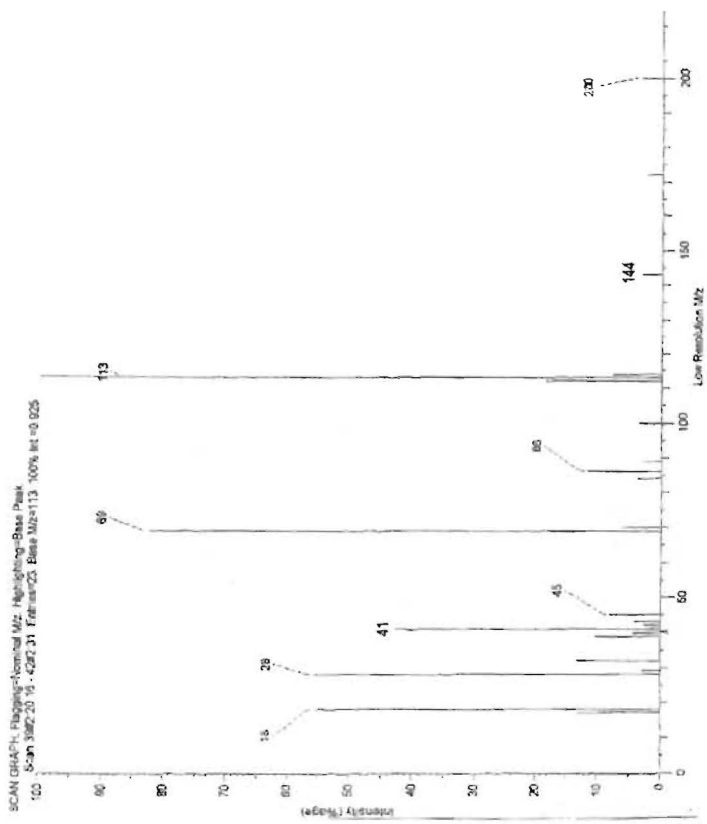


Figure A3.6 Mass spectrum of 3155A

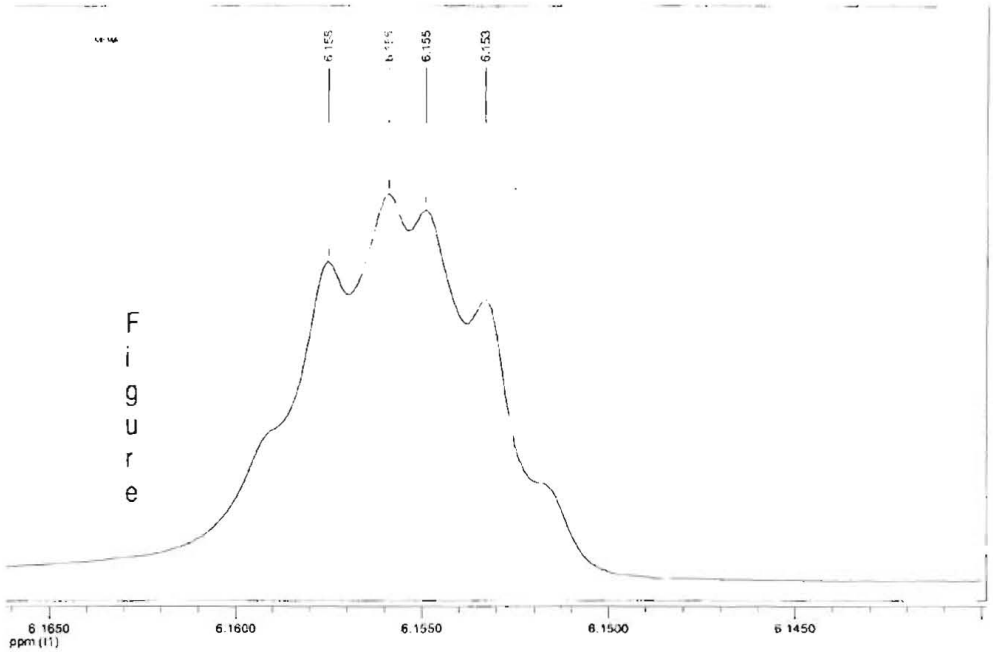


Figure A3.7. Expansion of 1H NMR spectrum of MEMA at δ 6.1

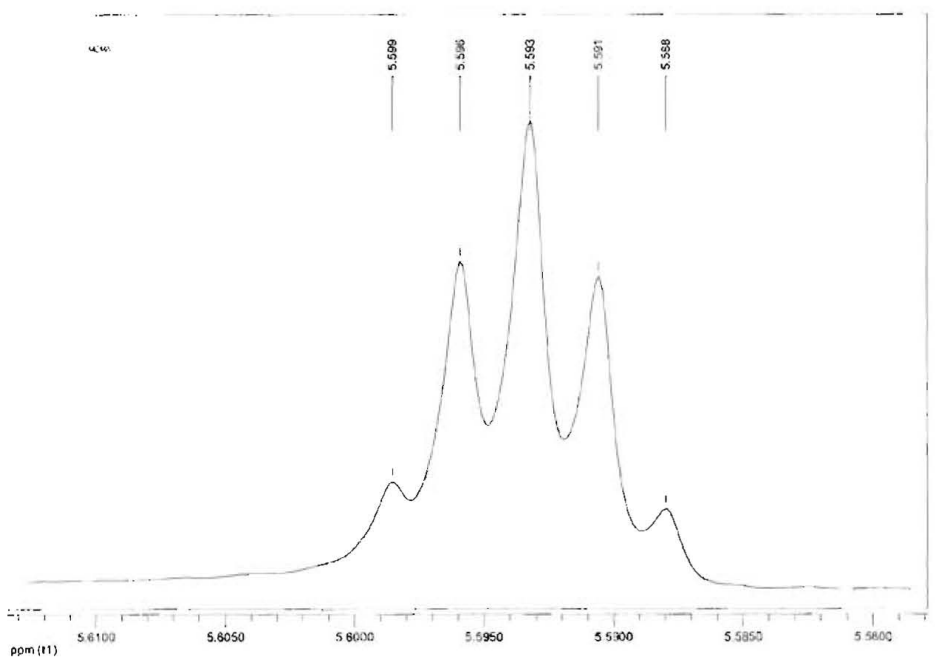


Figure A3.8. Expansion of ^{1}H NMR spectrum of MEMA at δ 5.6.

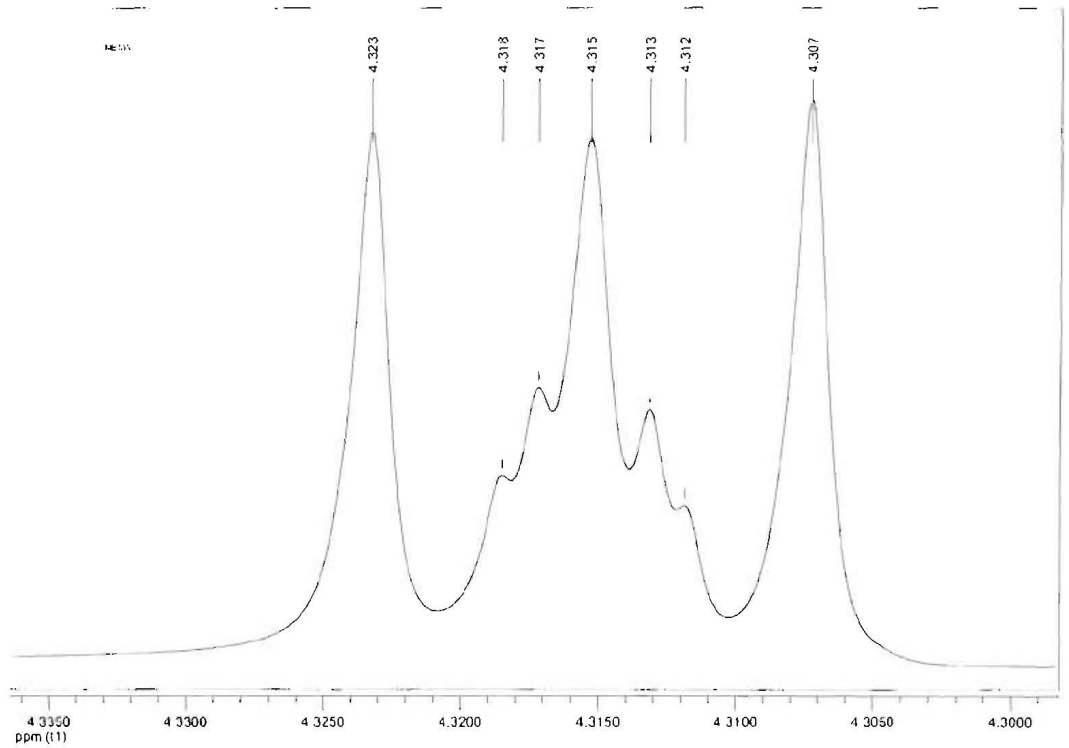


Figure A3.9. Expansion of ^{1}H NMR spectrum of MEMA at δ 4.3.

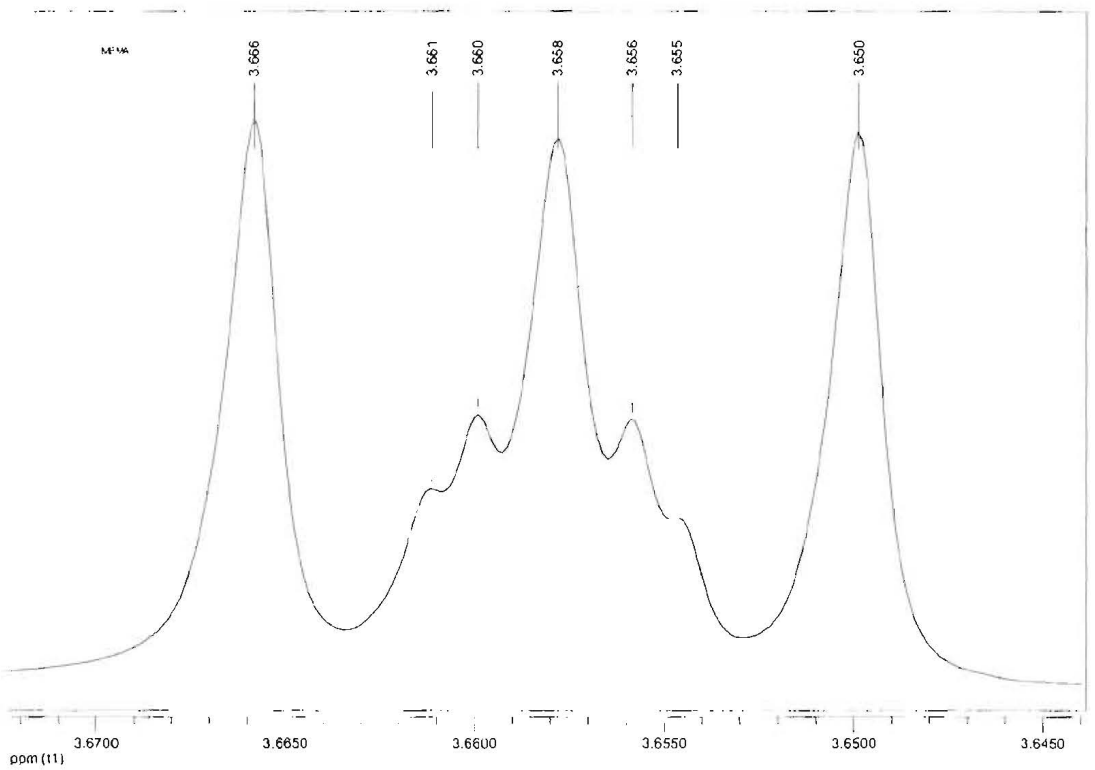


Figure A3.10. Expansion of ^{1}H NMR spectrum of MEMA at δ 3.6.

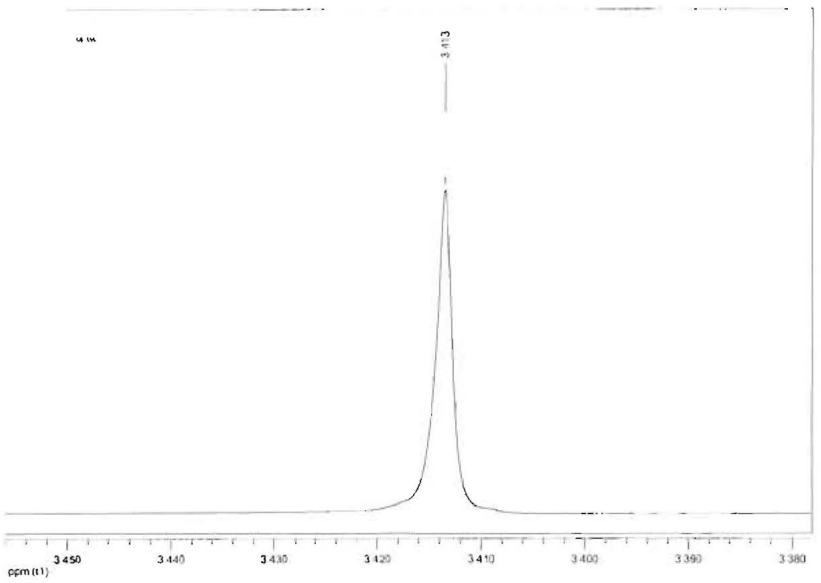


Figure A3.11. Expansion of ^{1}H NMR spectrum of MEMA at δ 3.4.

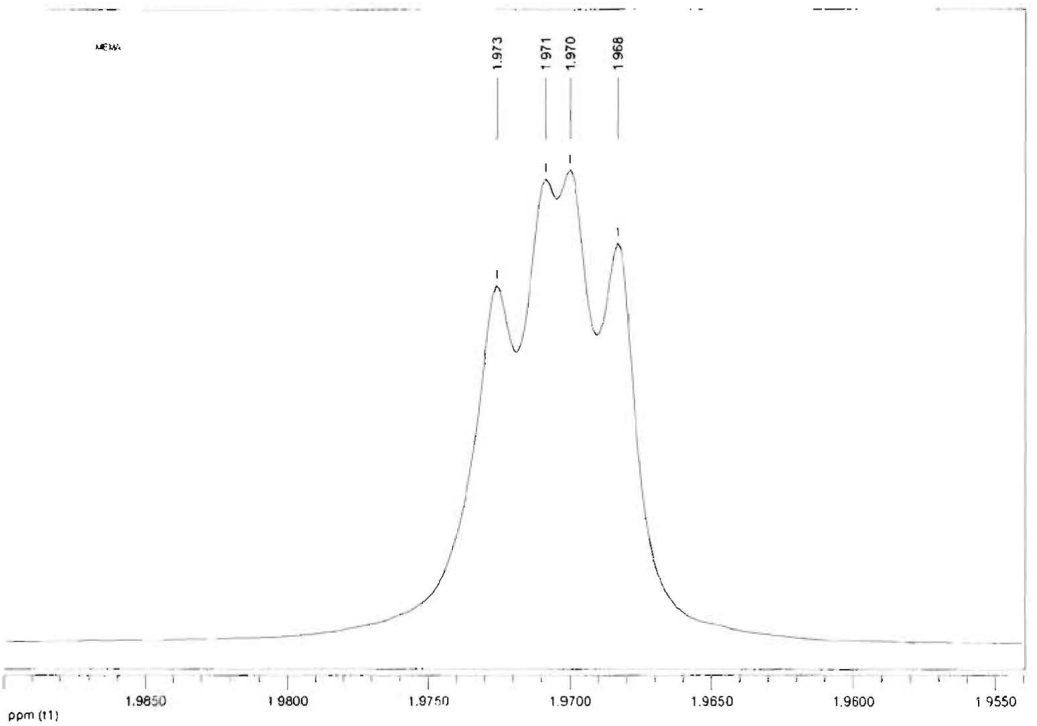


Figure A3.12. Expansion of 1H NMR spectrum of MEMA at δ 1.9.

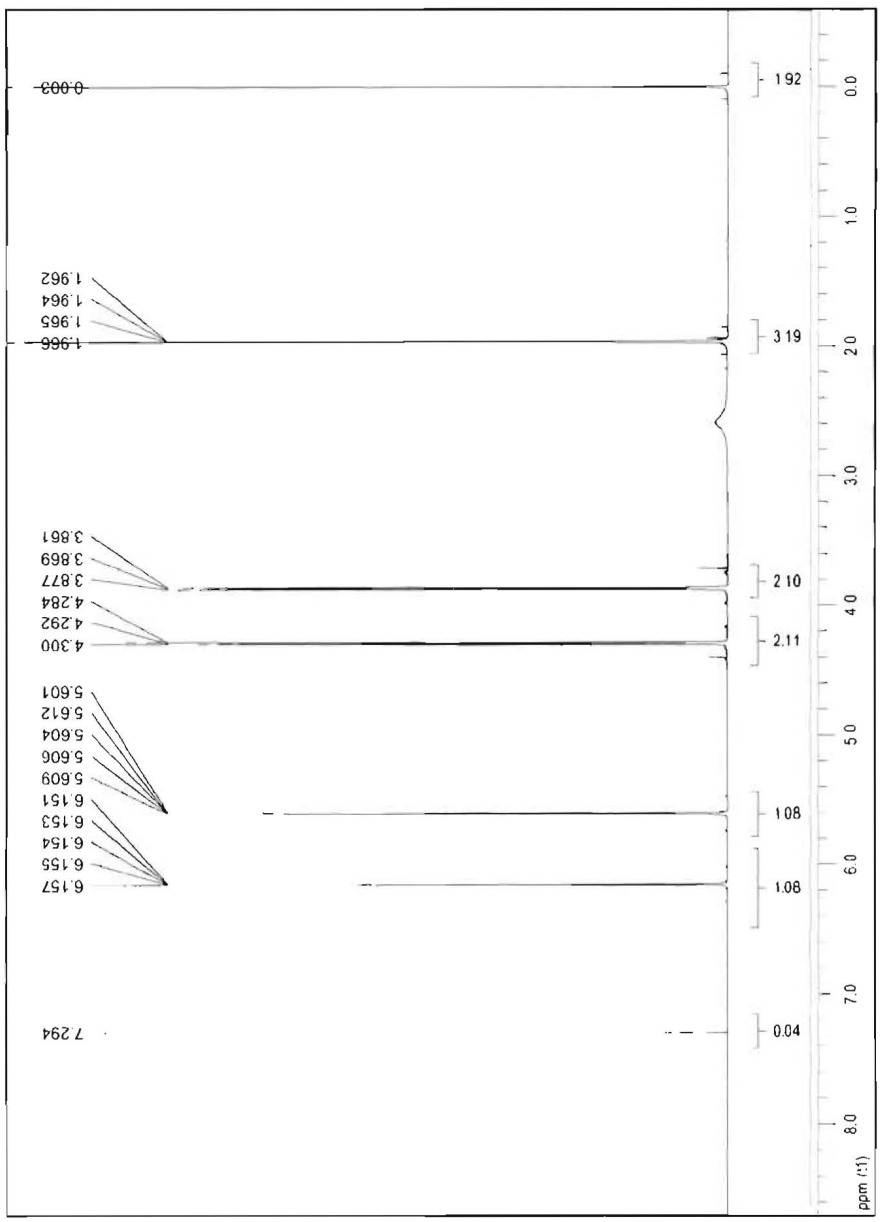


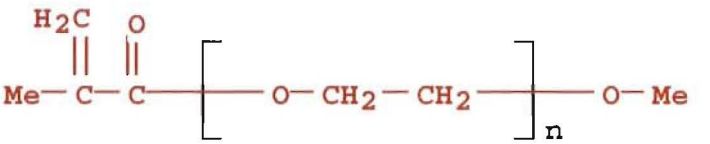
Figure A3.13 ^{1}HNMR spectrum of HEMA, 600 MHz

Appendix 4

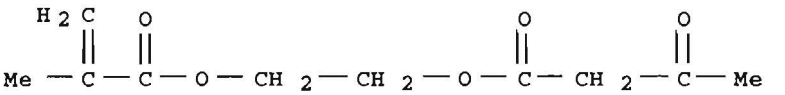
PATENTED DERIVATIVES OF MEMA

The following derivatives were obtained from a SciFinder 2004 search.

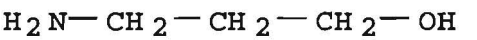
Component Registry Number: 26915-72-0
Formula: (C2 H4 O)n C5 H8 O2



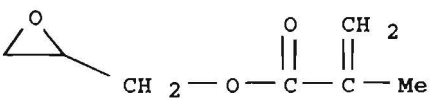
Component Registry Number: 21282-97-3
Formula: C10 H14 O5



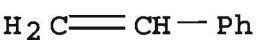
Component Registry Number: 156-87-6
Formula: C3 H9 N O



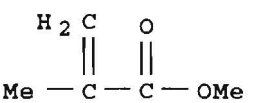
Component Registry Number: 106-91-2
Formula: C7 H10 O3



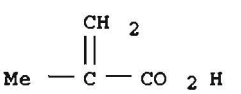
Component Registry Number: 100-42-5
Formula: C8 H8



Component Registry Number: 80-62-6
Formula: C5 H8 O2

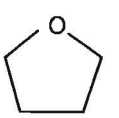


Component Registry Number: 79-41-4
Formula: C4 H6 O2



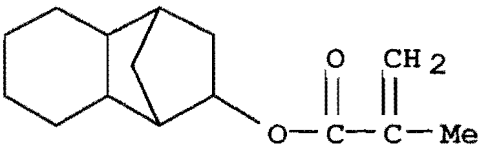
Component Registry Number: 27637-03-2
Formula: (C4 H8 O . C2 H4 O)x

Component Registry Number: 109-99-9
Formula: C4 H8 O

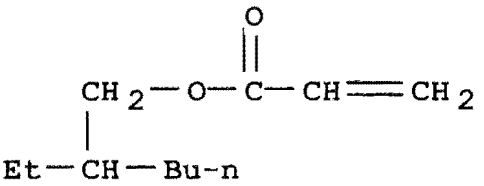


Component Registry Number: 75-21-8
Formula: C2 H4 O

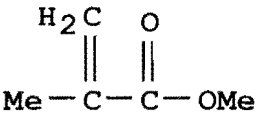
Component Registry Number: 124424-70-0
Formula: C15 H22 O2



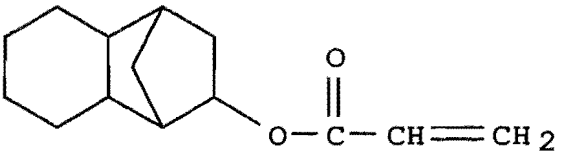
Component Registry Number: 103-11-7
Formula: C11 H20 O2



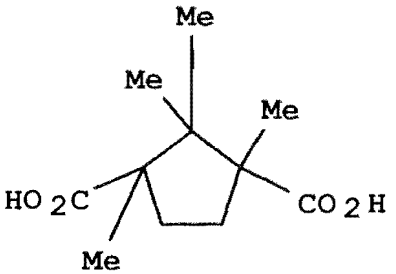
Component Registry Number: 80-62-6
Formula: C5 H8 O2



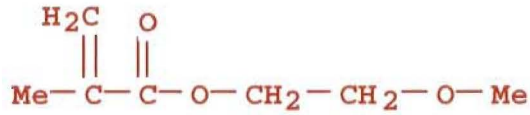
Component Registry Number: 178824-27-6
Formula: C14 H20 O2



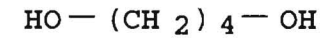
Component Registry Number: 98900-82-4
Formula: C11 H18 O4



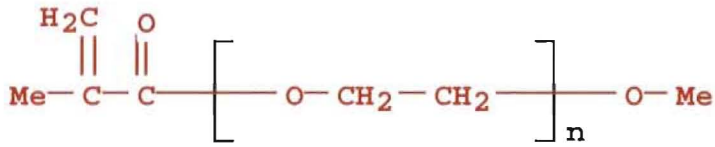
Component Registry Number: 6976-93-8
Formula: C7 H12 O3



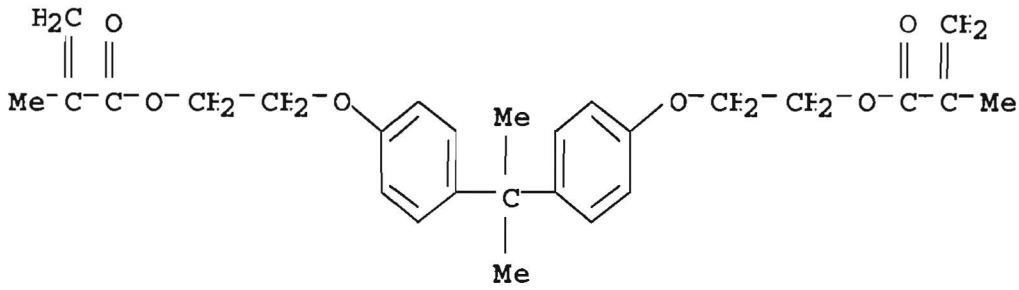
Component Registry Number: 110-63-4
Formula: C4 H10 O2



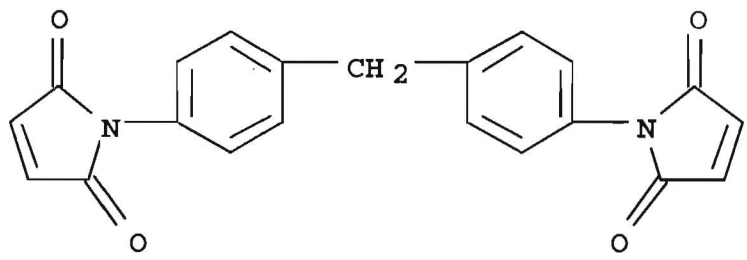
Component Registry Number: 26915-72-0
Formula: (C2 H4 O)n C5 H8 O2



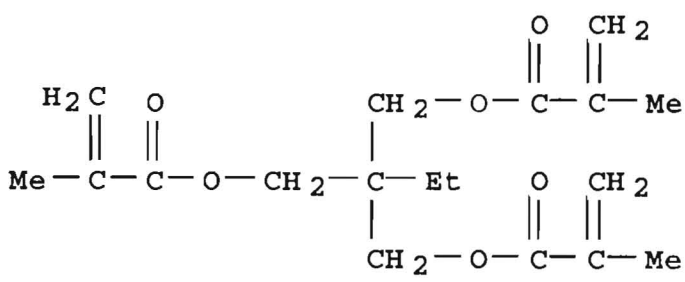
Component Registry Number: 24448-20-2
Formula: C27 H32 O6



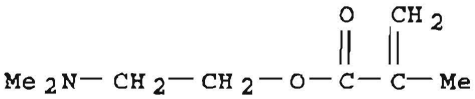
Component Registry Number: 13676-54-5
Formula: C21 H14 N2 O4



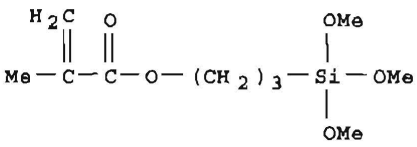
Component Registry Number: 3290-92-4
Formula: C18 H26 O6



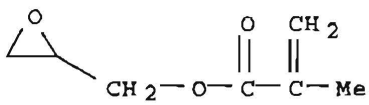
Component Registry Number: 2867-47-2
Formula: C8 H15 N O2



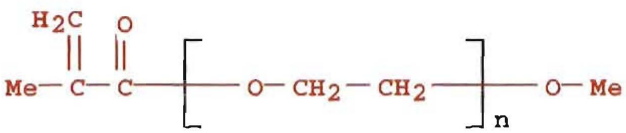
Component Registry Number: 2530-85-0
Formula: C10 H20 O5 Si



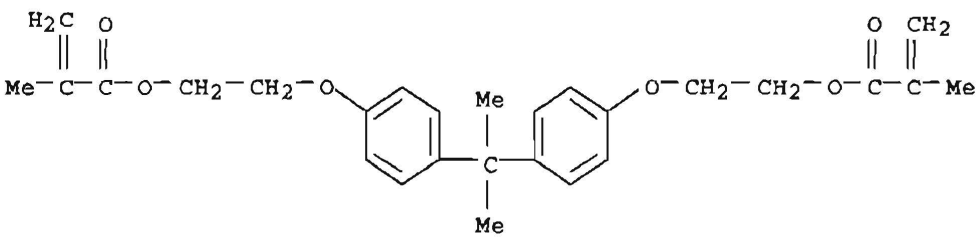
Component Registry Number: 106-91-2
Formula: C7 H10 O3



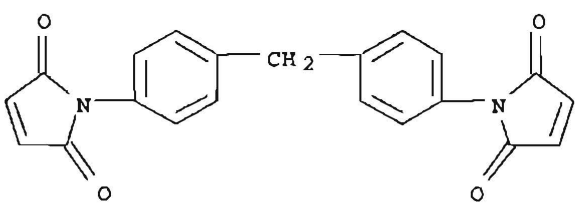
Component Registry Number: 26915-72-0
Formula: (C2 H4 O)n C5 H8 O2



Component Registry Number: 24448-20-2
Formula: C27 H32 O6



Component Registry Number: 13676-54-5
Formula: C21 H14 N2 O4



Appendix 5

DIRECT BIOAUTOGRAPHY

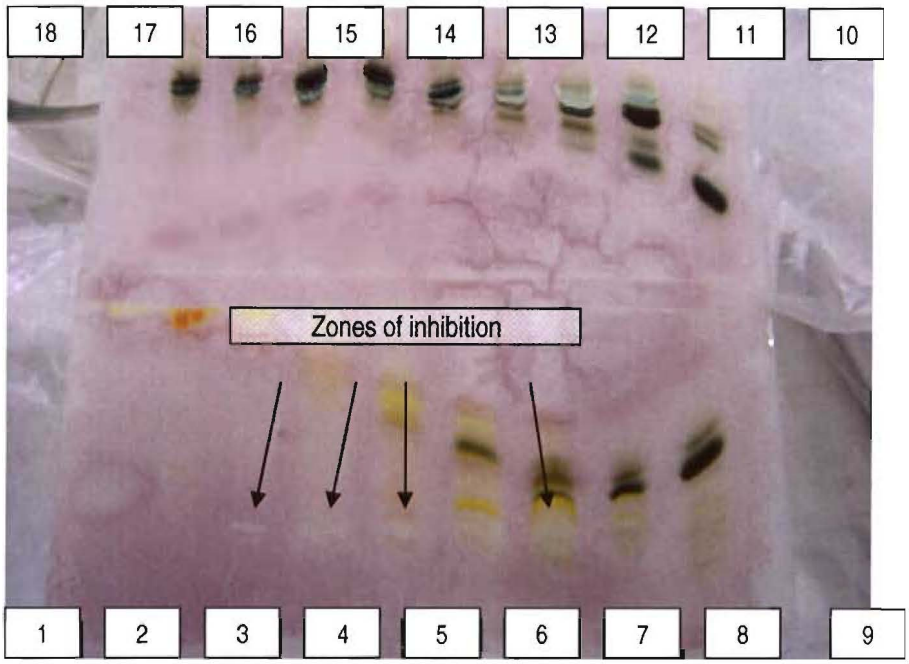


Figure A5.1. Inhibition zones obtained against *Mycobacterium aurum* in the direct bioautography method. Numbers 1 to 18 indicate the sample numbers as assigned in Table 7.2.

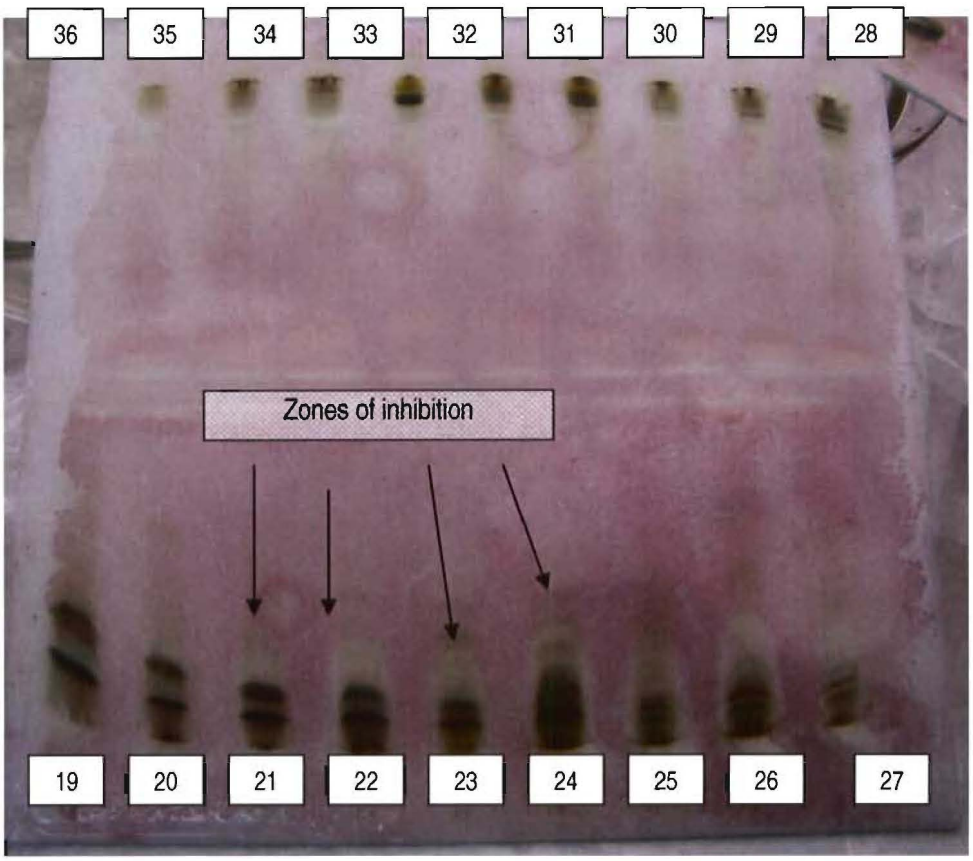


Figure A5.2. Inhibition zones obtained against *Mycobacterium aurum* in the direct bioautography method. Numbers 19 to 36 indicate the sample numbers as assigned in Table 7.2.

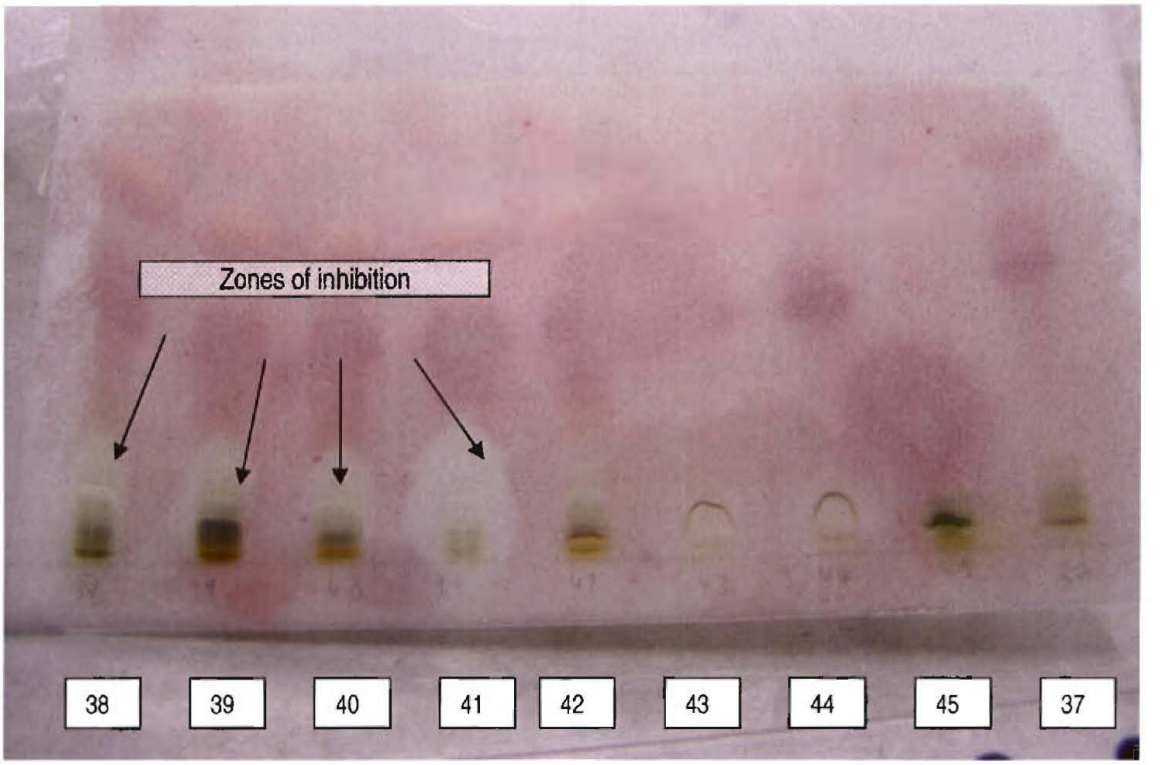


Figure A5.3. Inhibition zones obtained against *Mycobacterium aurum* in the direct bioautography method. Numbers 37 to 45 indicate the sample numbers as assigned in Table 7.2.

Appendix 6

GC-MS ANALYSIS RESULTS

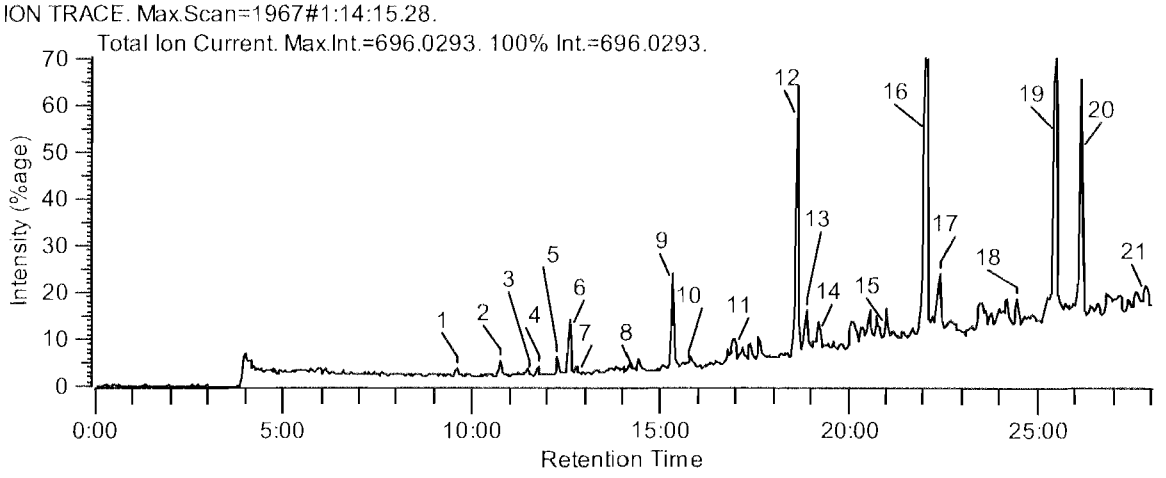
Submitted by: Denise Saravanakumar (UCT, Dept. of Pharmacology; Prof. Peter Smith)
Analysis date: 31 January 2006
Instrument: AMD 604 High Resolution Mass Spectrometer
Sample: **1730/11E**
Data folder: 200601 January
Calibration: 2006\0060119_CAL500_res1000
Scan: m/z 25-500

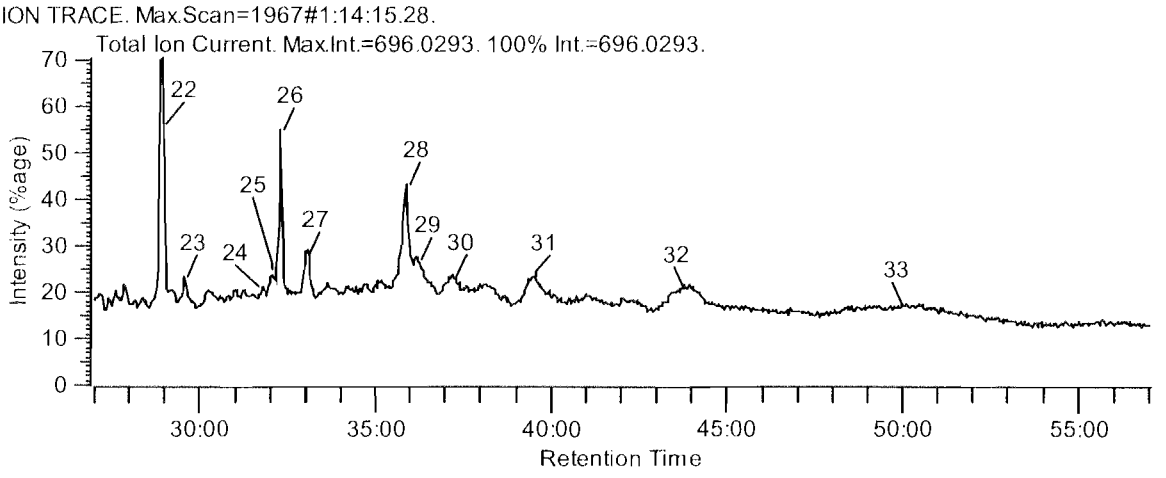
Sample preparation: The sample solution was injected (1 µl) directly into the gas chromatograph (GC) inlet.
Inlet method: Gas Chromatography

- Instrument
  - Carlo Erba GC 6000 Vega Series
  - Transfer line temperature: 250°C
- Column
  - LECUS, PS089-OH (DB-5 equivalent) silanol-terminated (95%)-methyl-(5%)-phenylpolisiloxane copolymer stationary phase
  - 40 m x 0.3 mm I.D.
  - Column number: P201
- GC Inlet
  - He pressure: *ca.* 40 kPa (linear velocity: 28.2 cm/sec at 40°C)
  - Split flow: 20 ml/min (septum purge: 5 ml/min)
  - Temperature: 250°C
- Oven
  - 130 - 270°C at 3°C/min, hold 10 min

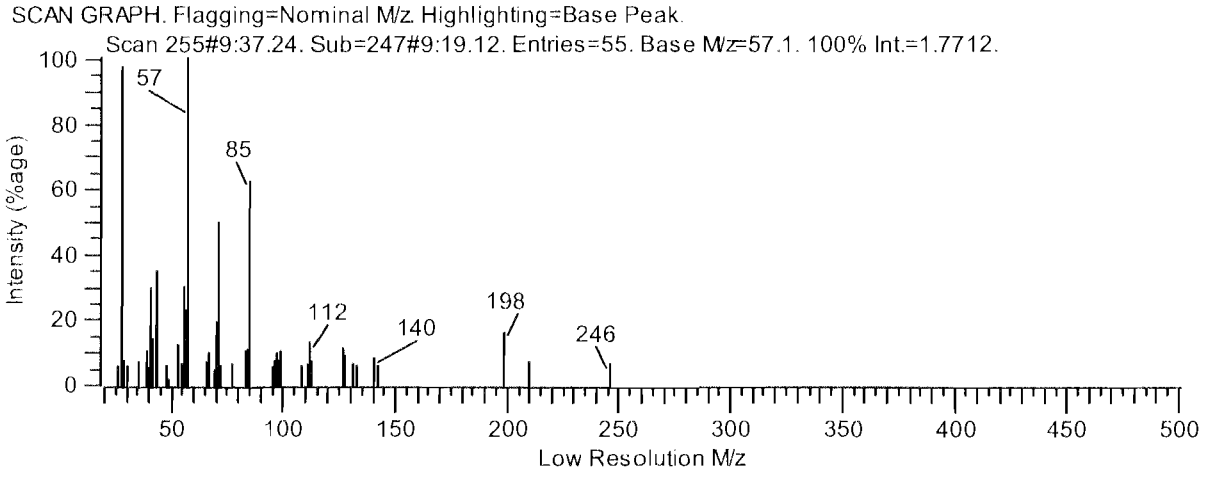
Ionization technique: EI^+ (Electron Energy: 70eV Source: 200°C)

Total Ion Current Chromatogram:

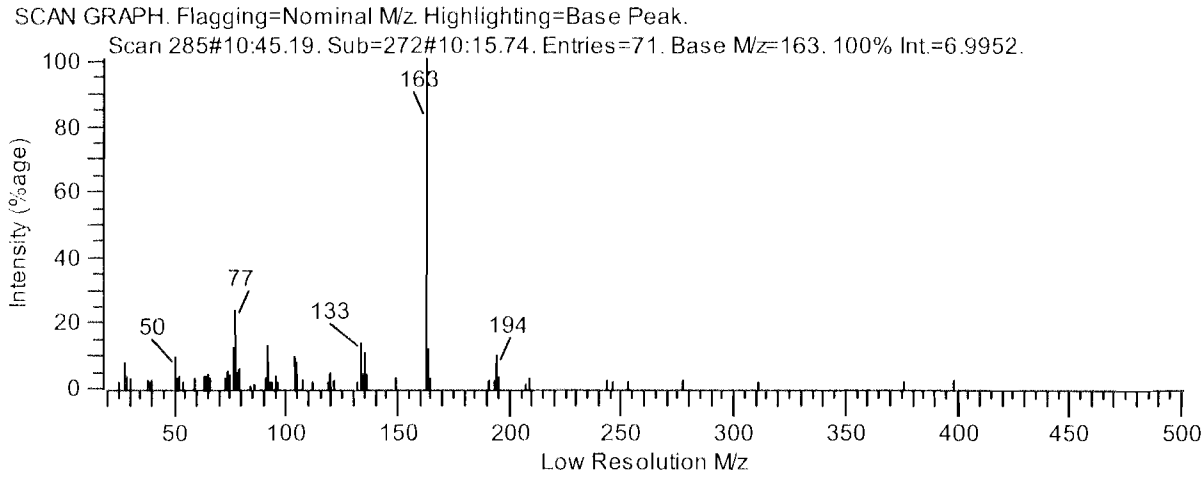




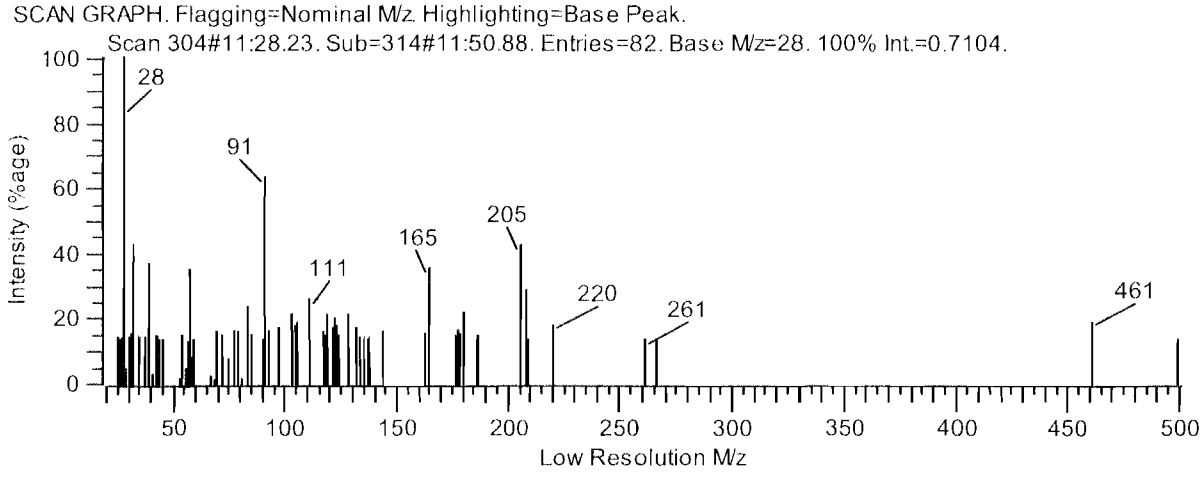
Low resolution EI⁺ mass spectra of the sample components, providing their tentative identities:



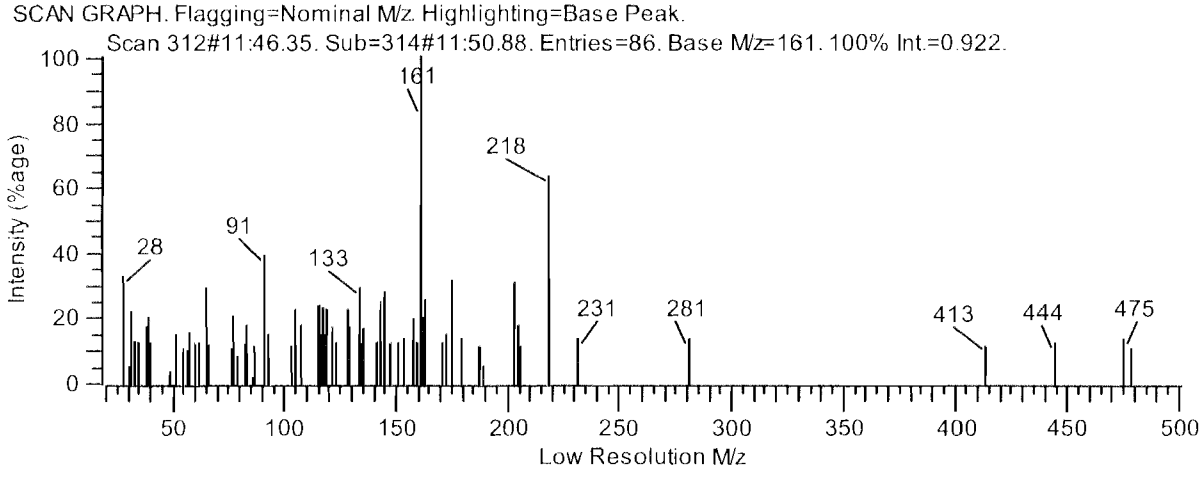
1. EI mass spectrum of an aliphatic hydrocarbon.



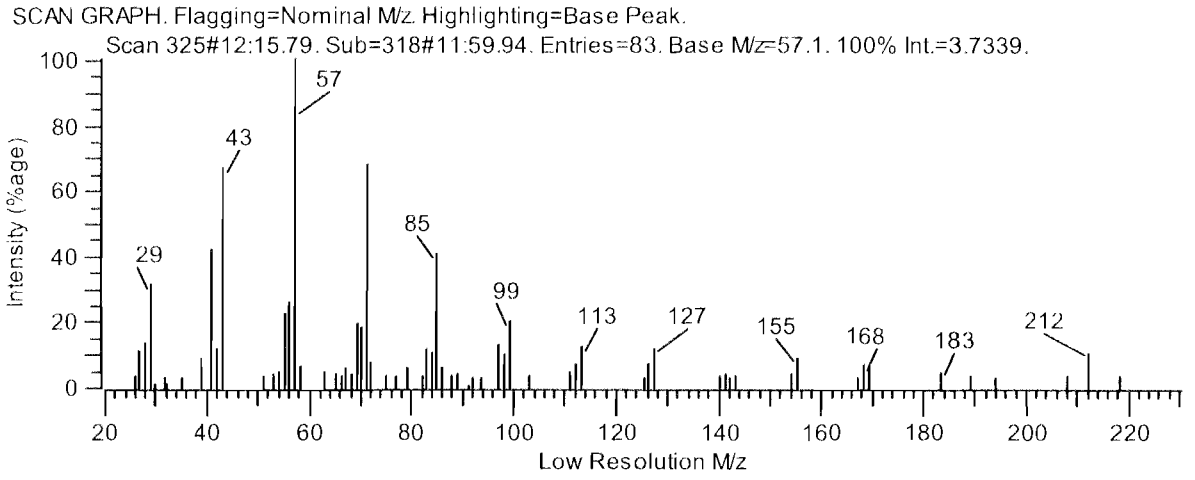
2. EI mass spectrum of dimethyl phthalate.



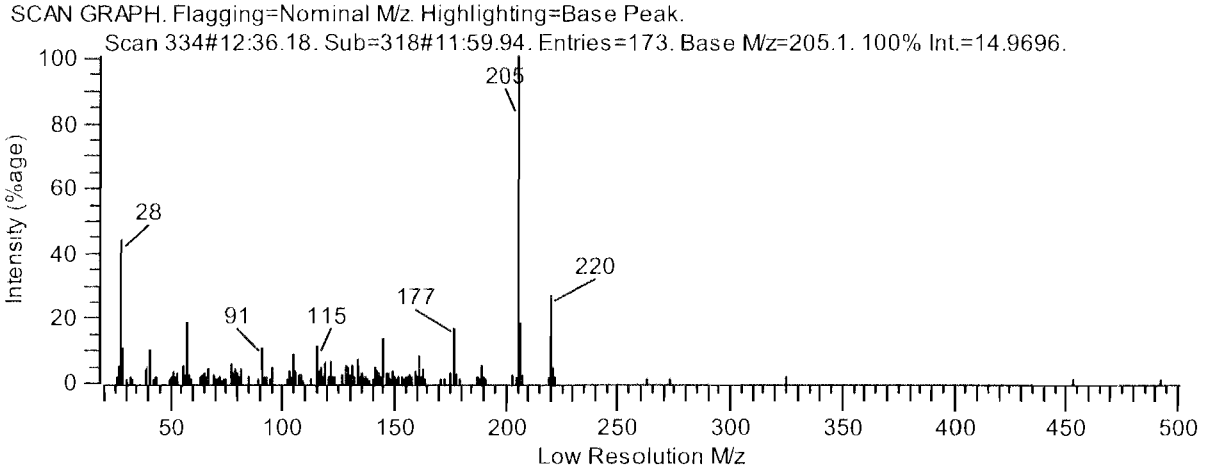
3. EI mass spectrum of an unknown compound.



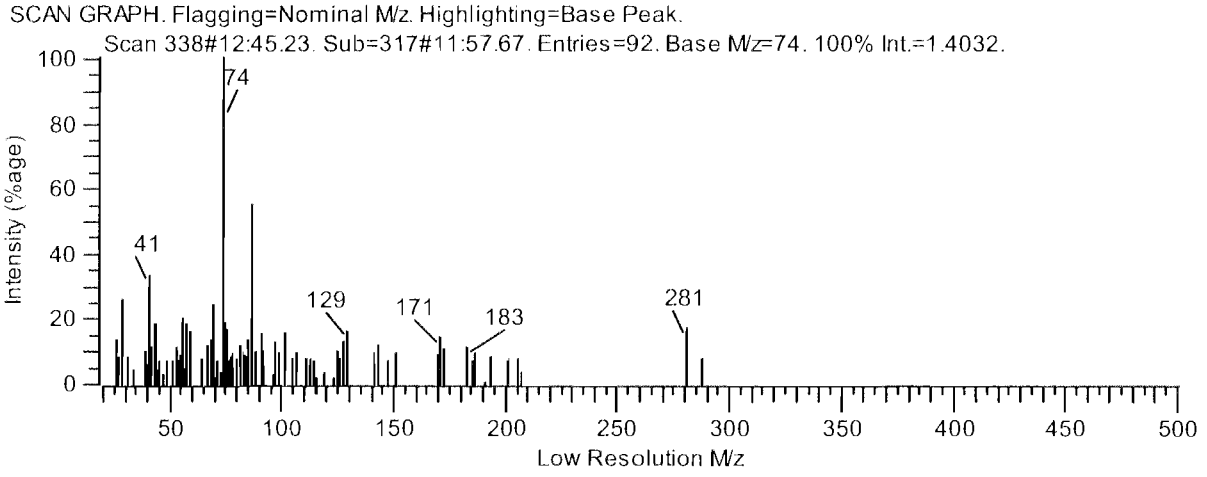
4. EI mass spectrum of an unsaturated cyclic hydrocarbon (could contain hydroxyl and/or carbonyl groups).



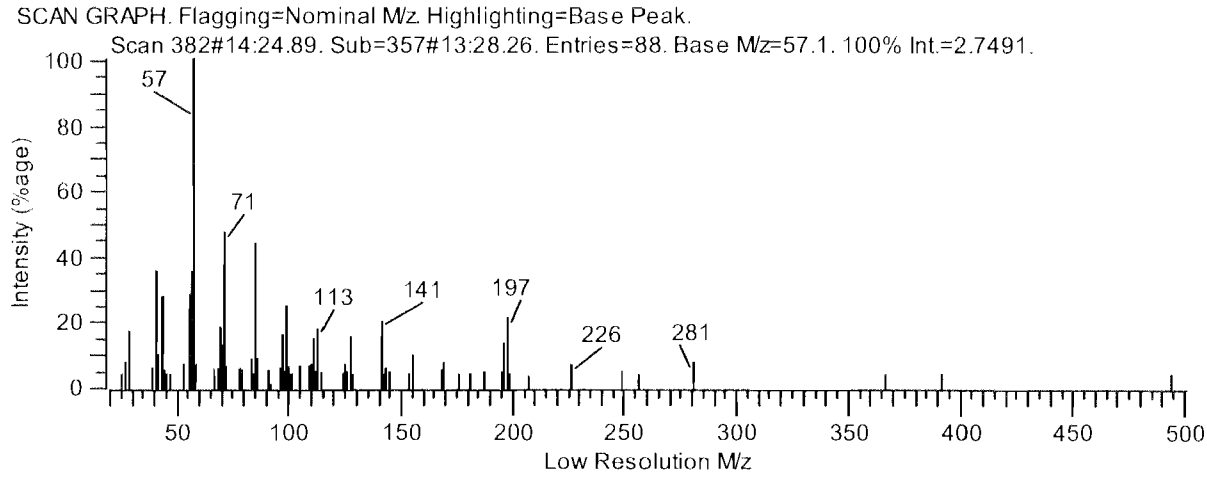
5. EI mass spectrum of pentadecane.



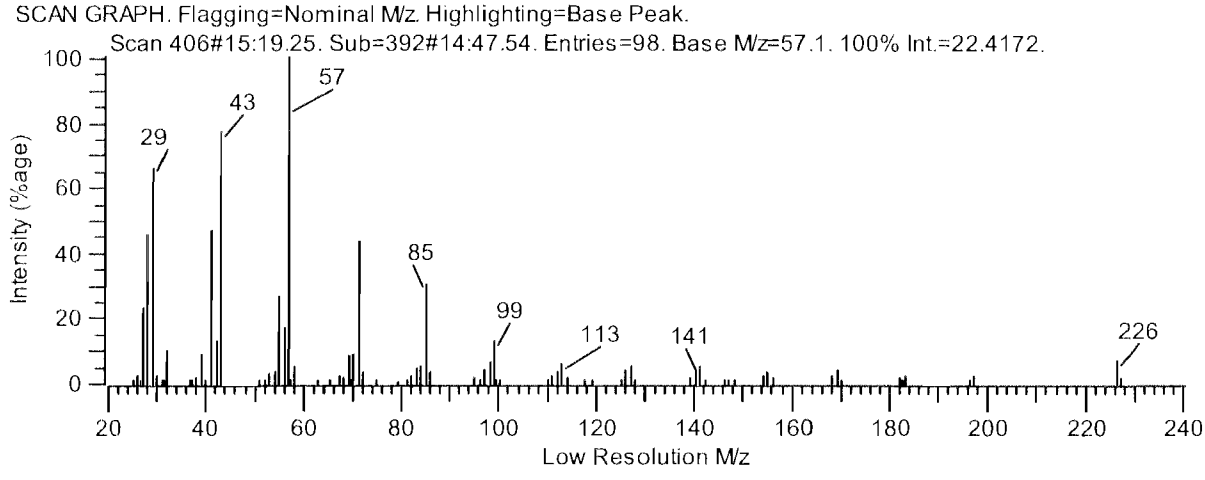
6. EI mass spectrum of butylated hydroxytoluene.



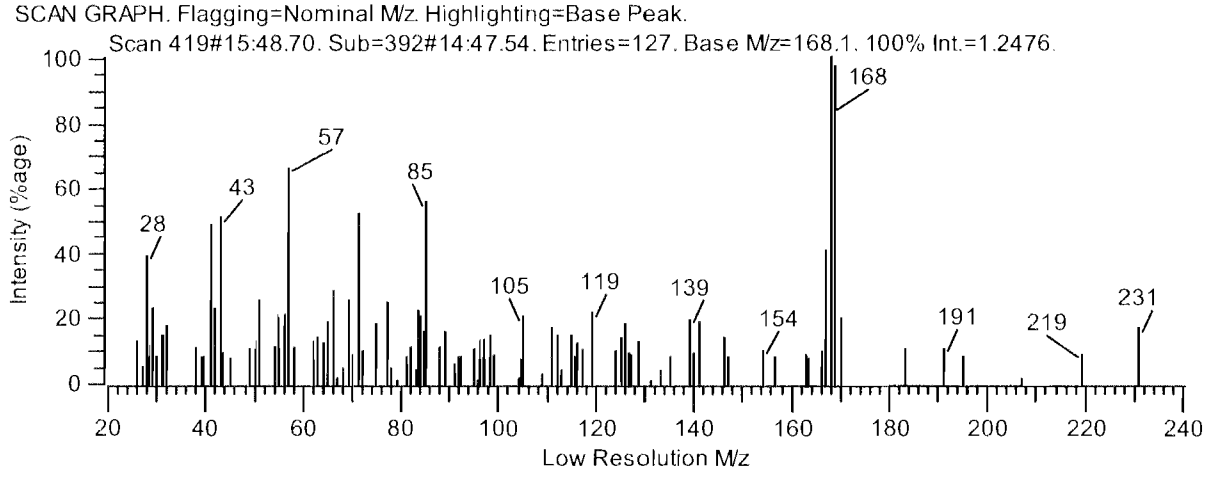
7. EI mass spectrum of methyl dodecanoate.



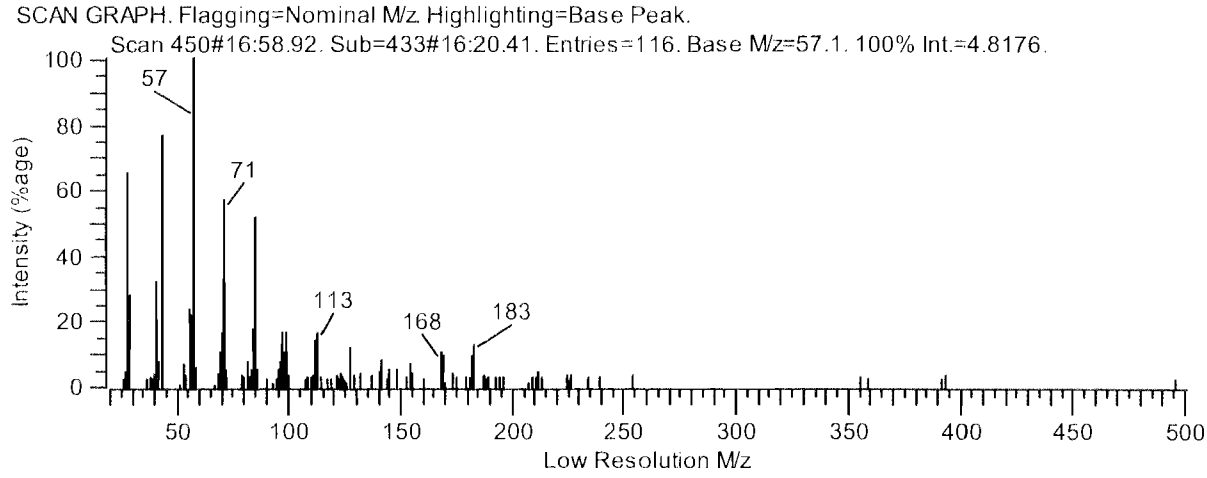
8. EI mass spectrum of an aliphatic hydrocarbon.



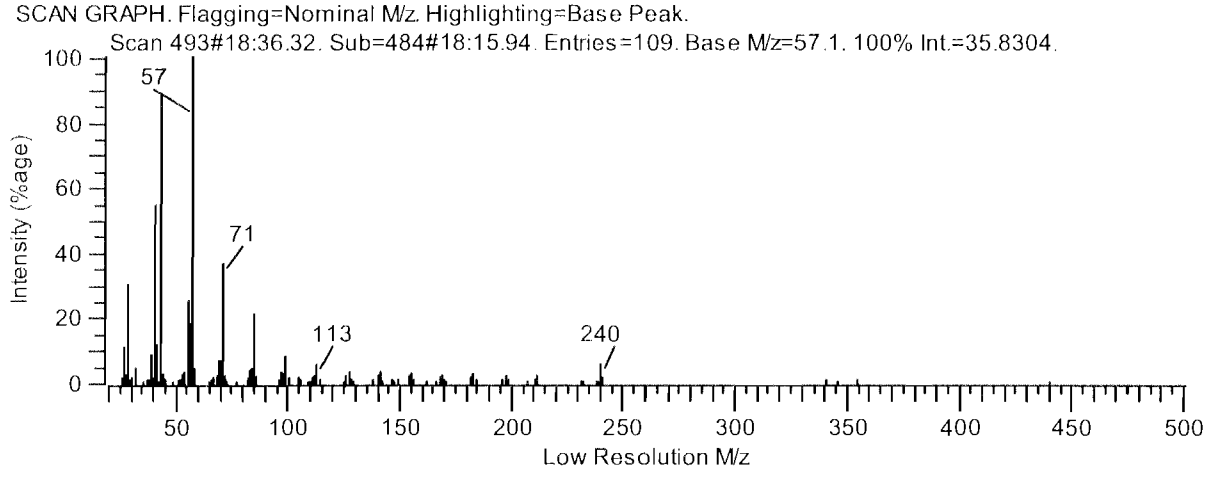
9. EI mass spectrum of a hexadecane.



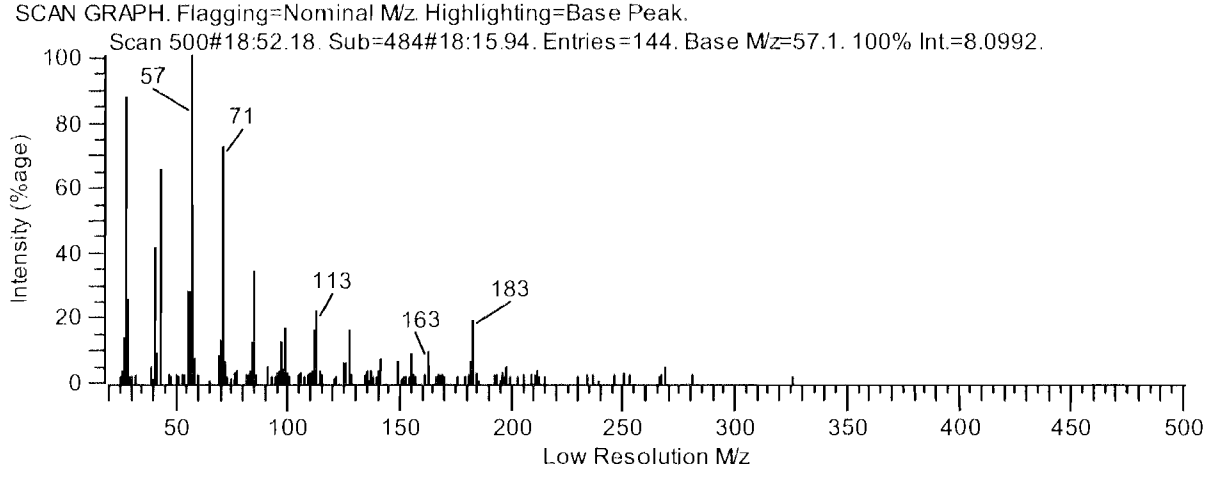
10. EI mass spectrum of diphenylamine.



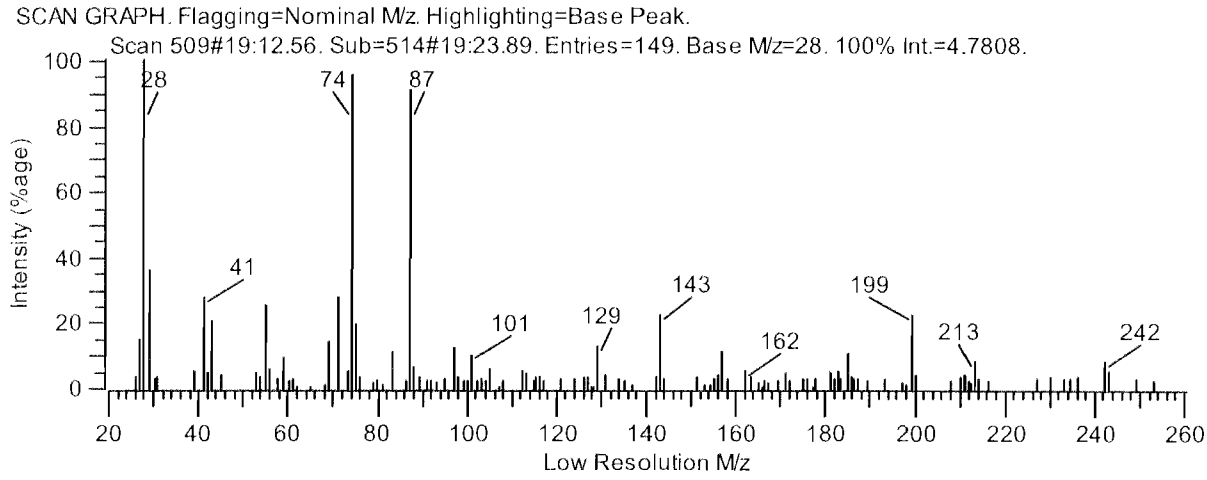
11. EI mass spectrum of an aliphatic hydrocarbon (the surrounding peaks from 16 min to 18 min have similar mass spectra).



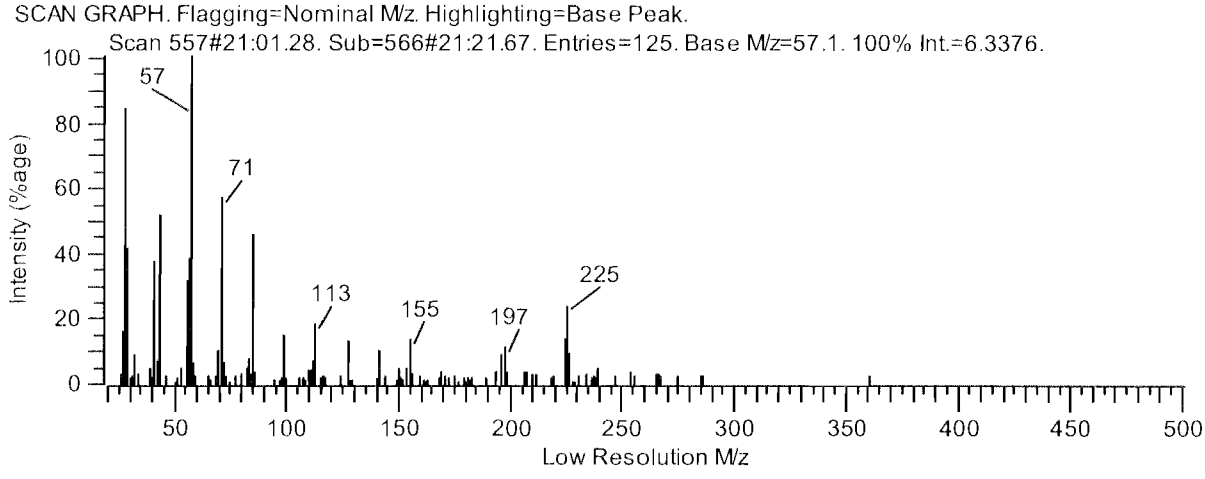
12. EI mass spectrum of heptadecane.



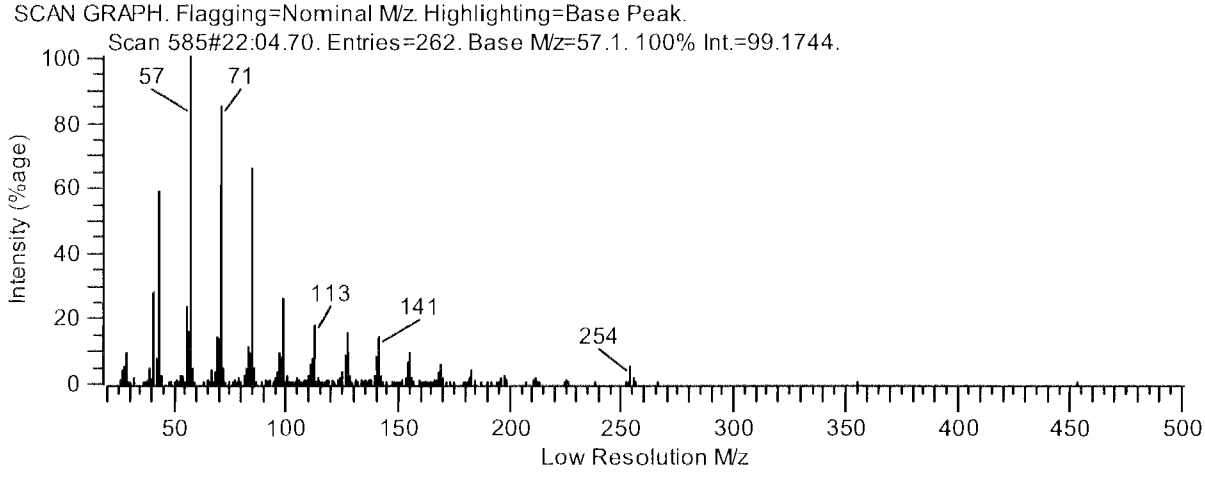
13. EI mass spectrum of an aliphatic hydrocarbon.



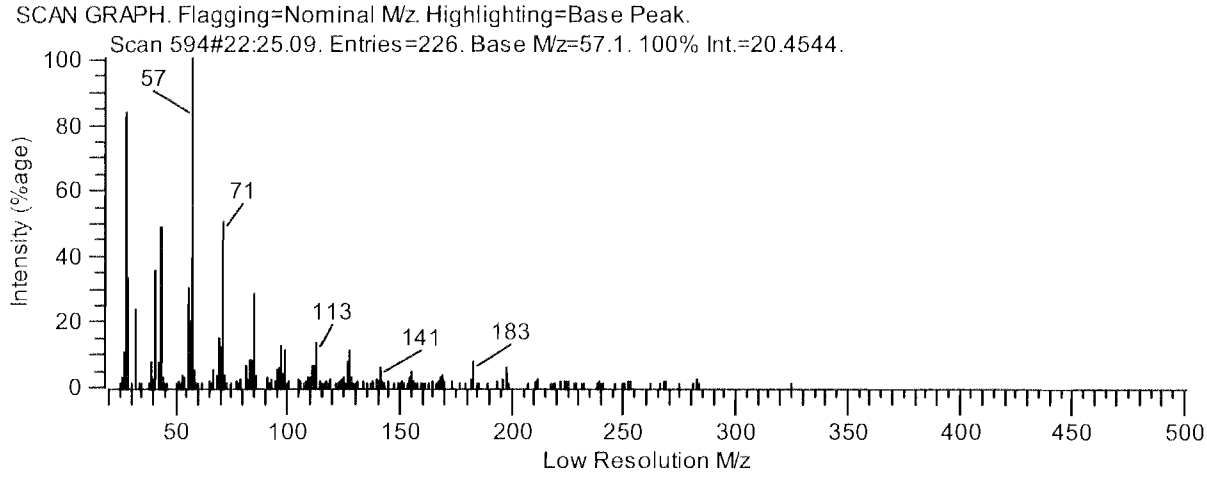
14. EI mass spectrum of methyl tetradecanoate.



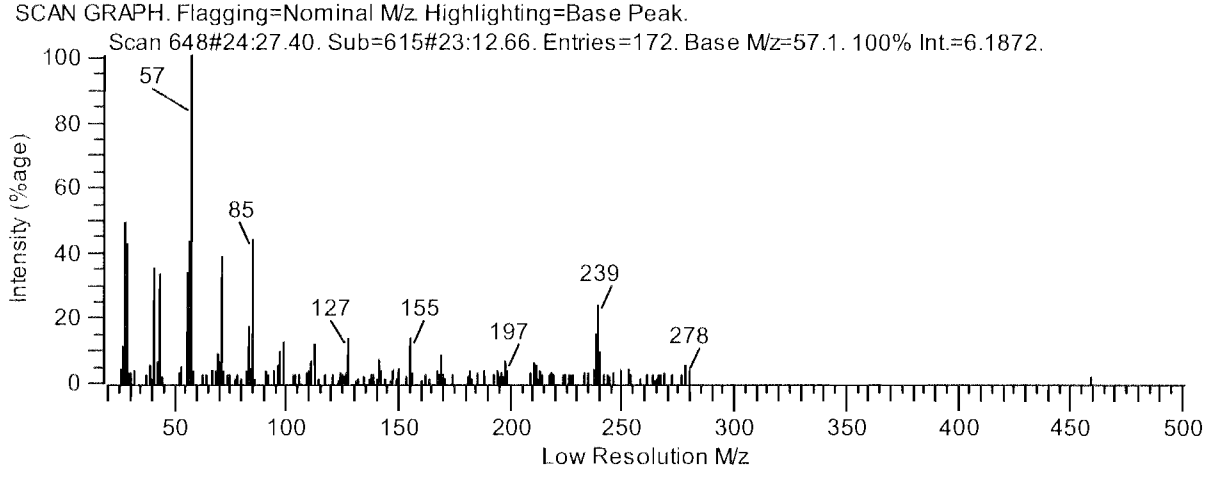
15. EI mass spectrum of an aliphatic hydrocarbon (the surrounding peaks from 20 min to 22 min have similar mass spectra).



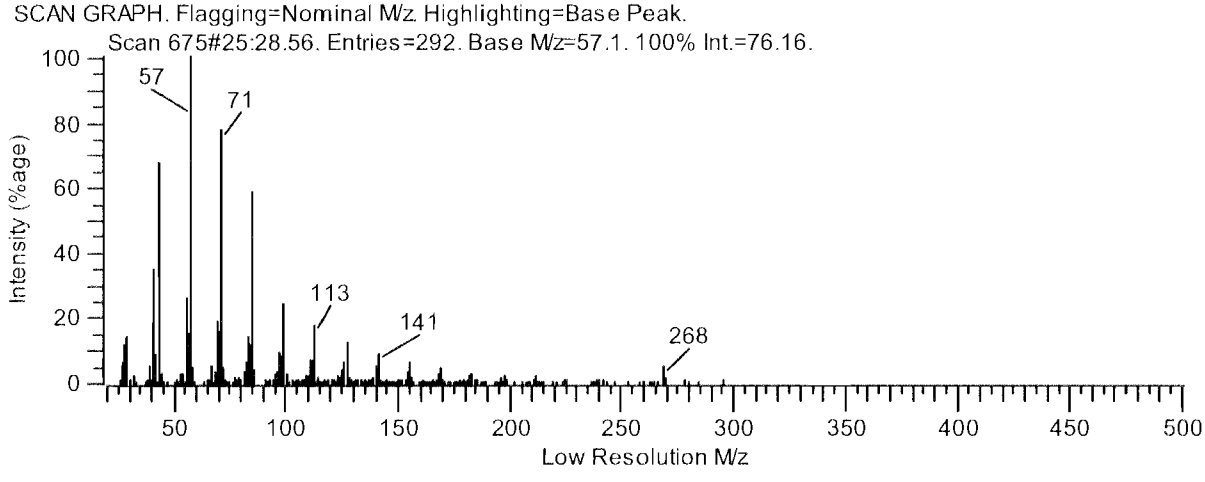
16. EI mass spectrum of octadecane.



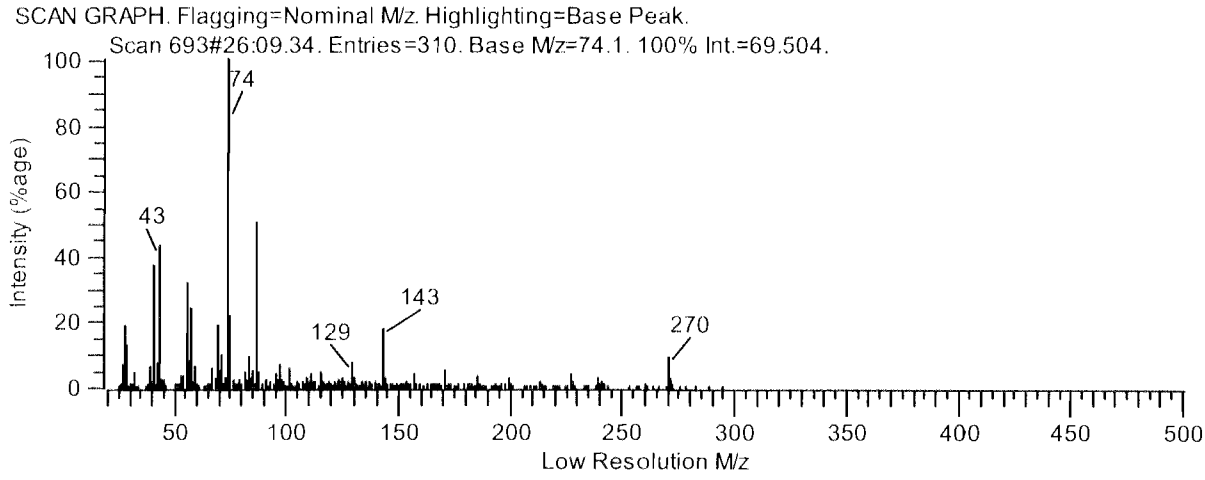
17. EI mass spectrum of an aliphatic hydrocarbon.



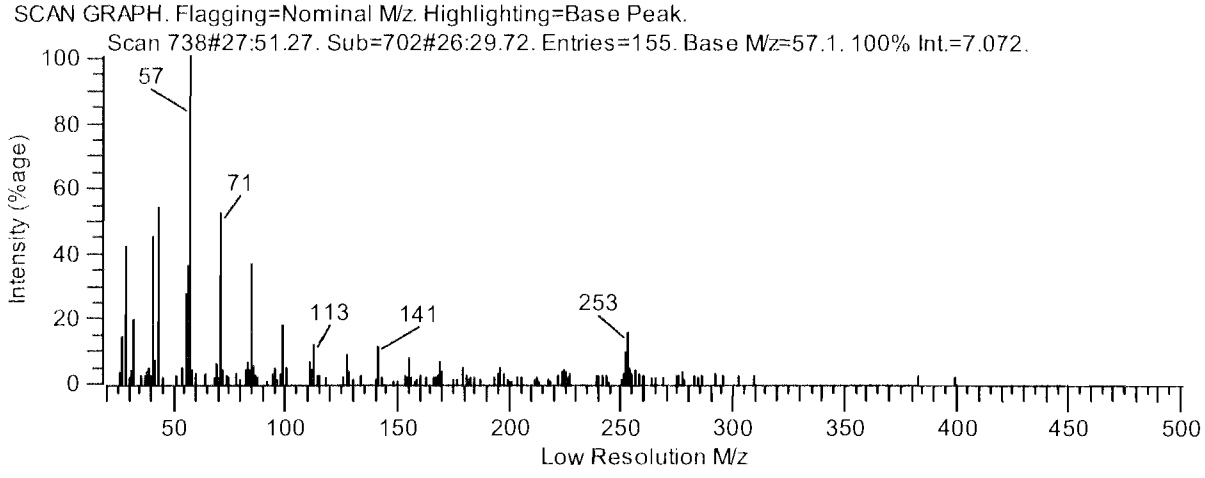
18. EI mass spectrum of an aliphatic hydrocarbon (possibly branched) (the surrounding peaks from 23 min to 25 min have similar mass spectra).



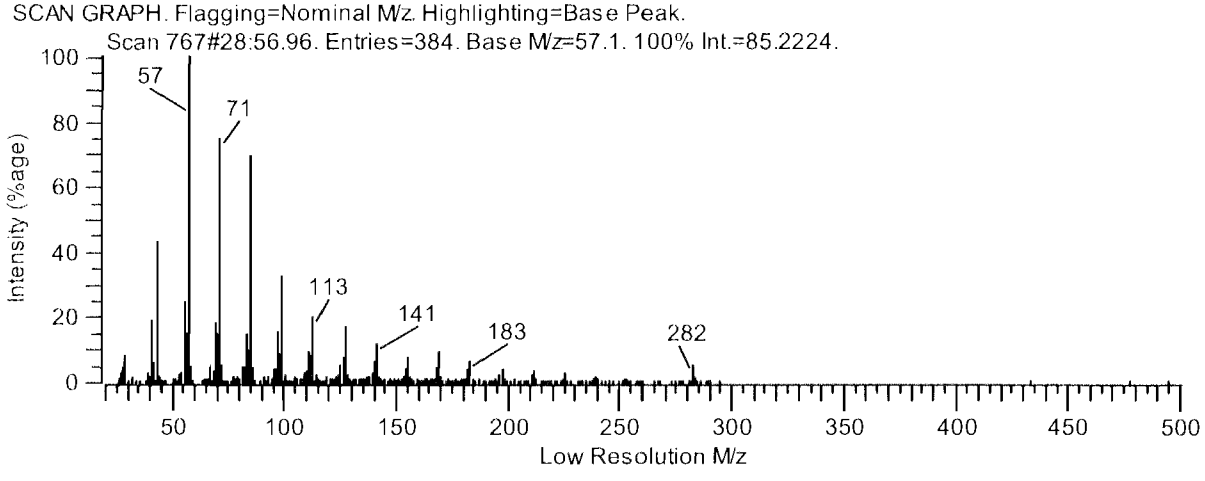
19. EI mass spectrum of nonadecane.



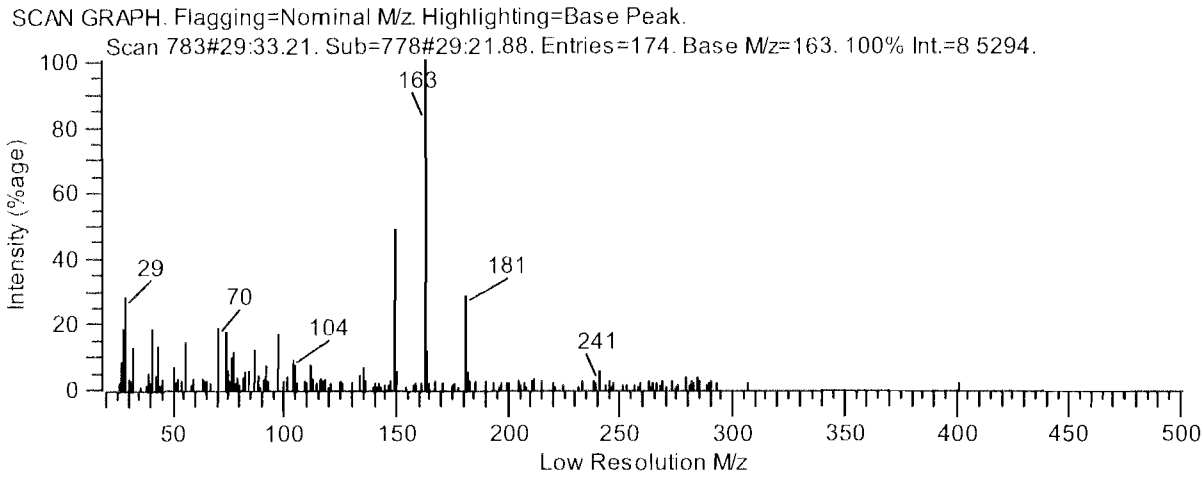
20. EI mass spectrum of methyl hexadecanoate.



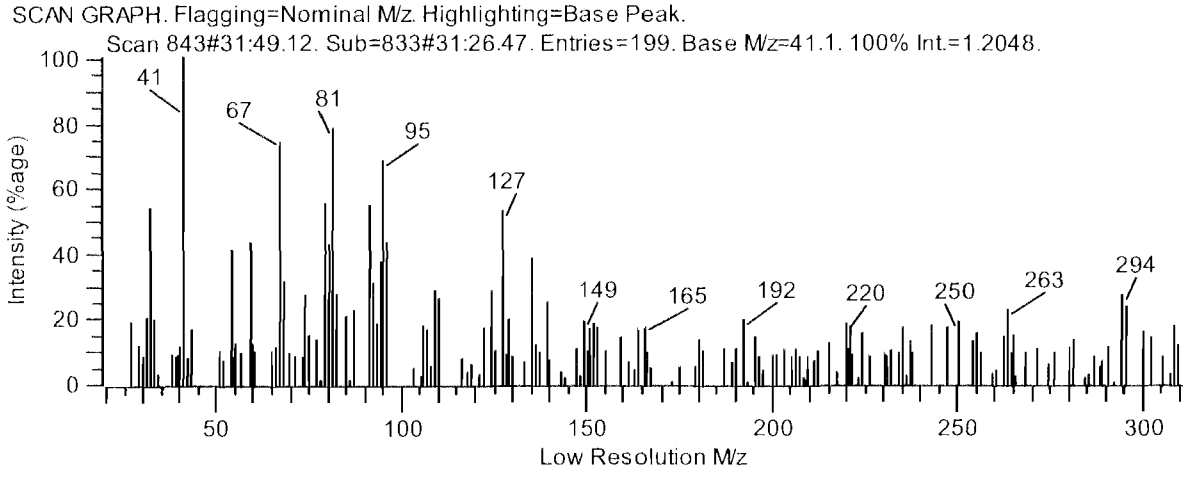
21. EI mass spectrum of an aliphatic hydrocarbon (possibly branched) (the surrounding peaks from 26 min to 28 min have similar mass spectra).



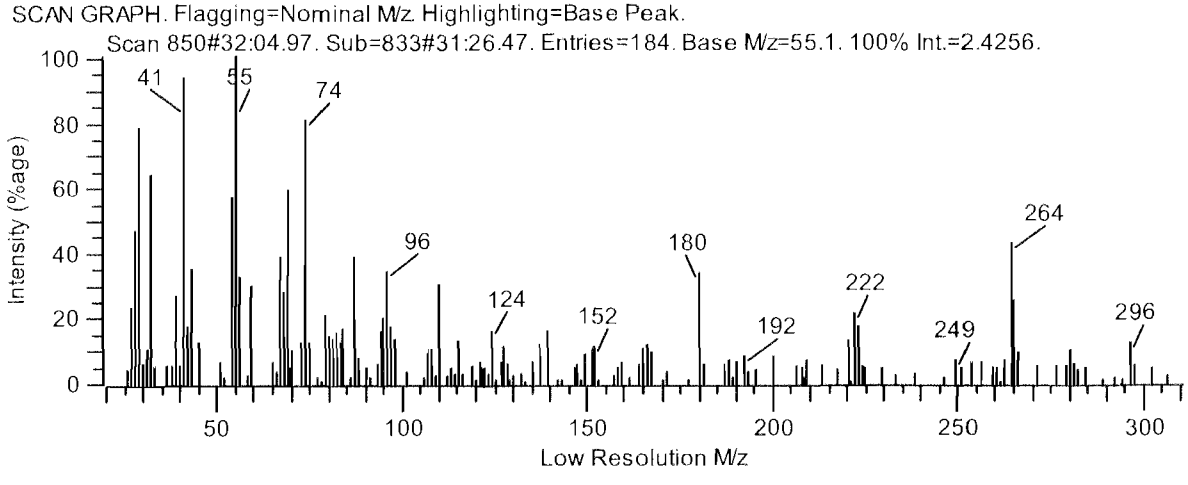
22. EI mass spectrum of icosane.



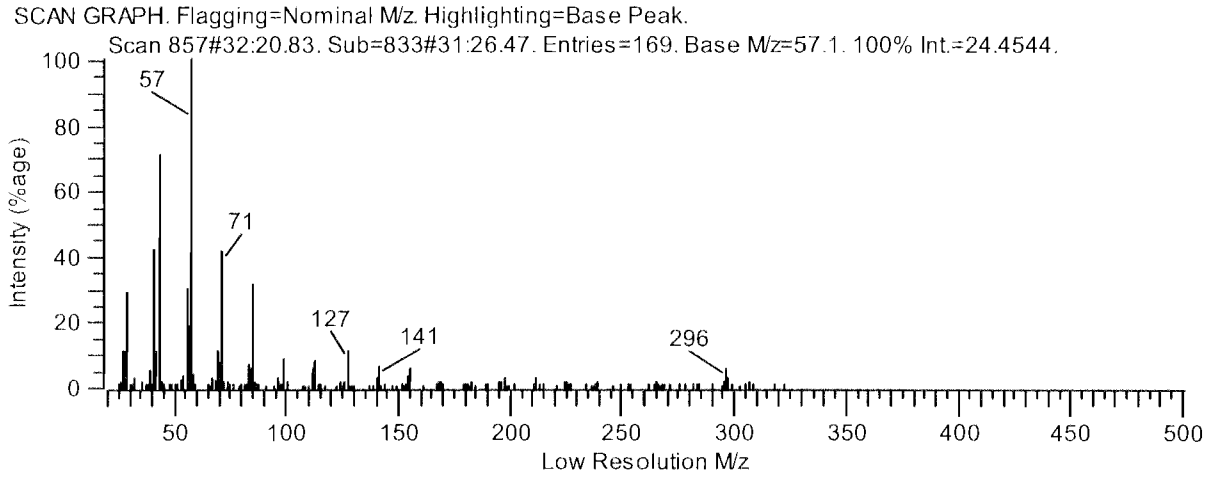
23. EI mass spectrum a phthalate.



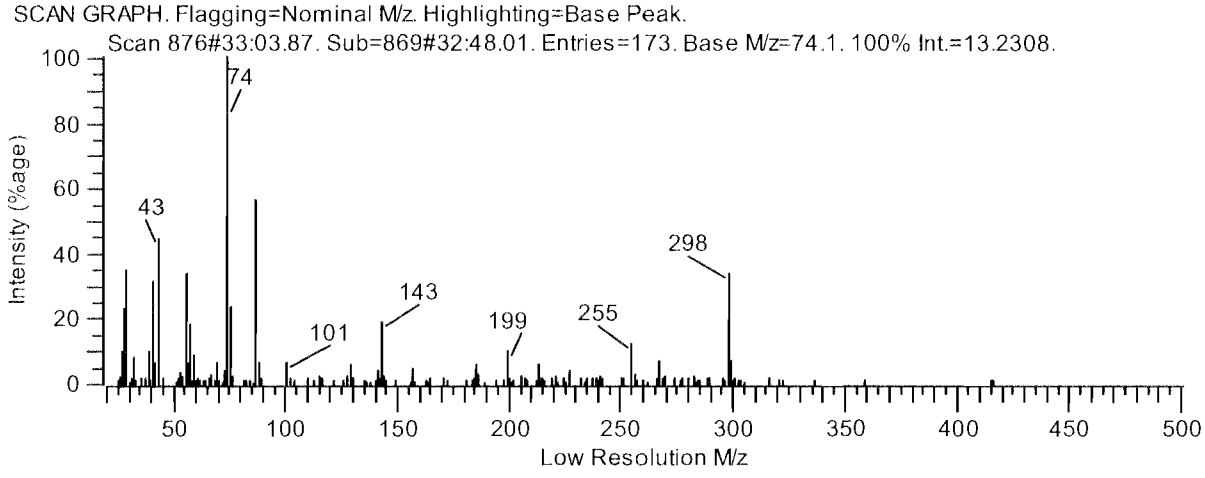
24. EI mass spectrum of methyl (Z,Z)-9,12-octadecadienoate.



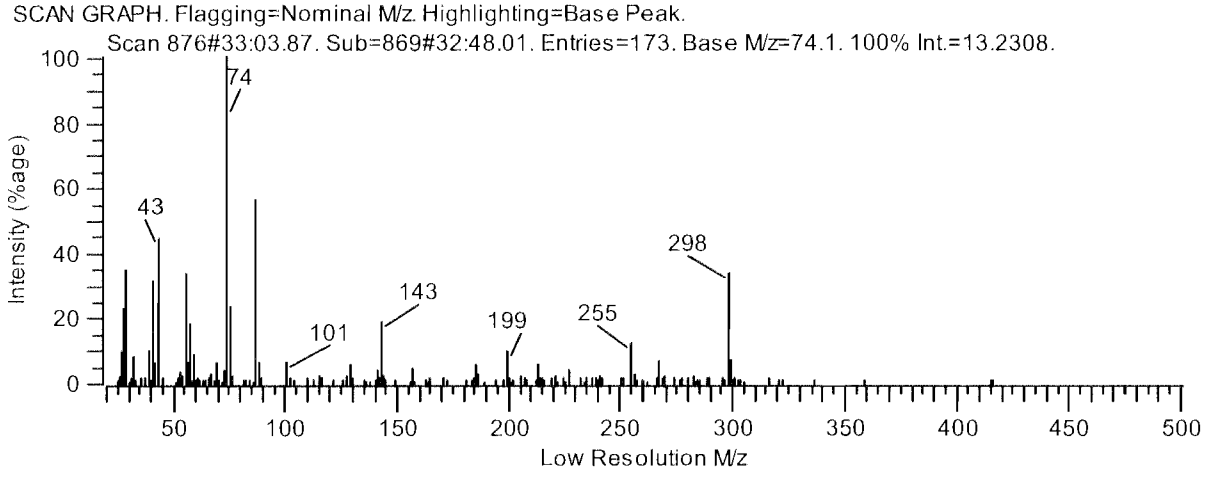
25. EI mass spectrum of methyl Z-9-octadecenoate.



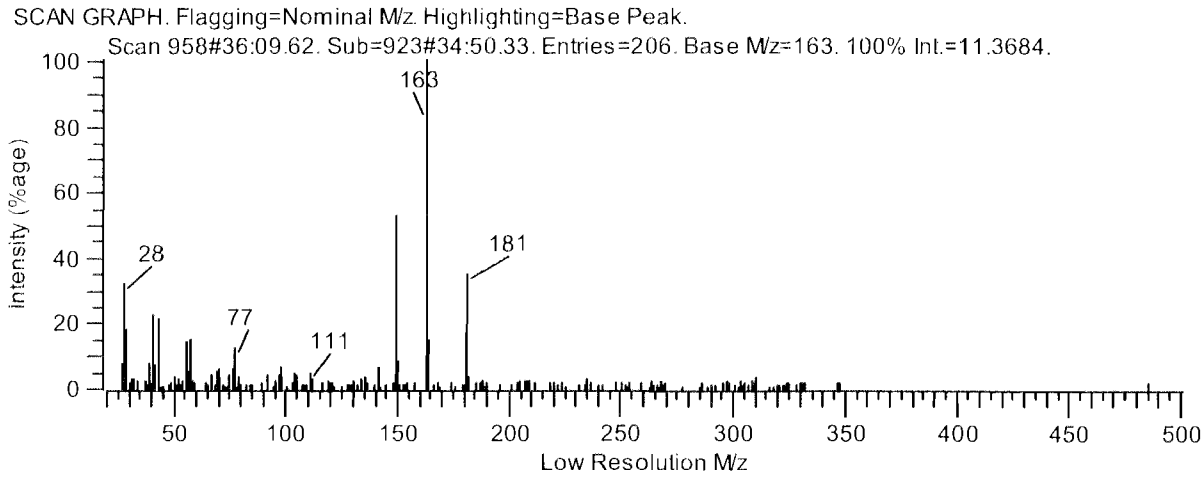
26. EI mass spectrum of henicosane.



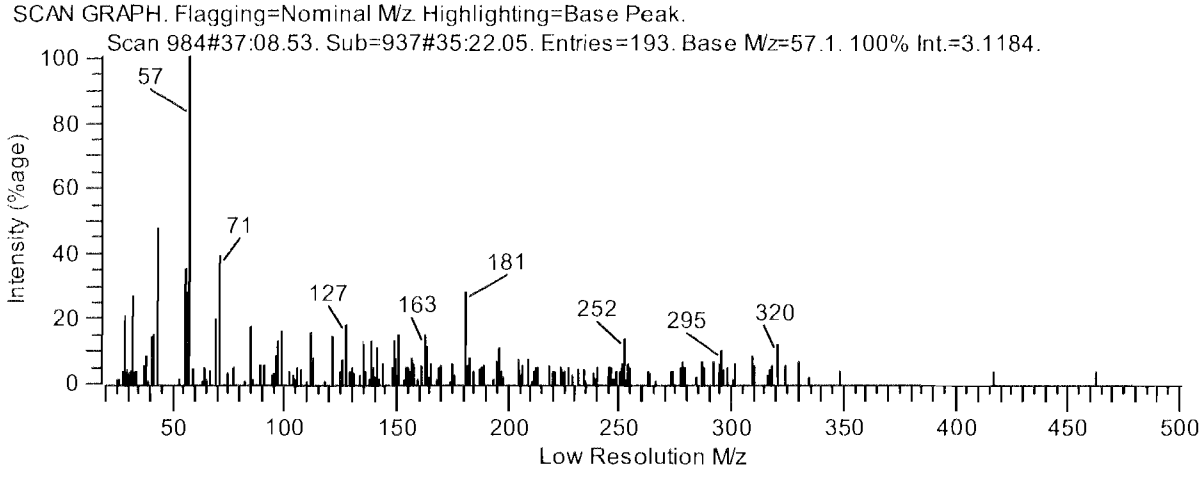
27. EI mass spectrum of methyl octadecanoate.



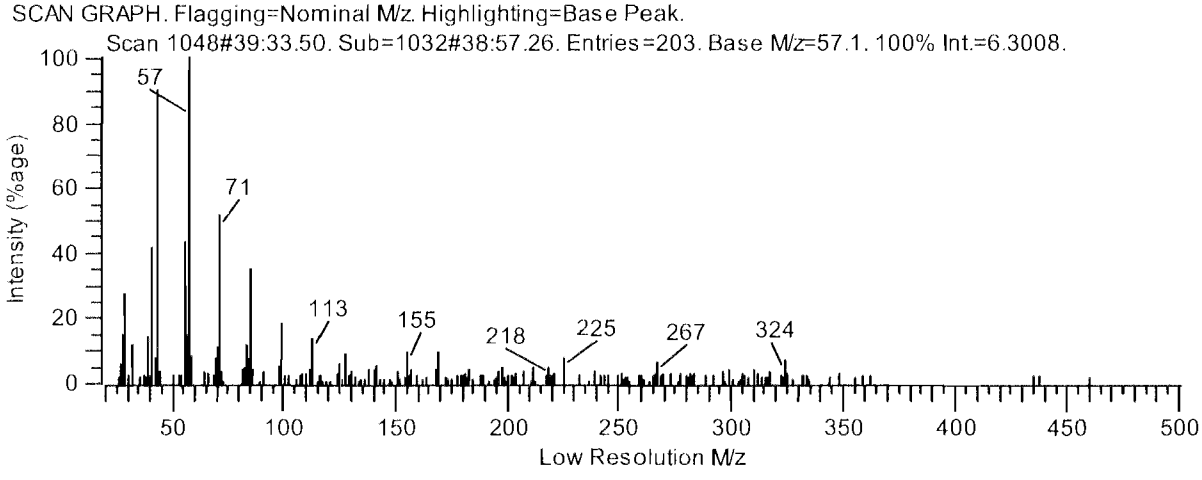
28. EI mass spectrum of docosane.



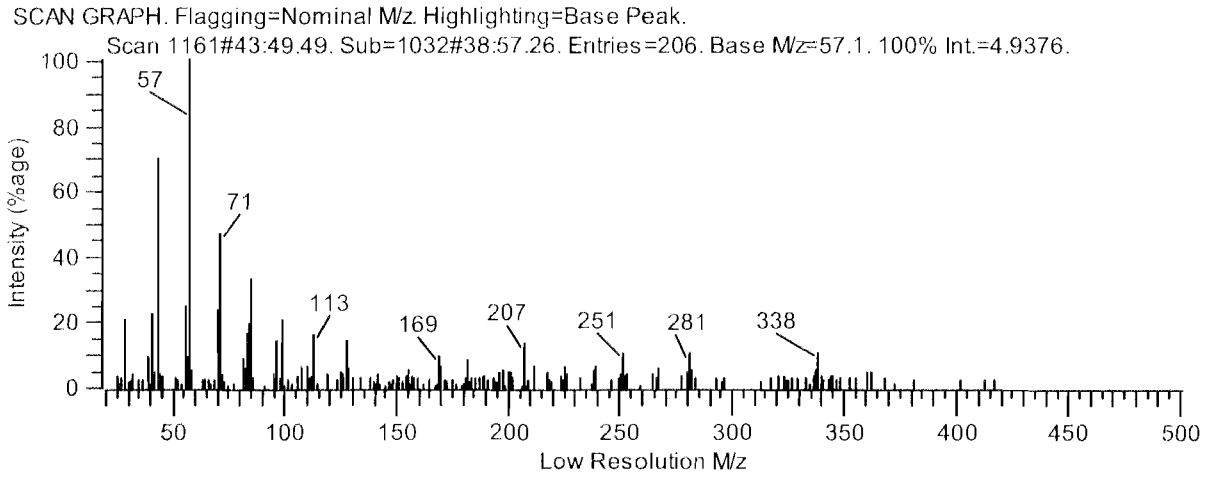
29. EI mass spectrum of a phthalate.



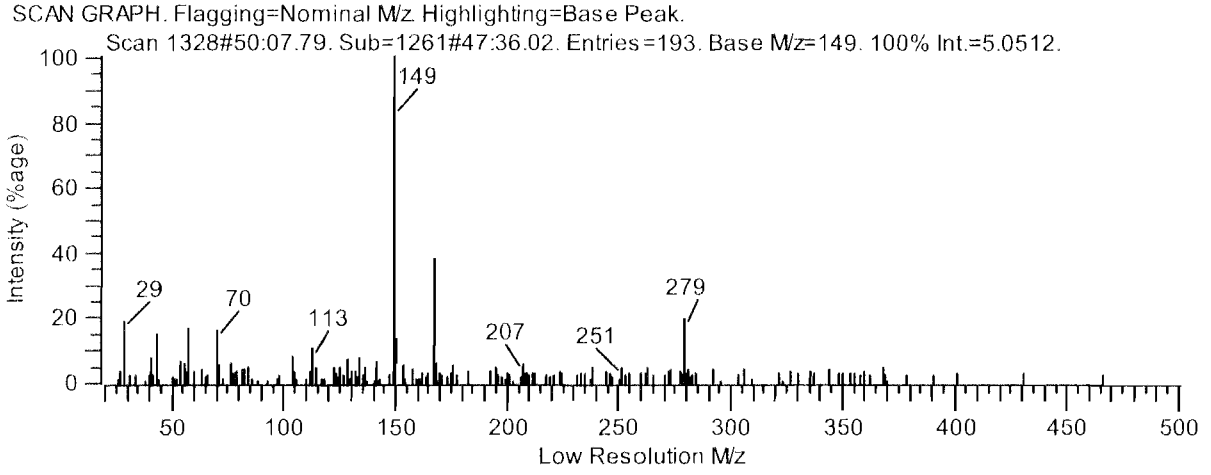
30. EI mass spectrum of aliphatic hydrocarbon.



31. EI mass spectrum of tricosane.



32. EI mass spectrum of tetracosane.



33. EI mass spectrum of a phthalate.

Submitted by: Denise Saravanakumar (UCT, Dept. of Pharmacology; Prof. Peter Smith)
Analysis date: 31 January 2006
Instrument: AMD 604 High Resolution Mass Spectrometer
Sample: **C1730E**
Data folder: 200601 January
Calibration: 2006\0060119_CAL500_res1000
Scan: m/z 25-500

Sample preparation: The sample solution was injected (1 μl) directly into the gas chromatograph (GC) inlet.
Inlet method: Gas Chromatography

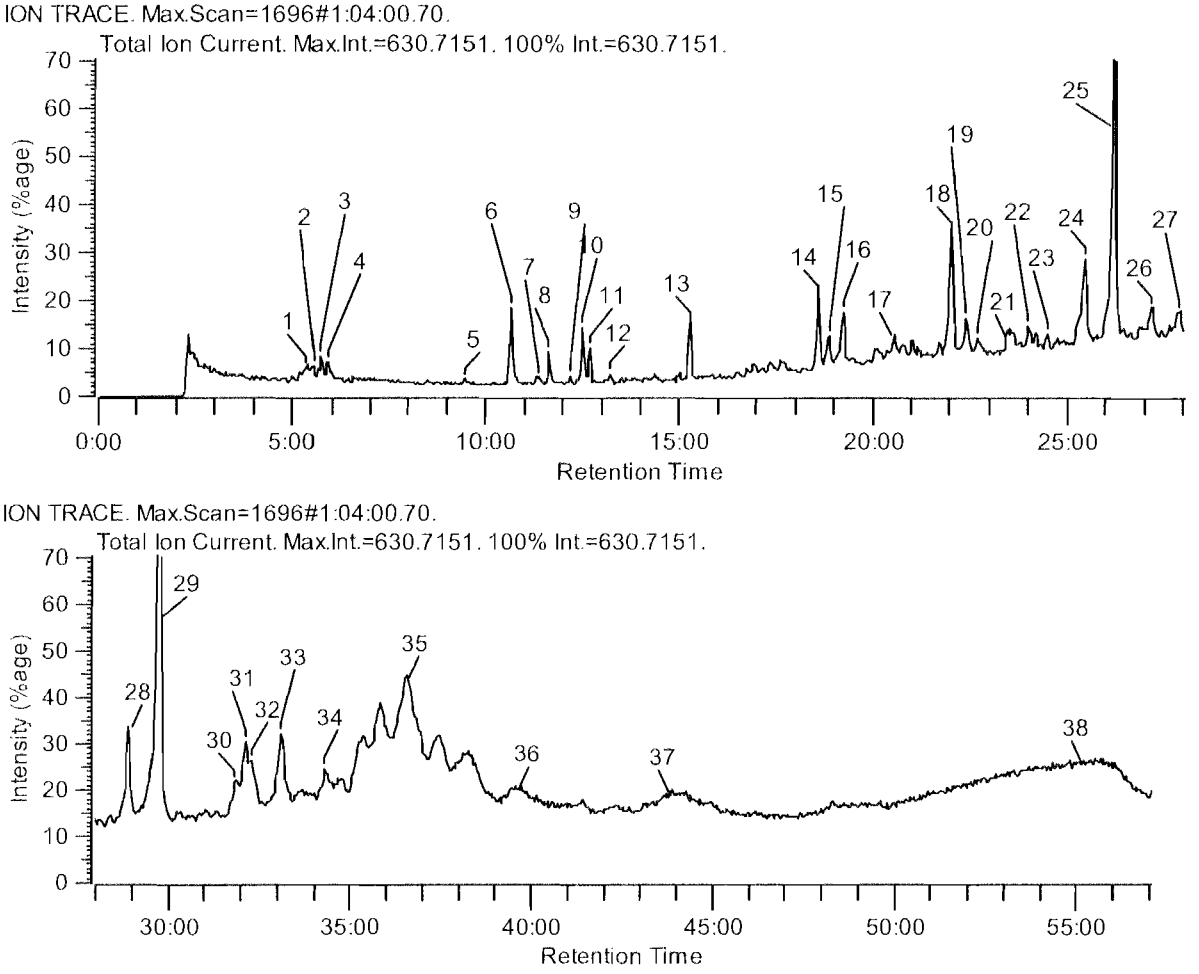
- Instrument
  - Carlo Erba GC 6000 Vega Series
  - Transfer line temperature: 250°C
- Column
  - LECUS, PS089-OH (DB-5 equivalent) silanol-terminated (95%)-methyl-(5%)-phenylpolisiloxane copolymer stationary phase
  - 40 m x 0.3 mm I.D.
  - Column number: P201
- GC Inlet

- He pressure: *ca.* 40 kPa (linear velocity: 28.2 cm/sec at 40°C)
- Split flow: 20 ml/min (septum purge: 5 ml/min)
- Temperature: 250°C

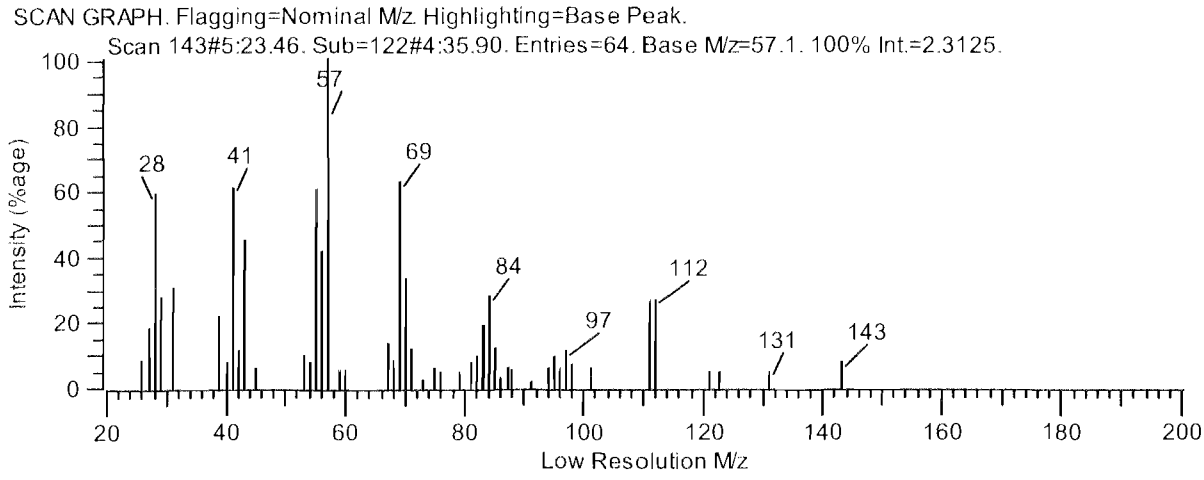
- Oven
  - 130 - 270°C at 3°C/min, hold 10 min

Ionization technique: EI^+ (Electron Energy: 70eV Source: 200°C)

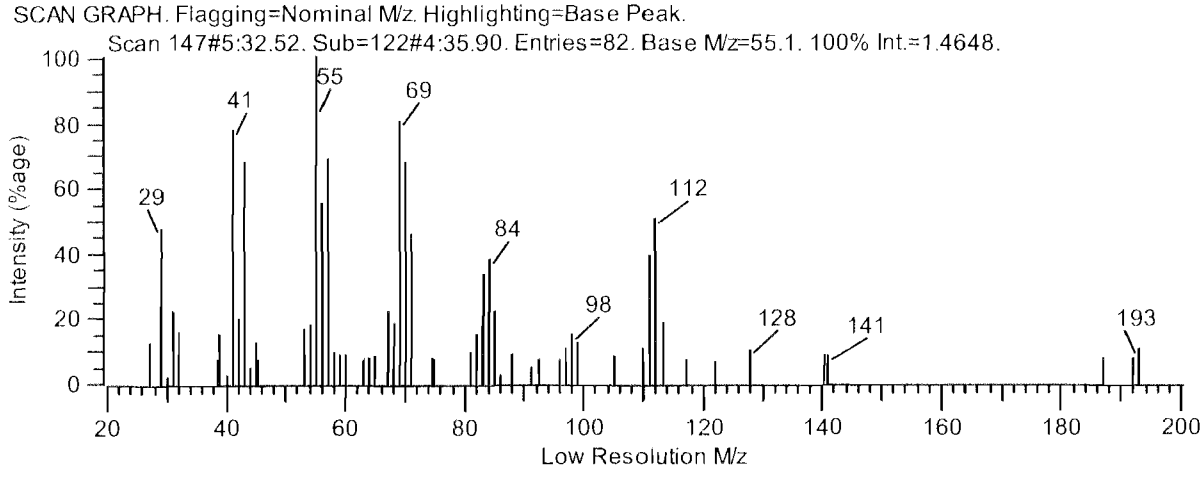
Total Ion Current Chromatogram:



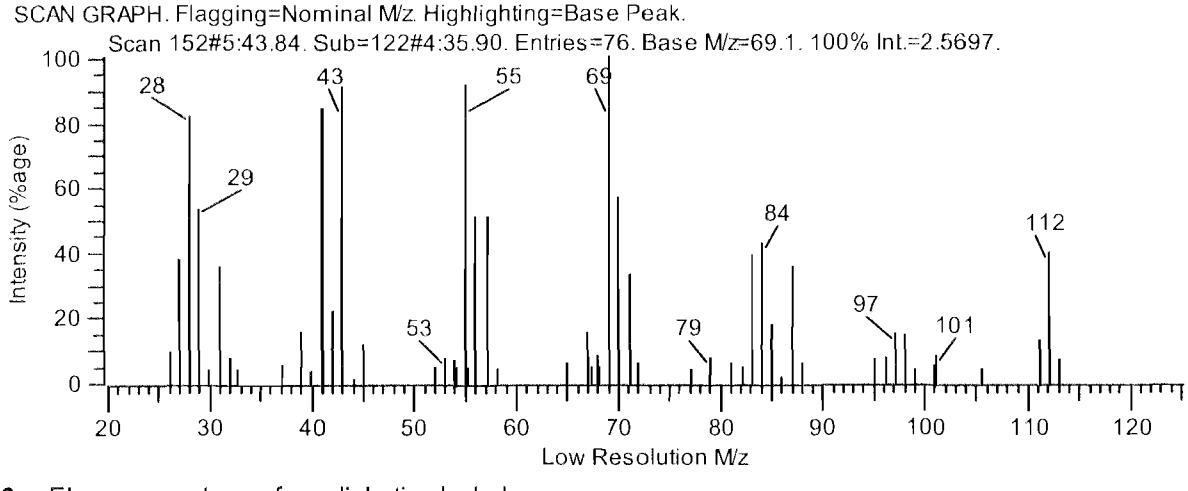
Low resolution EI^+ mass spectra of the sample components, providing their tentative identities:



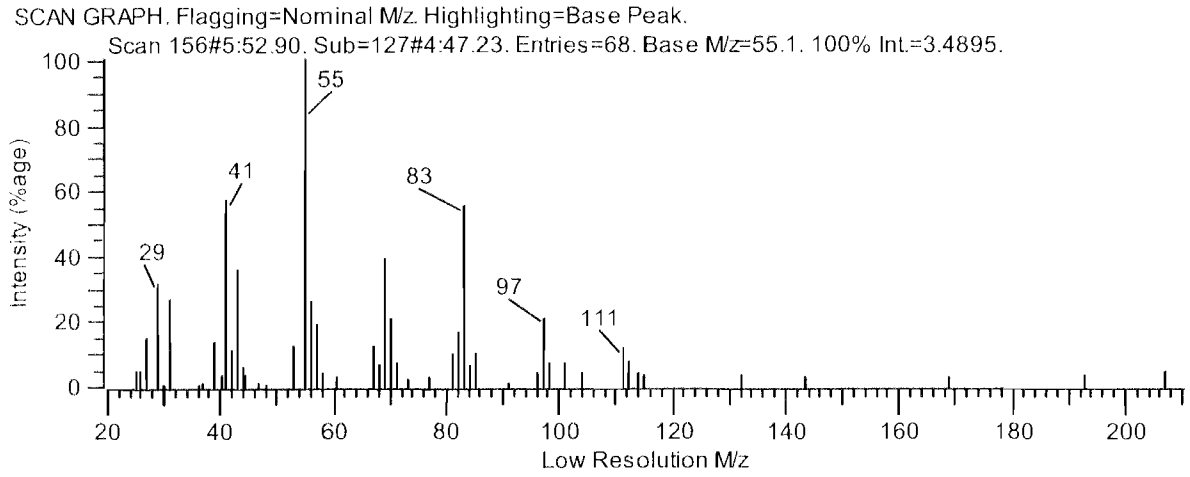
1. EI mass spectrum of an aliphatic alcohol.



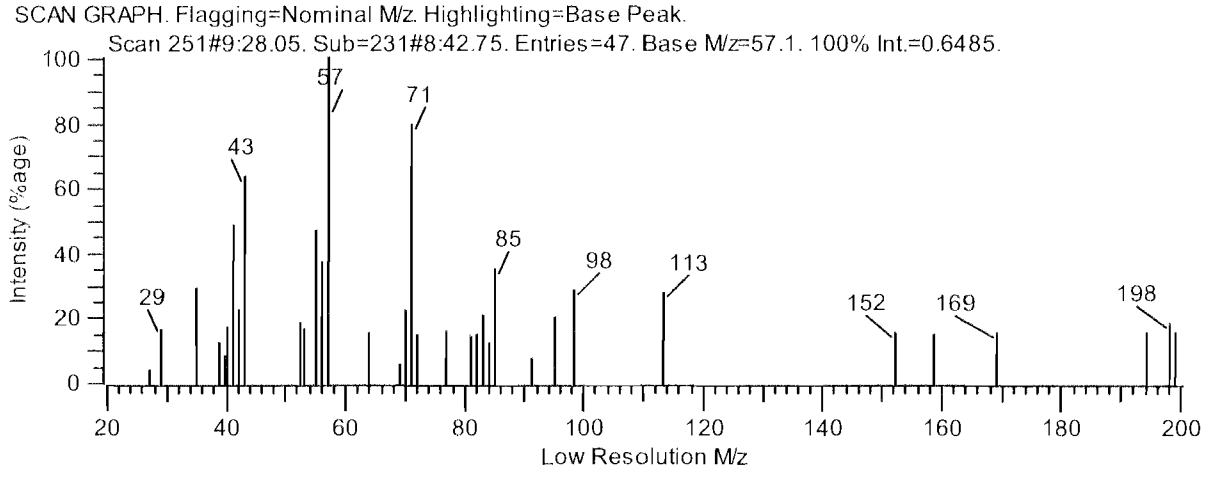
2. EI mass spectrum of an unsaturated aliphatic hydrocarbon.



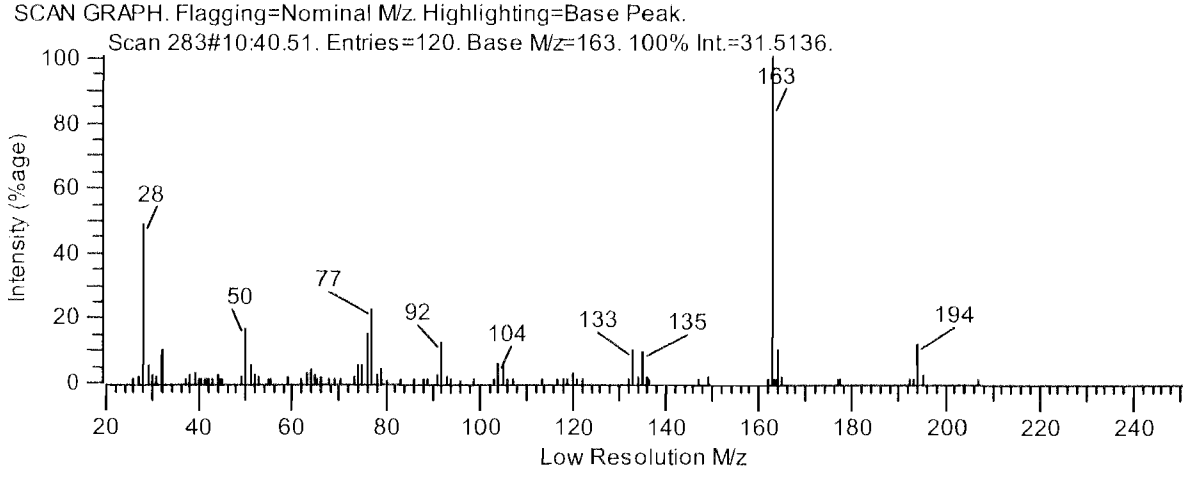
3. EI mass spectrum of an aliphatic alcohol.



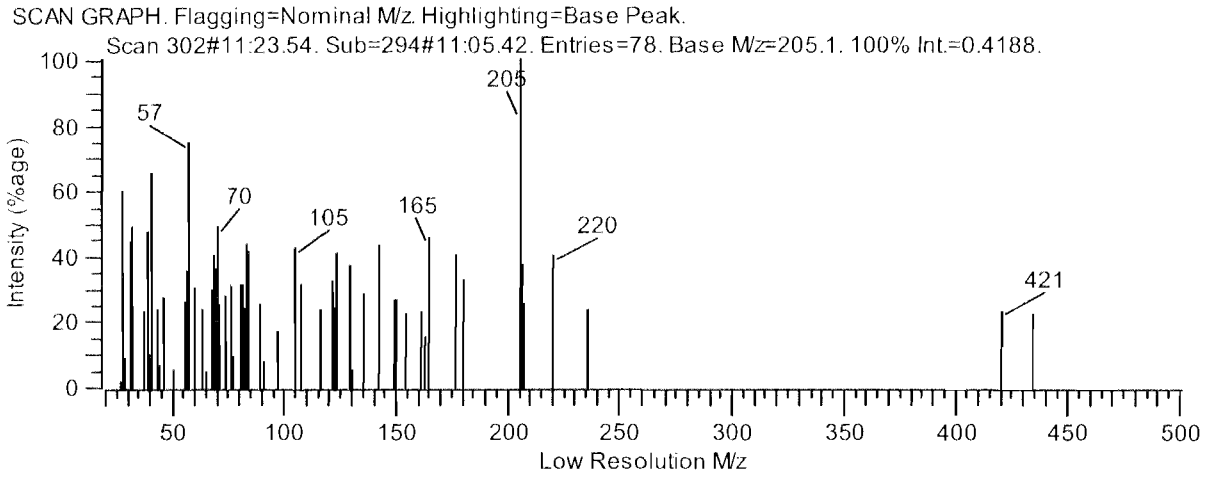
4. EI mass spectrum of an unsaturated aliphatic hydrocarbon.



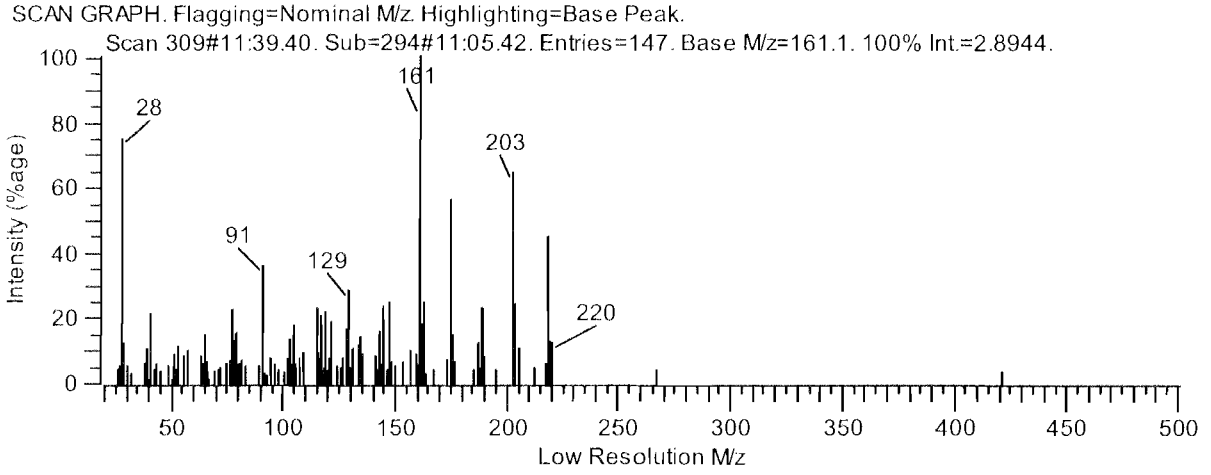
5. EI mass spectrum of an aliphatic hydrocarbon.



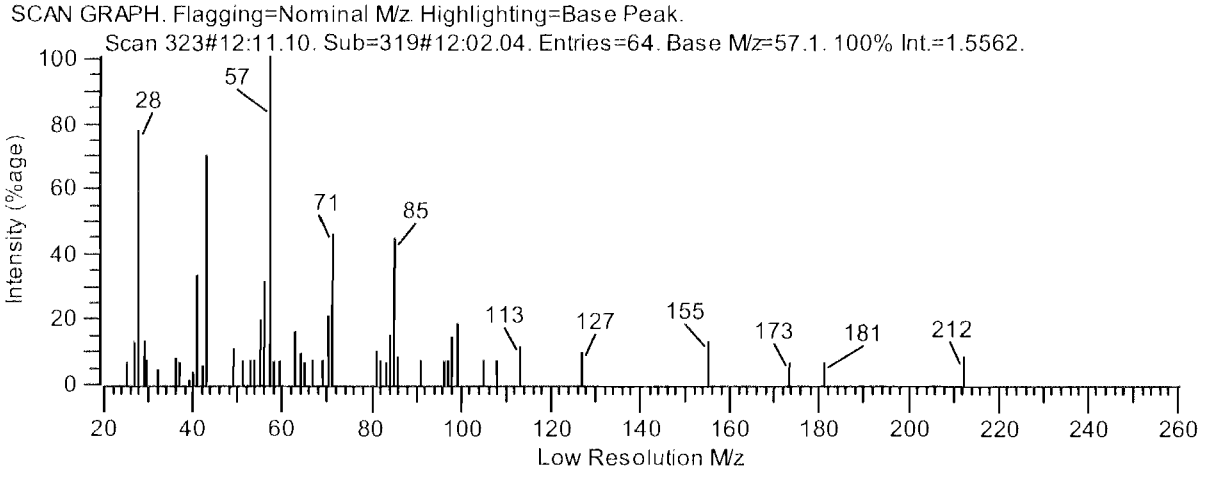
6. EI mass spectrum of dimethyl phthalate.



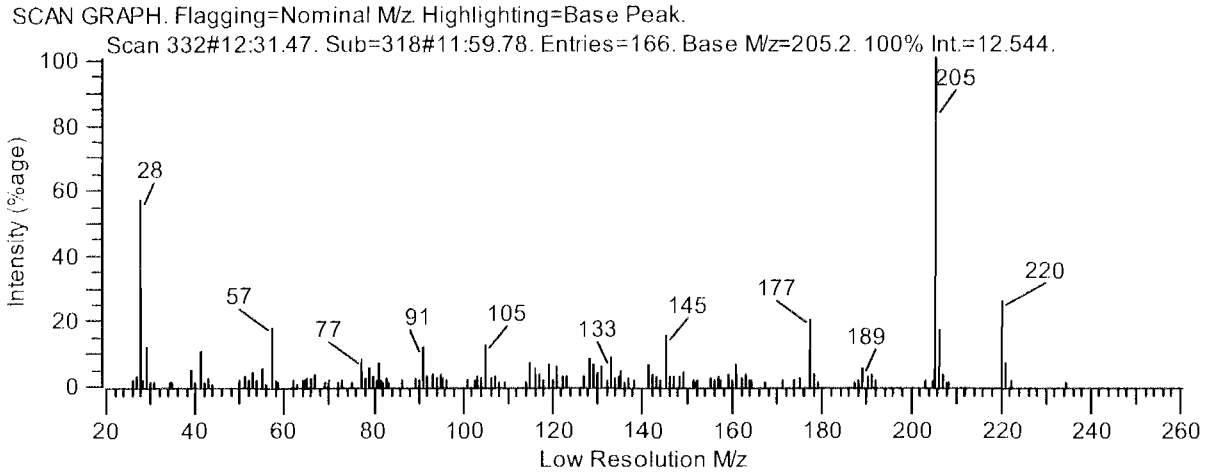
7. EI mass spectrum of an unknown compound.



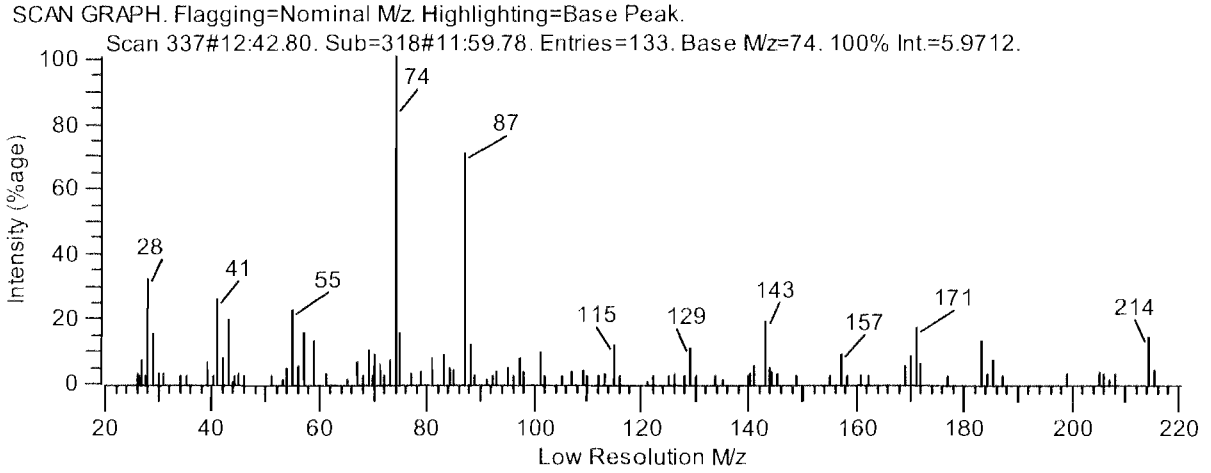
8. EI mass spectrum of an unsaturated cyclic hydrocarbon (could contain hydroxyl and/or carbonyl groups).



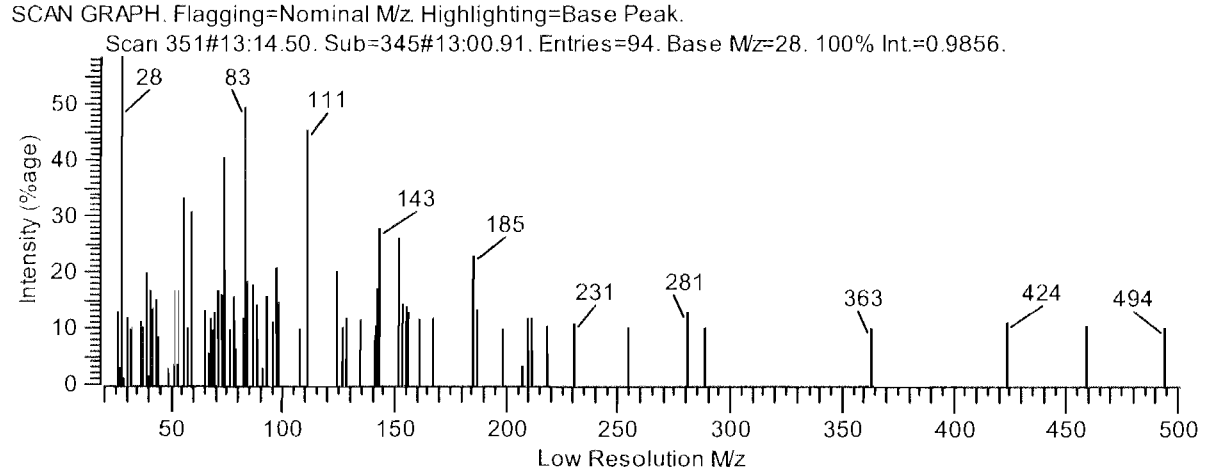
9. EI mass spectrum of an aliphatic hydrocarbon.



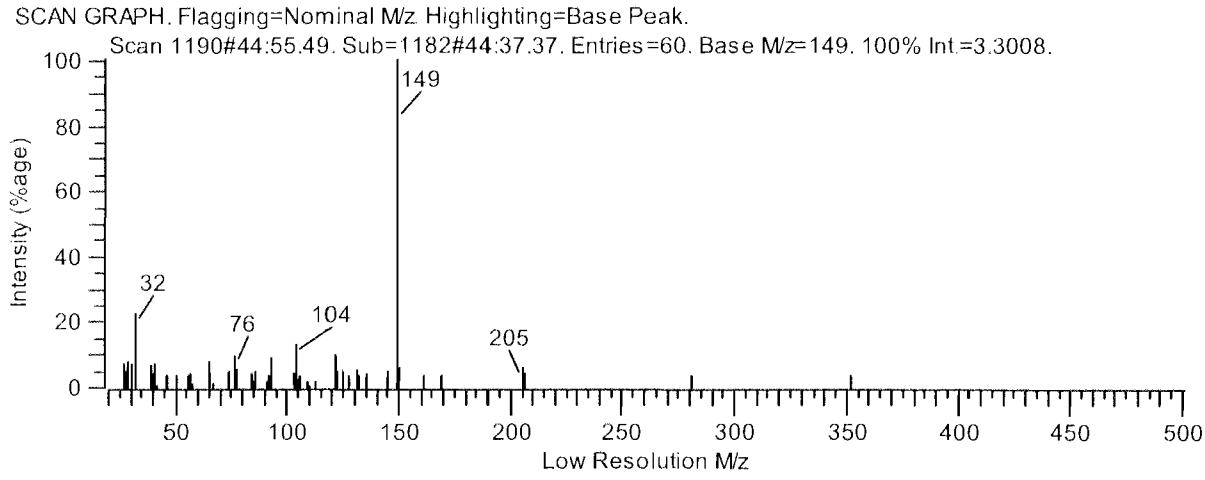
10. EI mass spectrum of butylated hydroxytoluene.



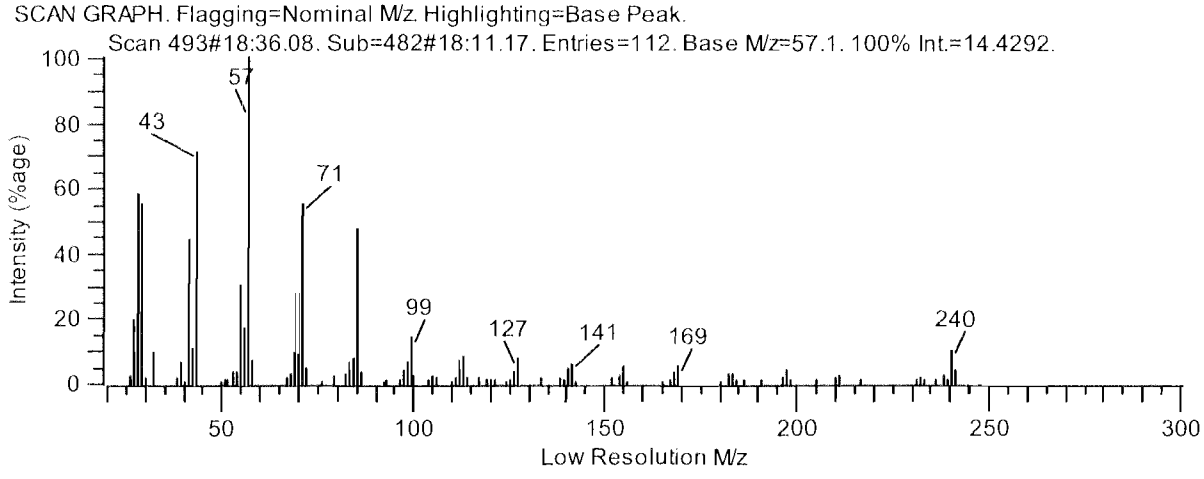
11. EI mass spectrum of methyl dodecanoate.



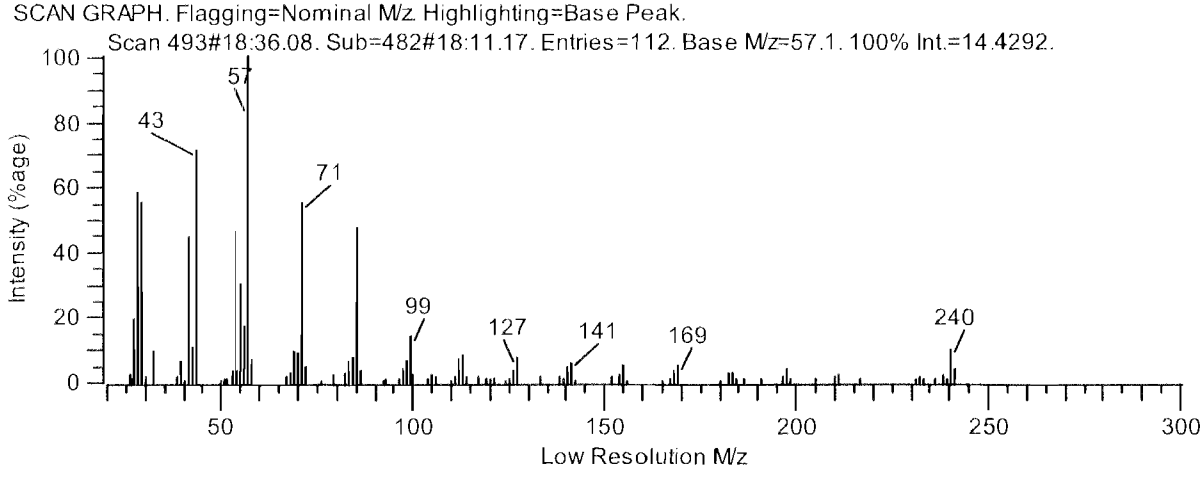
12. EI mass spectrum of an unknown compound.



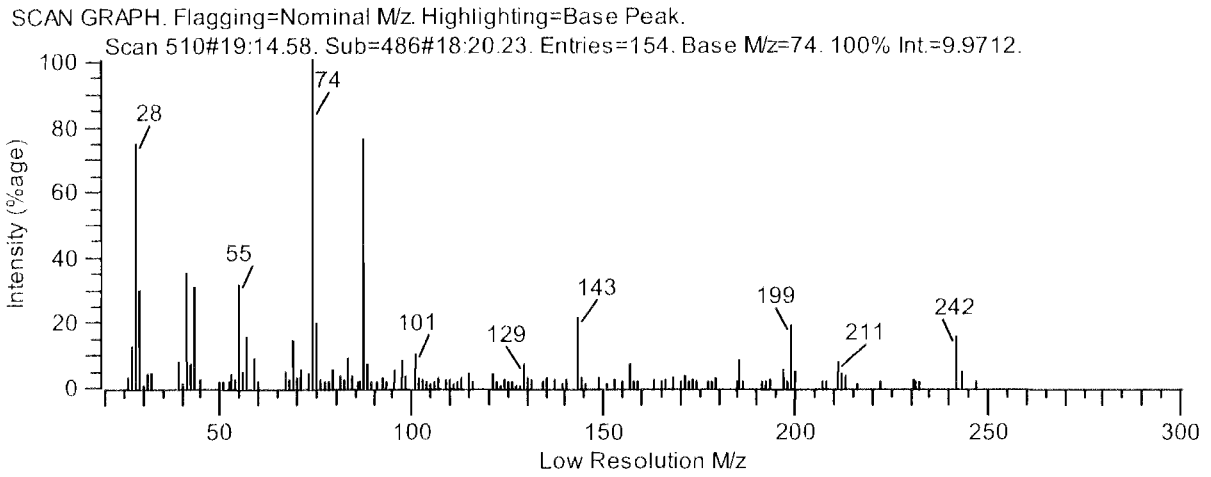
13. EI mass spectrum of a phthalate



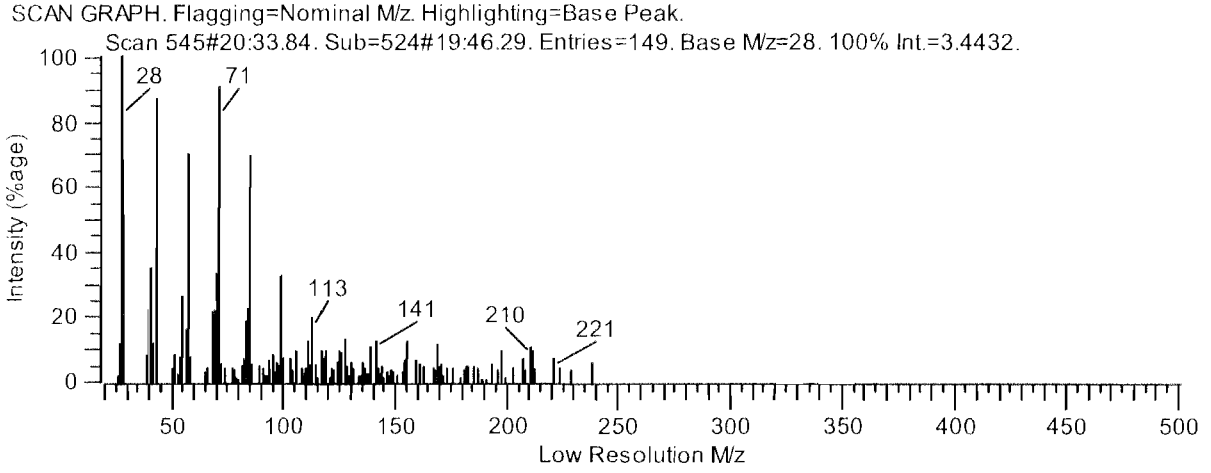
14. EI mass spectrum of heptadecane.



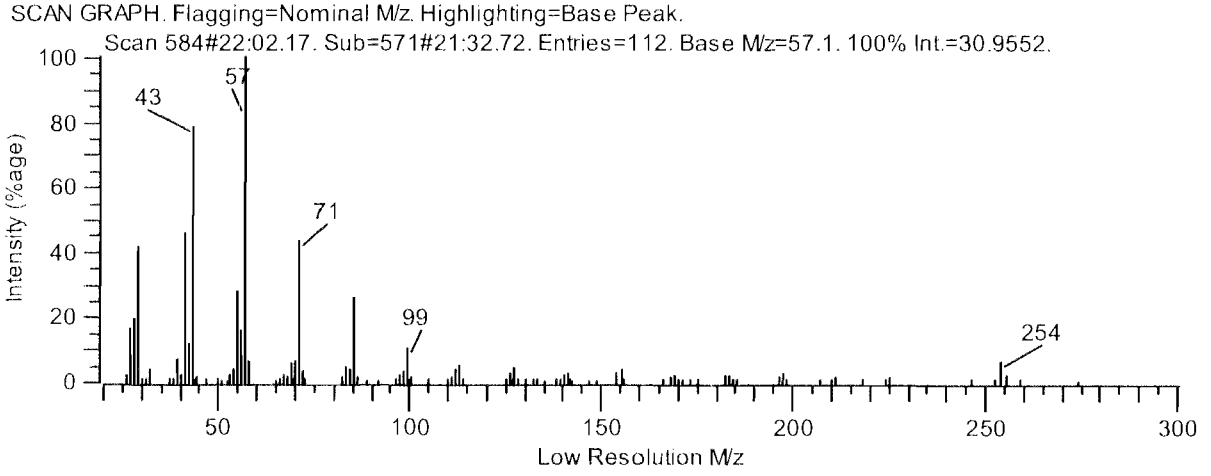
15. EI mass spectrum of a phthalate.



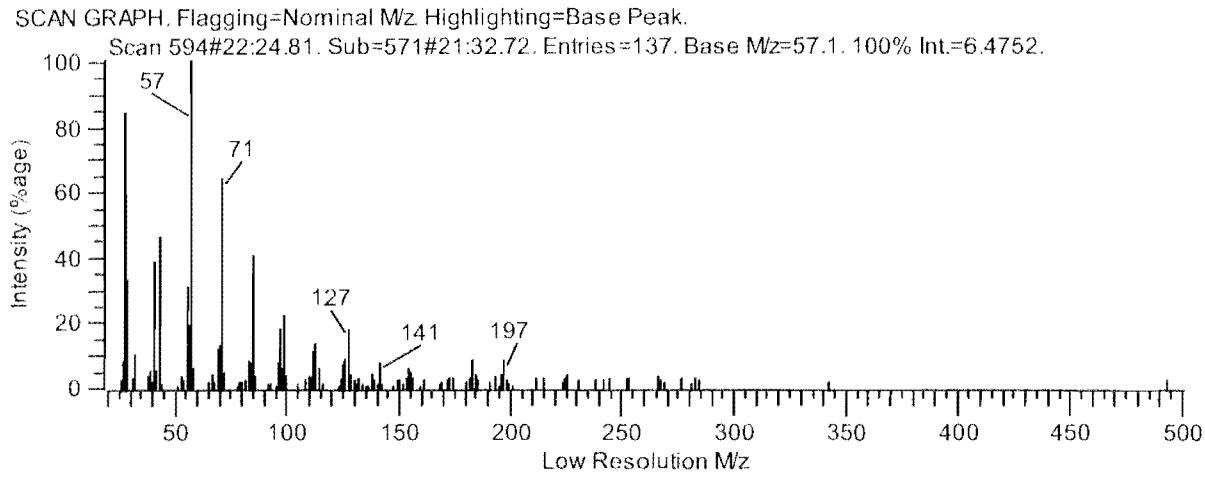
16. EI mass spectrum of methyl tatredacanoate.



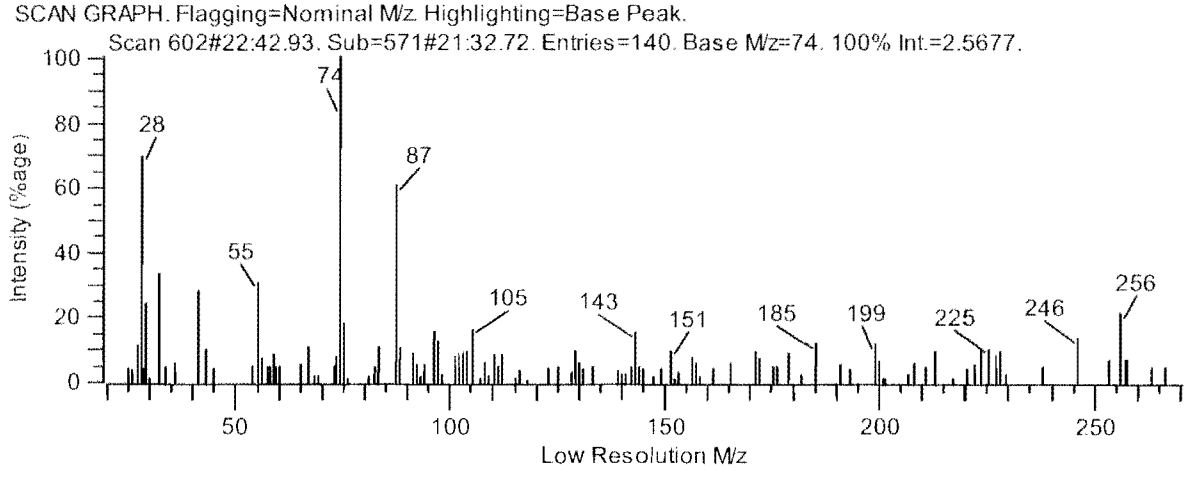
17. EI mass spectrum of an aliphatic hydrocarbon.



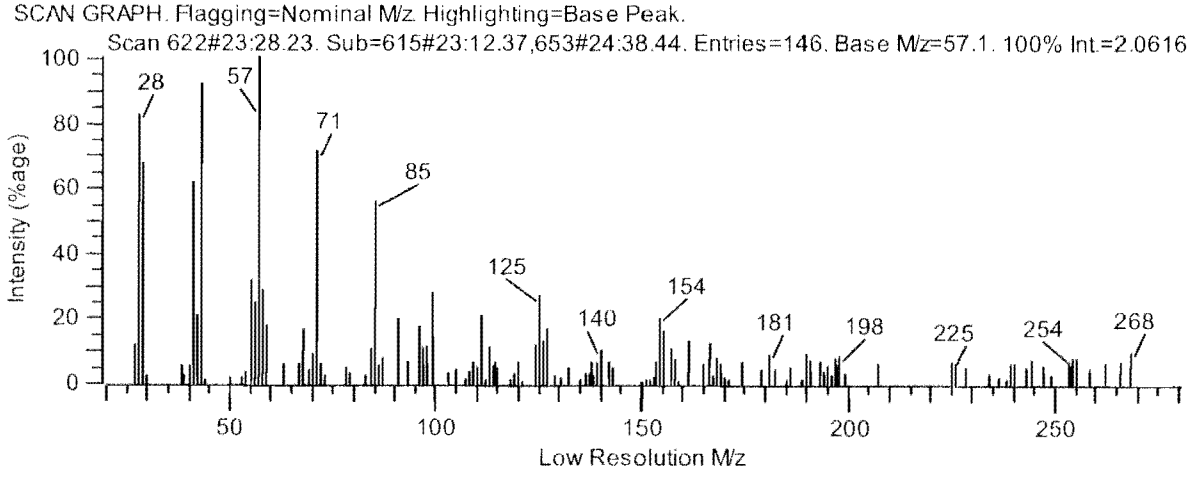
18. EI mass spectrum of octadecane.



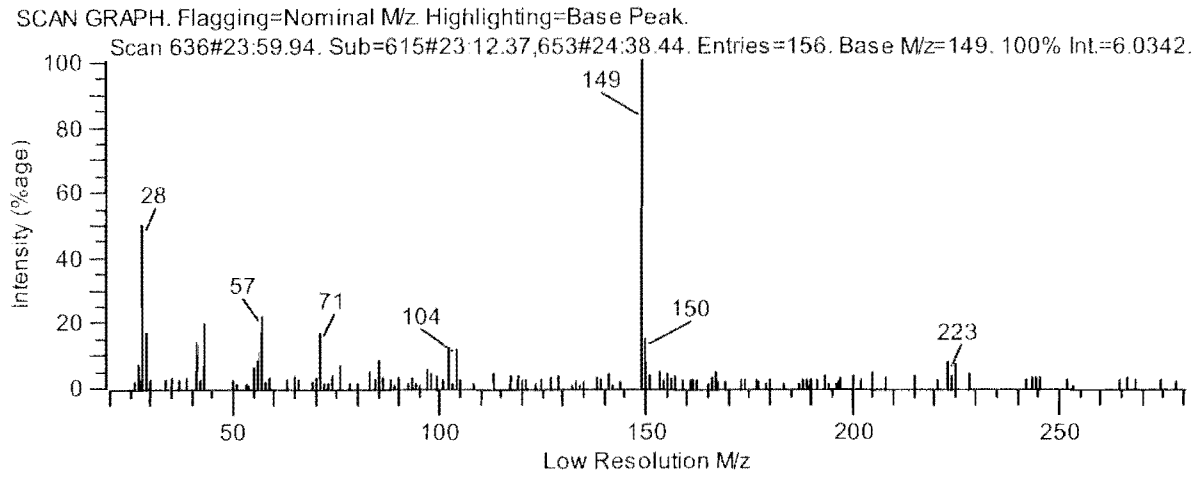
19. EI mass spectrum of an aliphatic hydrocarbon.



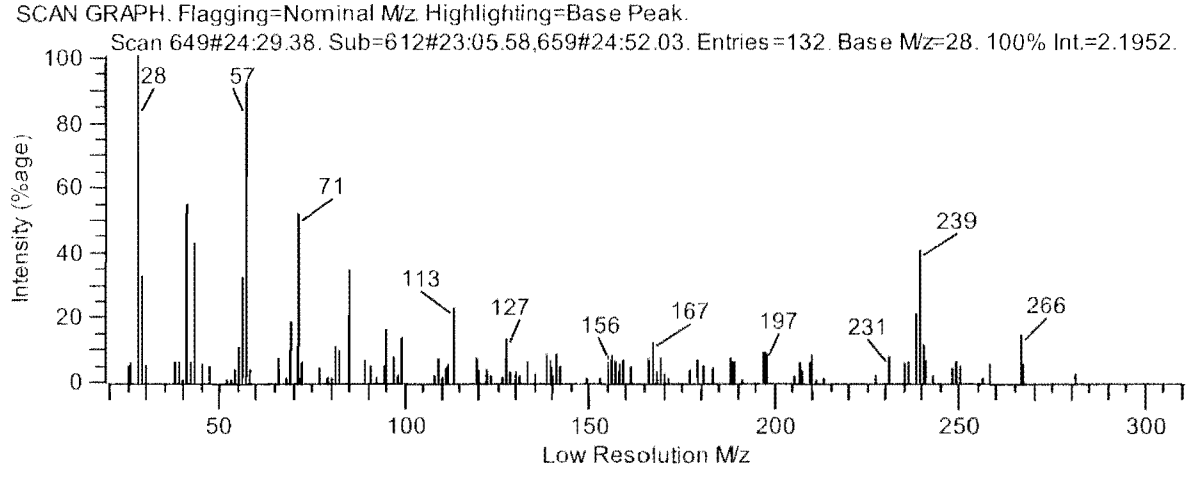
20. EI mass spectrum of methyl pentadecanoate.



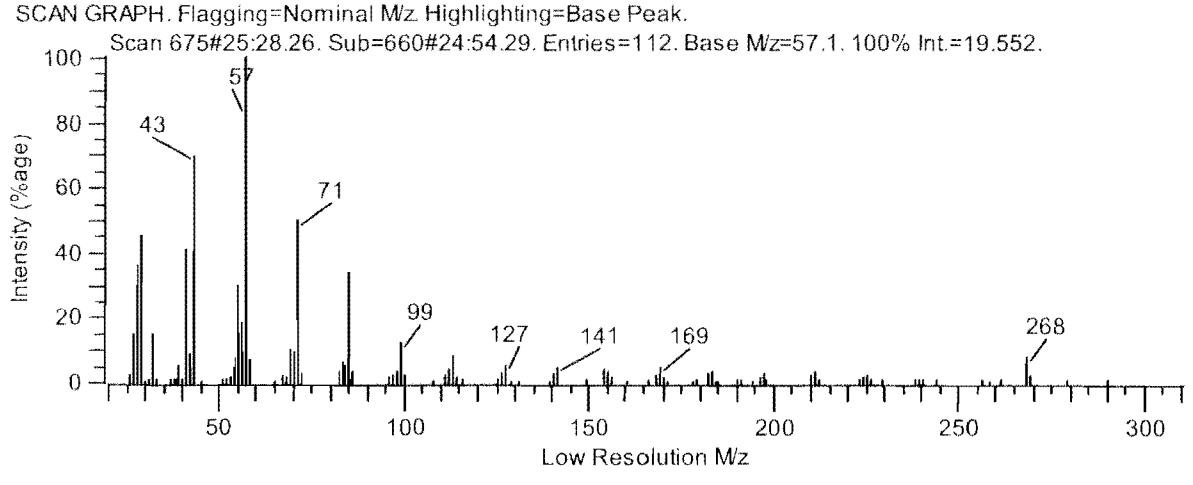
21. EI mass spectrum of an aliphatic hydrocarbon.



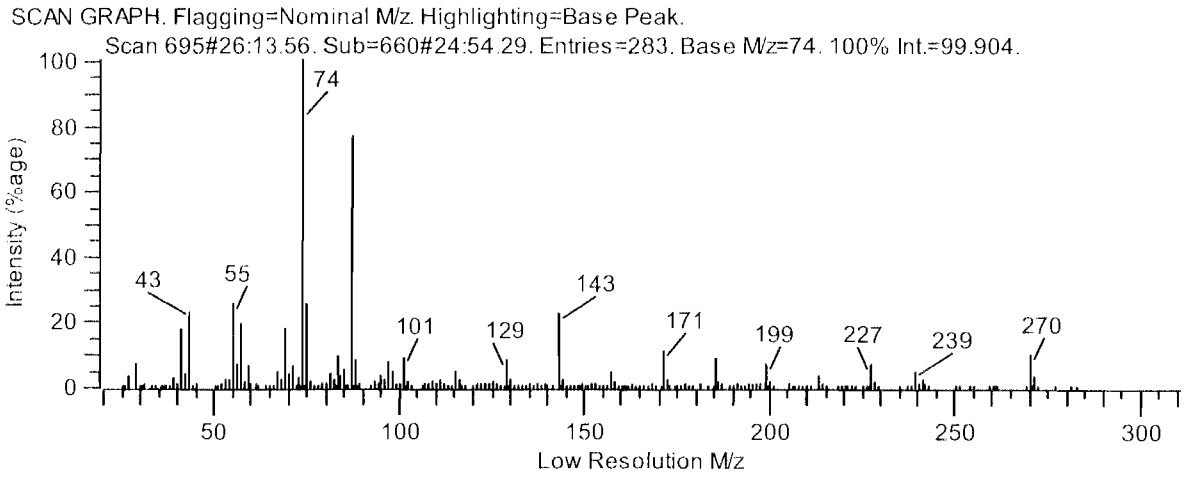
22. EI mass spectrum of a phthalate.



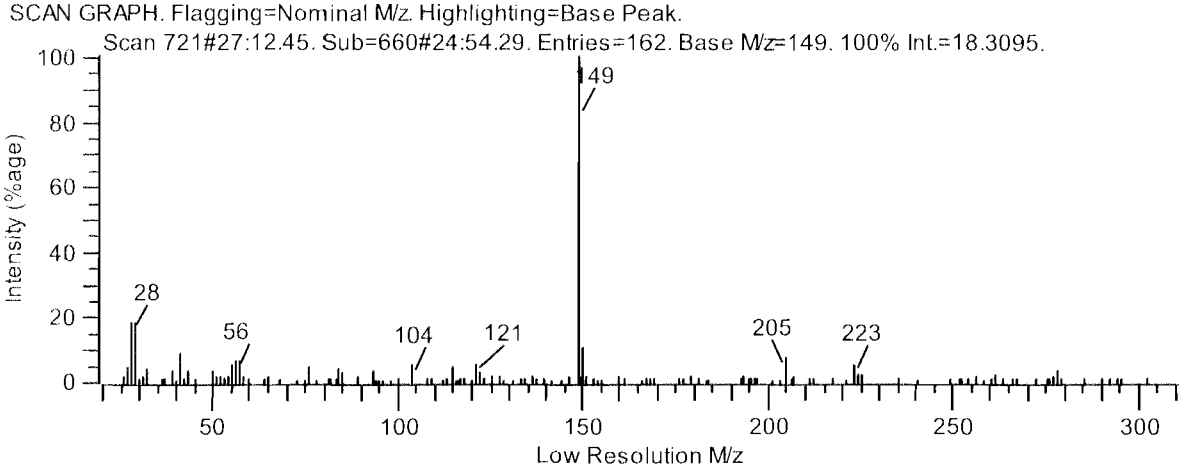
23. EI mass spectrum of an aliphatic hydrocarbon (possibly branched).



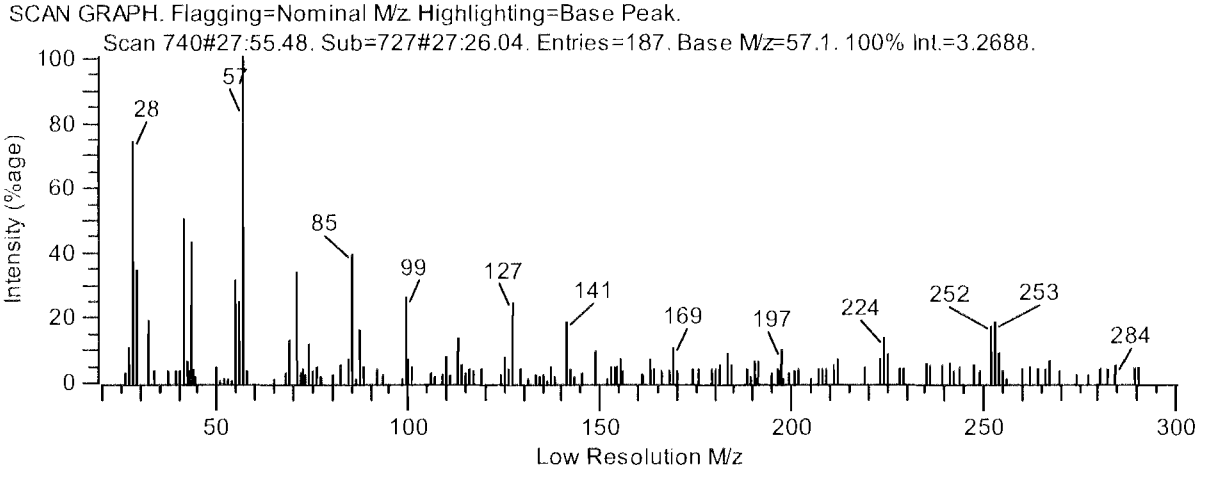
24. EI mass spectrum of nonadecane.



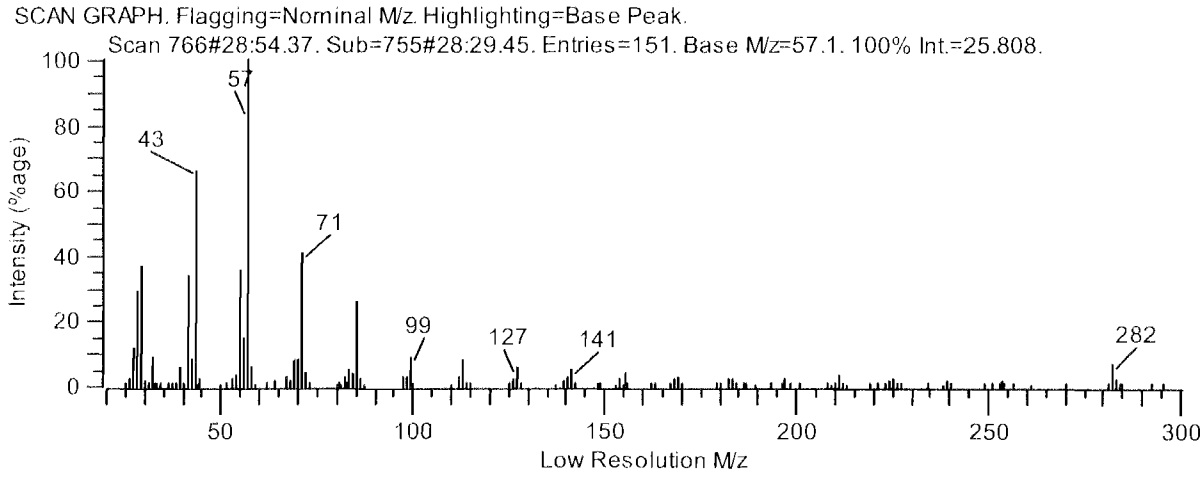
25. EI mass spectrum of methyl hexadecanoate.



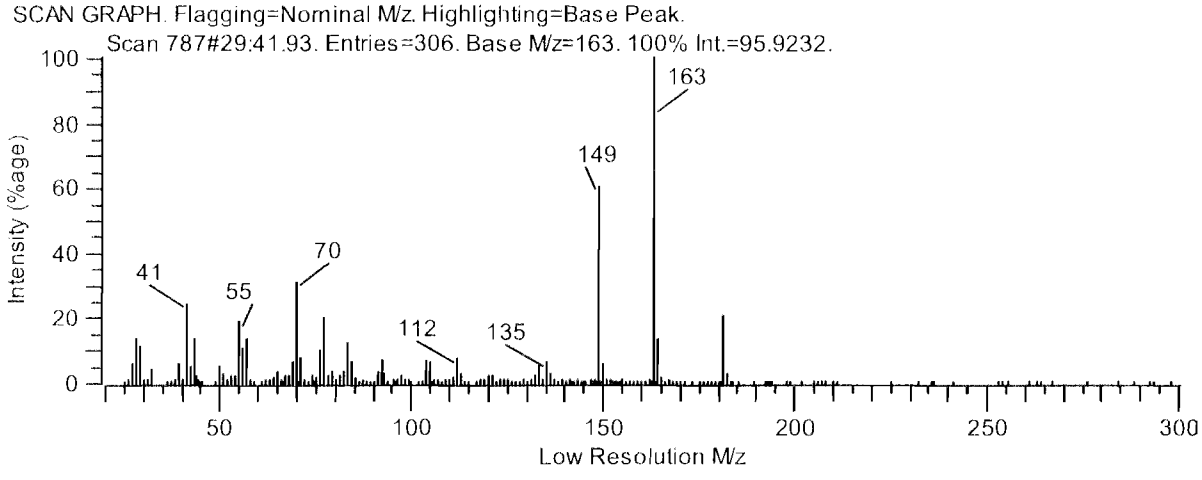
26. EI mass spectrum of a phthalate.



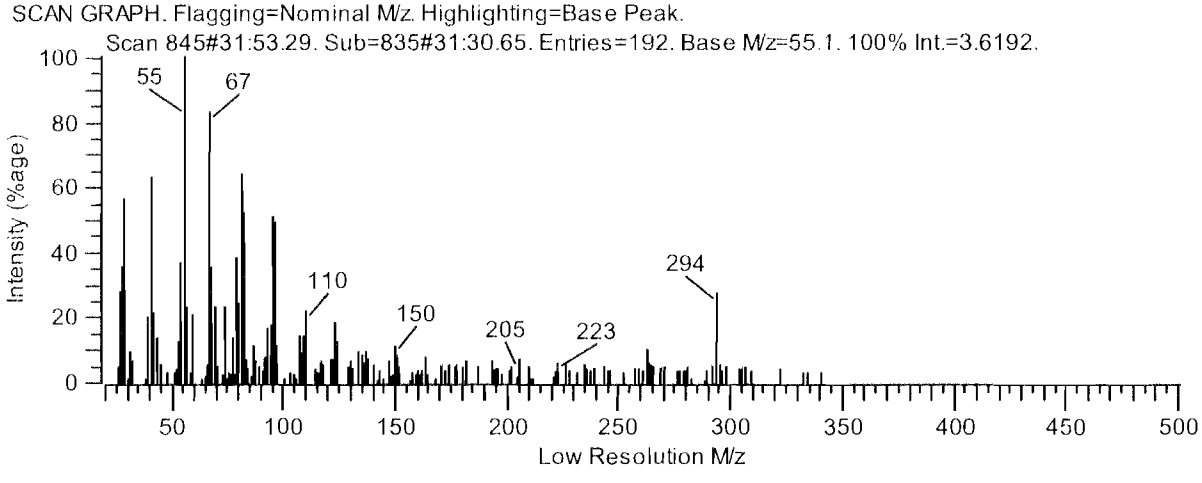
27. EI mass spectrum of an aliphatic hydrocarbon.



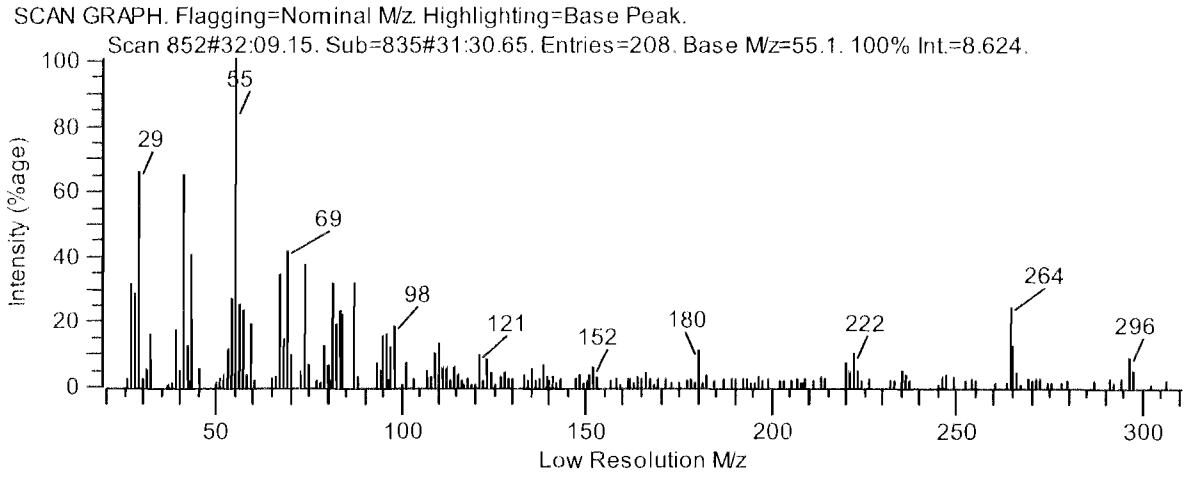
28. EI mass spectrum of icosane.



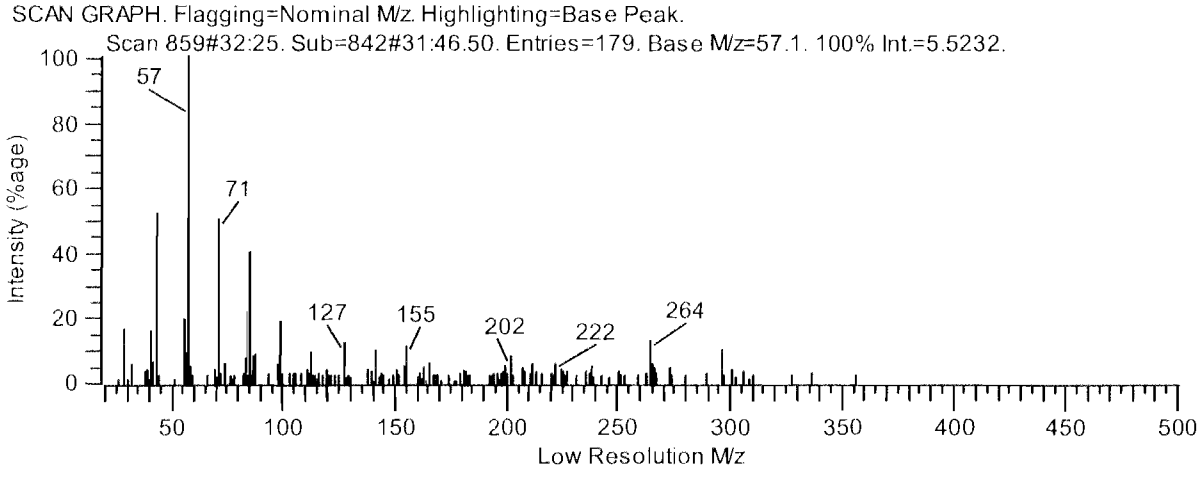
29. EI mass spectrum of a phthalate.



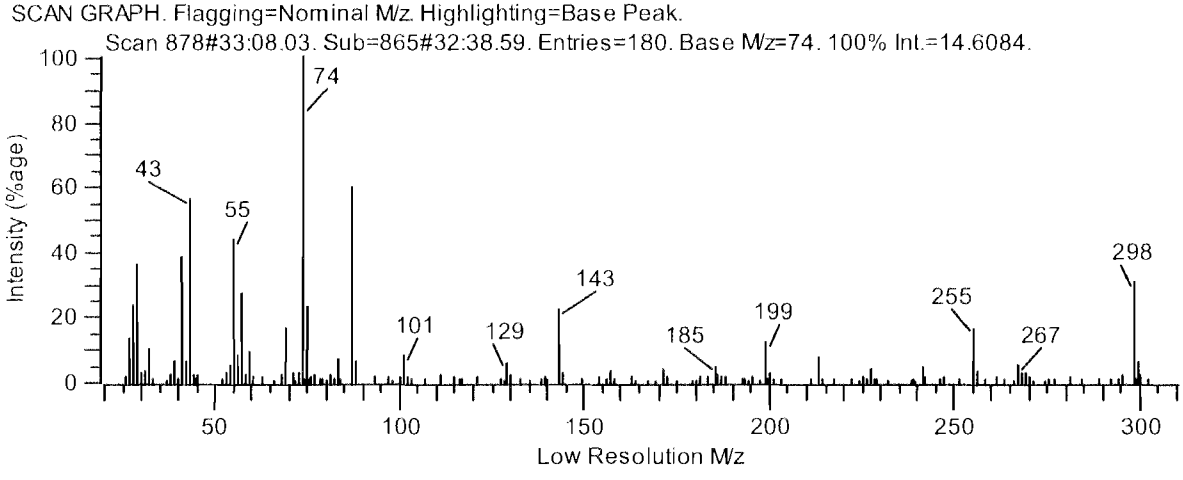
30. EI mass spectrum of methyl (Z,Z)-9,12-octadecadienoate.



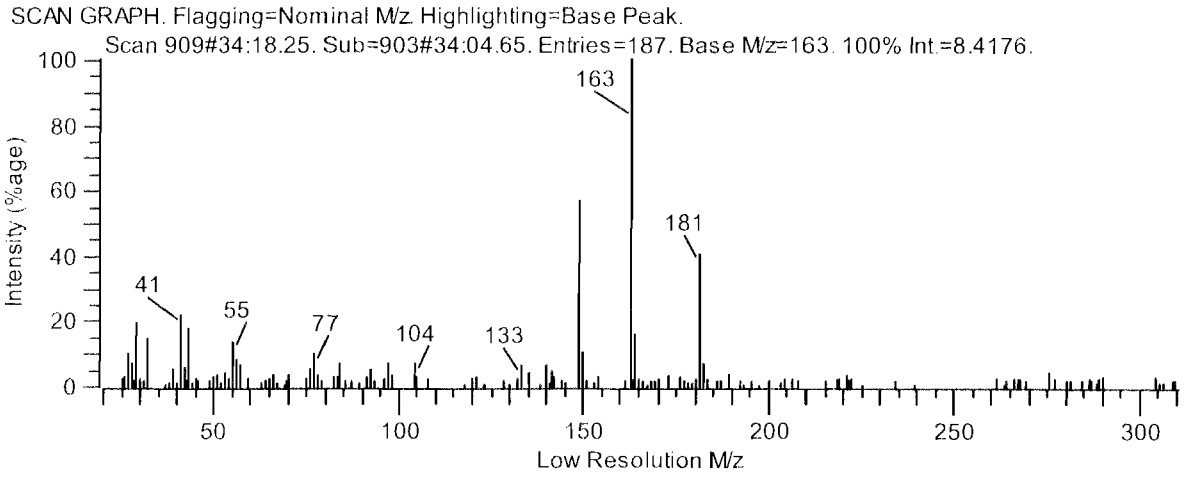
31. EI mass spectrum of methyl Z-9-octadecenoate.



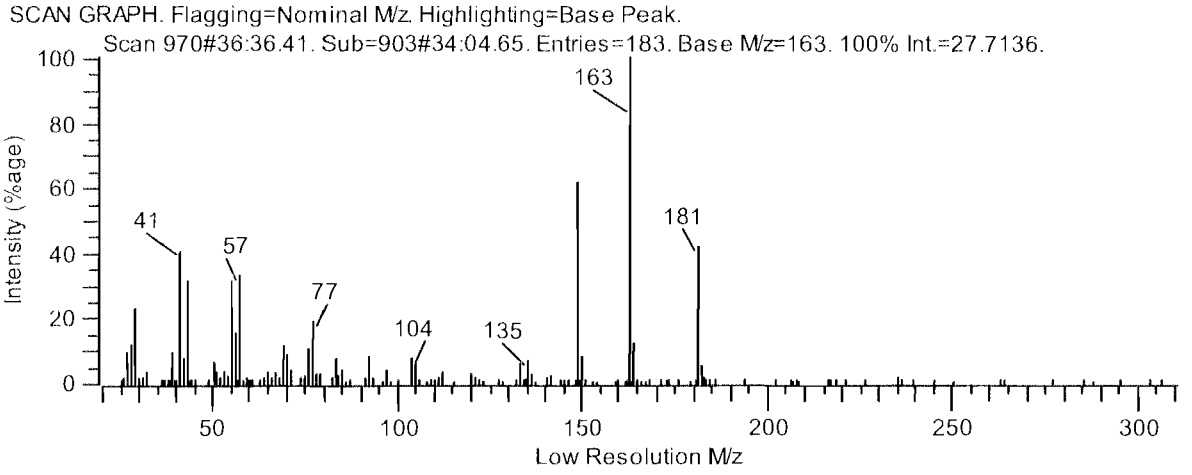
32. EI mass spectrum of an aliphatic hydrocarbon.



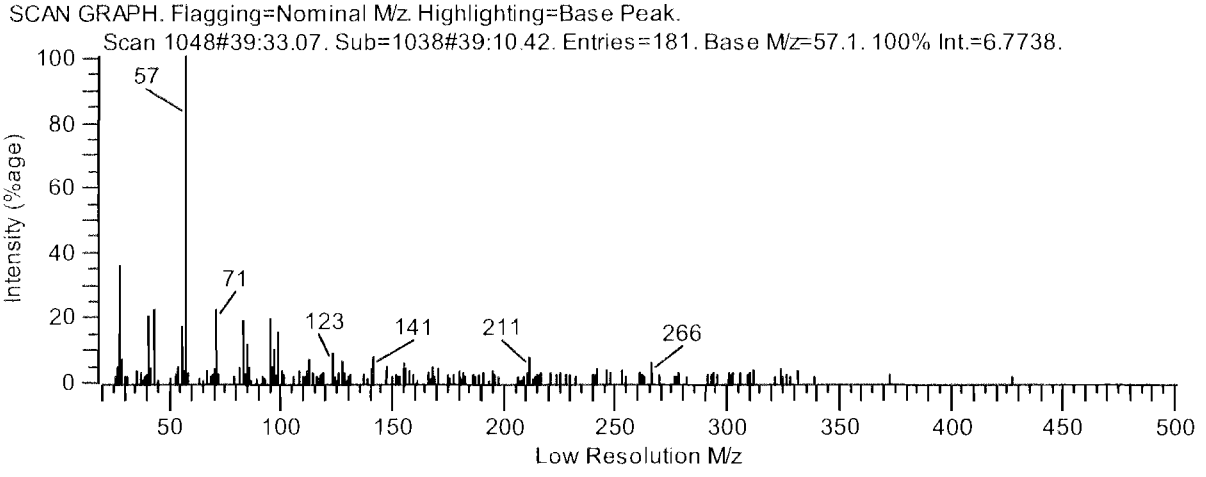
33. EI mass spectrum of methyl octadecanoate.



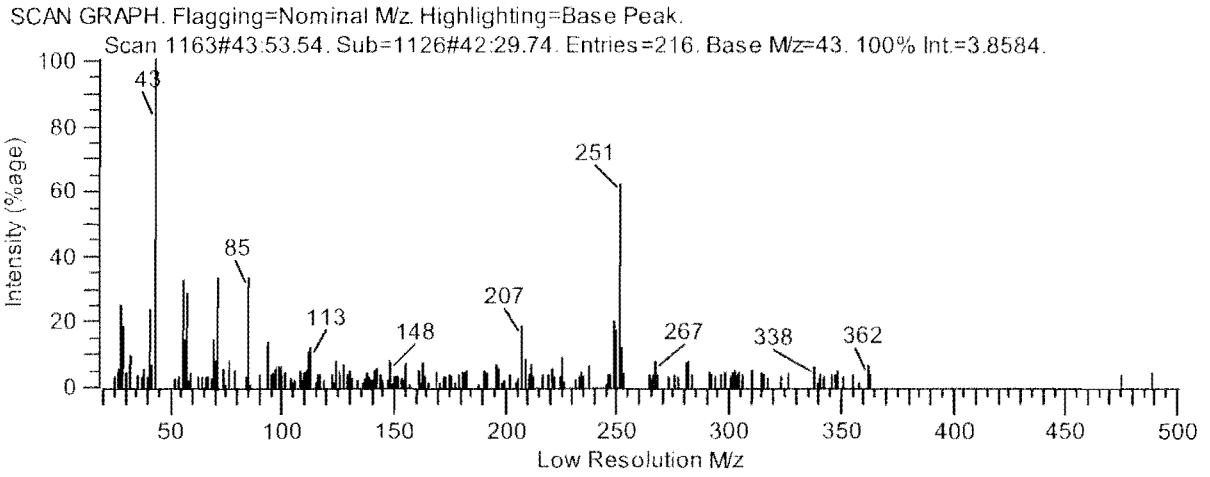
34. EI mass spectrum of a phthalate.



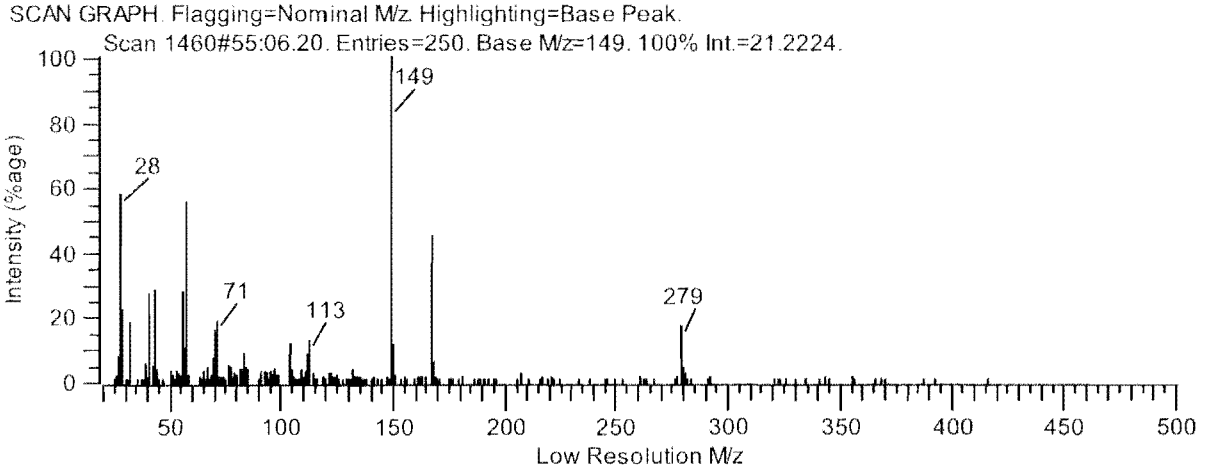
35. EI mass spectrum of a phthalate (the surrounding peaks from 35 min to 39 min have similar mass spectra).



36. EI mass spectrum of an aliphatic hydrocarbon.



37. EI mass spectrum of an aliphatic hydrocarbon (possibly branched).



38. EI mass spectrum of a phthalate.

Reported by:

Dr. Stefan Louw

Gas Chromatography Analysis Report – Summary Page

Submitted by: Denise Saravanakumar (UCT, Dept. of Pharmacology; Prof. Peter Smith)
Analysis date: 29 March 2006
Instrument: HP 5890 Series II Gas Chromatograph
Sample: **C1730E**
Data folder: HPCHEM\2006\DATA\060329

SUMMARY OF RESULTS:

The identities of the following sample components were confirmed by retention time comparison by the co-injection of the sample with reference standard solutions into a gas chromatograph.

Component number	Compound
11	methyl dodecanoate
16	methyl tetradecanoate
20	methyl pentadecanoate
25	methyl hexadecanoate
30	methyl (Z,Z)-9,12-octadecadienoate
31	methyl Z-9-octadecenoate
33	methyl octadecanoate

Reported by:

Dr. Stefan Louw