The isolation of antibacterial compounds from the marine diatom *Phaeodactylum tricornutum*

Ralph Bickerdike

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This Ph.D. is dedicated to my mother Patricia, and brother Leigh, as it would not have been possible without their love and support over the years.

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Multum in parvo

Abstract

A range of extraction and chromatographic methods were used to isolate at least 2 antibacterial compounds in cell lysates of the marine diatom *Phaeodactylum tricornutum* (Bolin). One was not identified or fully characterised, but appeared to be a fatty acid, and was active against a Gram-positive marine bacterium. The other antibacterial factor was found to be a tetrabutyl-ammonium phenolate. This was routinely purified by reversed-phase high performance liquid chromatography from cellular methanol extracts, and was characterised by a series of one, and two-dimensional, nuclear magnetic resonance spectroscopy experiments (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC). The molecular mass of the ammonium cation was determined by electrospray mass spectrometry to be 242.4 Da, but the molecular mass of the phenolate anion was not obtained. Ambiguity remains with regard to the functional group at *para*-substitution on the phenolate. To the best of my knowledge, this is the first report of the isolation of a tetrabutyl-ammonium phenolate from a natural source. It was found to be active against Gram-positive and Gram-negative marine and non-marine bacteria, including a pathogenic strain of methicillin-resistant *Staphylococcus aureus,* but does not appear to be haemolytic against mammalian erythrocytes. As such, it may have an application in pharmacology as a novel antimicrobial agent. The possible role in marine chemical ecology of the production of a tetrabutyl-ammonium phenolate by *P. tricornutum* is discussed.

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Abbreviations

The following abbreviations are used throughout this thesis:

%	percent
× g	times force of gravity
°C	degrees Centigrade
µg	microgram(s)
cm	centimetre(s)
d	day
et al.	et alia (and others)
g	gram(s)
kDa	kilo Daltons
l	litre(s)
M	molar
mg	milligram(s)
min	minute
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
nm	nanometre(s)
OD	optical density
PPFD	photosynthetic photon flux density
SD	standard deviation
sp.	Species
TMS	trimethylsilyl ester
MRSA	methicillin-resistant *Staphylococcus aureus*
TBAP	tetrabutyl-ammonium phenolate
AMP	antimicrobial peptide
Ac.	acetate ($C_2H_3O_2$)
ACN	acetonitrile
TFA	trifluoracetic acid

Chemical formulae and element symbols have their conventional meanings.

Chapter 1

General Introduction

1.1 Eukaryotic microalgae

Eukaryotic microalgae are single-celled, oxygen producing non-vascular organisms considered as part of the kingdom Protista, although some groups may be more closely related to the kingdom Plantae (Lee 1999). The main classes of eukaryotic microalgae generally include; the Bacillariophyceae (diatoms), Dinophyceae (dinoflagellates), Chlorophyceae and Prasinophyceae (both planktonic green algae), Euglenophyceae (euglenoid flagellates), Chrysophyceae and Prymnesiophyceae (both brown coloured phytoflagellates) and the Cryptophyceae (Lee 1999). However, the taxonomic classification of microalgae is still a source of contention. There are also the prokaryotic microalgae, termed cyanobacteria or blue-green algae (Radmer 1996), but for the purposes of this study only the eukaryotic microalgae are to be investigated.

Most eukaryotic microalgae are photoautotrophic in that they obtain their energy directly from the sun using the process of photosynthesis to split water molecules and form complex organic molecules. Eukaryotic microalgae exhibit a wide range of size and organisation from unicells to colonial and filamentous species (Radmer 1996). They are considered to have high structural diversity, often showing dramatic variation in morphology throughout their life cycle (Lee 1999).

Microalgae are highly photosynthetically efficient (Aaronson and Dubinsky, 1982). High growth rates have also been reported for microalgae. Droop (1974) reported growth rates as high as 5.5 divisions per day. It has been estimated that altogether the algae probably account for more than half the total primary production worldwide (Van den Hoek *et al.* 1995). Moreover, the microalgae form the basis of the food chain for more than 70 % of the world's biomass (Andersen 1996; Day *et al.* 1999). Although microalgae are found in abundance within the photic zone of oceans, they have traditionally been regarded as playing a significant ecological role in surface associated habitats (Costerton *et al.* 1987; Characklis and Marshall 1990; Hoagland *et al.* 1993). Thus, microalgae not only make an important contribution to the overall primary productivity of a water mass but have greater, far reaching significance on a global scale.

1.2 Biologically active compounds from eukaryotic microalgae

It is well known that microalgae can produce a wide range of extracellular products, which are released to their surroundings, either actively or passively, whilst living, during senescence or subsequent decomposition after death (Sharp 1977; Hoagland *et al.* 1993; Vasconcelos *et al.* 2002). These products include amino acids and peptides, carbohydrates, polyalcohols, vitamins, enzymes and various toxins (Bell and Mitchell 1972; Hoagland *et al.* 1993).

Historically, since many of these compounds did not appear to resemble the classical primary metabolites they were termed secondary metabolites. However this terminology is deceptive, as it is now known that some of these compounds may contribute as much as primary metabolites to the survival of the organism, reviewed

by Harper *et al.* (2001). These so called 'secondary metabolites' are typically classified by the metabolic pathways by which they are derived, specifically: isoprenoid, acetogenin, amino acid, shikimate, nucleic acid, carbohydrate and mixed biogenesis (Munro and Blunt 1999). A recent review of the phylogenetic distribution of these compounds from marine organisms is given in Harper *et al.* (2001). Many authors have suggested that the release of these compounds may stimulate or inhibit the activities of other organisms (Lucas 1947; Sieburth and Mac, 1968; Bell and Mitchell, 1972; Bell *et al.*, 1974; Al-Ogily and Knight-Jones, 1977; Kogure *et al.*, 1979; Bakus *et al.*, 1986; Hay, 1996; Faulkner, 1998).

The inhibitory activities by microalgae include antigrazing (Shaw *et al.* 1995a), antibacterial (Jorgensen 1962; Berland *et al.* 1972; Cooper *et al.* 1983; Viso *et al.* 1987; Cannell *et al.* 1988), antiviral (Cardellina II *et al.* 1994; Berge *et al.* 1999; Fabregas *et al.* 1999), antifungal (Pesando *et al.* 1979; Viso *et al.* 1987; Kellam *et al.* 1988) and antialgal functions (Proctor 1957a,b; Sharp *et al.* 1979; Chan *et al.* 1980; Goldman *et al.* 1981). Some microalgal species have also been found to be toxic to animals (reviewed by Metting and Pyne 1986; Kim *et al.* 2000). In particular, the potent neurotoxin, domoic acid, is known to be synthesized by species of the diatom *Pseudo nitzsschia* (reviewed by Hasle 2002), and has been associated with the death and injury of marine shore birds and mammals and poses a significant health risk to humans consuming contaminated seafood products (Ferdin *et al.* 2002). The potential significance of extracellular products being used as metabolic substrates should not be overlooked, particularly when it is appreciated that extracellular products can account for 1-20% of the total assimilated carbon by natural microalgae populations (Helleburst 1965; Bell and Mitchell 1972).

1.3 Biotechnological significance of eukaryotic microalgae

The high productivity values and the latest developments in biotechnology have led to the proposed exploitation of numerous commercially important properties of microalgae. Some of the potential applications of these properties include the production of a wide variety of useful products (summarised in Table 1.1). For example, microalgae are attracting great interest as sources of pharmaceuticals and other biologically active compounds as they have the potential to produce complex molecules, which are difficult or impossible to produce by chemical synthesis (reviewed by Borowitzka 1995). Most of the early research has investigated the biological activity of phycotoxins produced by cyanobacteria and dinoflagellates (reviews by Metting and Pyne 1986; Borowitzka 1995; Skulberg 2000). Some interesting activities of cyanobacteria have been shown to include neurotoxic (Skulberg *et al.* 1992), antiviral, and antineoplastic behavior (reviewed by Borowitzka 1995). Eukaryotic microalgae have only recently begun to receive the same attention as the cyanobacteria within this field of applied phycology. This is surprising due to the wide range of eukaryotic strains available in culture collections and the frequent reports of biological activity.

Certain useful ecosystem services of microalgae have also been suggested such as soil conditioning and wastewater nutrient removal (Rodriguez-Lopez 1983; Becker 1994; Craggs *et al.* 1995; Craggs *et al.* 1995; Craggs *et al.* 1997; reviewed by Day *et al.* 1999), biodegradation of organic pollutants (Semple *et al.* 1999). Microalgae have also been used as sentinel organisms for ecotoxicological assays (reviews by Becker, 1994; Day *et al.* 1999) and as breath test diagnostics for biomedical applications (Radmer and Parker 1994).

Table 1.1 Potential uses of microalgae biomass

Use	Citation(s)
Animal feedstuffs	Brune and Walz 1978; Shelef *et al.* 1978; Lincoln *et al.* 1978; Aaronson and Dubinsky 1982; Mahadevaswamy and Venkatamaran 1986; Becker 1988, 1994.
Aquaculture feeds	Paniagua-Michel *et al.* 1987; Pantastico 1987; Herrero *et al.* 1991; Benemann 1992; Dunstan *et al.* 1993; Becker 1994; Raymond and Maxey 1994; Muller-Feuga 2000.
Fine chemicals	Borowitzka *et al.* 1984; Hall and Rao 1989; Becker 1994; Borowitzka 1995.
Fuel (hydrocarbons)	Birch and Bachofen 1988; Hall and Rao 1989; Becker 1994.
Fuel (indirect)	Becker 1994.
Glycerol production	Chen and Chi 1981; Becker 1994.
Integrated aquaculture	Ryther *et al.* 1972; Proulx and de la Noue 1985a,b; Phang 1990; Becker 1994.
Mariculture feeds	Yufera 1990; Brown 1991; McLaughlin 2001.
Metal biosorption	Volesky 1990; Whiston 1996.
Nanotechnology	Parkinson and Gordon 1999.
Oils	Borowitzka 1988; Becker 1994.
Organic fertilisers	Rodriguez-Lopez 1983; Lembi and Waaland 1988; Becker 1994.
Pharmaceuticals	Borowitzka *et al.* 1984; Burton and Ingold 1984; Metting and Pyne 1986; Dunstan *et al.* 1993; Becker 1994; Borowitzka 1995.
Pigments	Aasen *et al.* 1969; Ben-Amotz and Avron 1982; Becker 1994; Borowitzka 1995.
Single cell protein (human consumption)	Cook 1962; Moraine *et al.* 1979; Becker 1988, 1994.
Stable isotope biochemicals	Radmer and Parker 1994.
Vitamins	Lem and Glick 1985; Richmond 1986; Fujimoto 1990; Becker 1994; Running *et al.* 1994; Borowitzka 1995.
Wastewater treatment	Craggs *et al.* 1995, 1997.

Although microalgae show great potential for exploitation of the products and processes described above, commercial microalgae production is still considered to be in its infancy and restricted to very few species producing high-value health food supplements or pigments (reviews by Laing 1991; Becker 1994; Radmer and Parker 1994, Radmer 1996; Day 1999). Contrary to commercial production, current

scientific research in microalgal biotechnology is focusing on the development of new products for mass commercial markets as opposed to the health food markets (reviews by Parker *et al.* 1994; Borowitzka 1995; Apt and Behrens 1999; Richmond 2000). In addition, the optimisation of culture conditions for more economical production of useful products is continually being studied and has recently started to employ new scientific disciplines such as genetic engineering (reviews by Raymond and Maxey 1994; Running *et al.* 1994; Kroger 2001).

The volume of literature where genetic engineering has been reported for the advancement of microalgal biotechnology has increased considerably over the last few years (Apt *et al.* 1994). Reproducible genetic transformation systems have been developed for a few microalgal species, including the green flagellate *Chlamydomonas reinhardtii* (reviewed by Kindle and Sodeinde 1994), the related colonial species *Volvox carteri* (Schiedlmeier *et al.* 1994), the diatoms *Cyclotella cryptica* and *Navicula saprophila* (Dunahay *et al.* 1995) and *Phaeodactylum tricornutum* (Zaslavskaia *et al.* 2000). Possibly the most significant achievement was the artificial introduction of DNA into diatom cells for trophic conversion. Zazlavskaia *et al.* (2001) showed that the obligate photoautothroph, *Phaeodactylum tricornutum,* could be genetically engineered to utilise exogenous glucose in the absence of light through the induction of a gene encoding a glucose transporter (*glut1* or *hup1*). This advance enables the potential use of non-light dependant fermentation technology for the large-scale commercial exploitation of naturally photoautotrophic microalgae for a range of useful products (Apt and Behrens 1999; Kroger 2001; Zazlavskaia *et al.* 2001). This is of particular interest for the production of

pharmaceutical compounds, which are often produced in very low concentrations by microalgae so are expensive to produce by light-dependant culture.

1.4 Antimicrobial activity by eukaryotic microalgae

One of the first reports of antimicrobial activity from a microalga was by Pratt and Fong (1940). In this paper, a crude extract of *Chlorella vulgaris* cells was shown to be auto-inhibitory (Pratt and Fong 1940). The active component, named chlorellin was later identified to be a mixture of fatty acids that were auto-inhibitory and antibacterial against Gram-positive and Gram-negative bacteria (Pratt *et al.* 1944). An increasing amount of research has followed this discovery. The early work was ecological, concerning the interactions between microalgae and other organisms or the regulation of the stimulating / inhibiting substance (Steeman-Nielsen 1955; Jorgensen 1956; Proctor 1957a, b; Sieburth and Mac 1959; Jorgensen and Nielsen 1961; Jorgensen 1962; Droop and Elson 1966; Duff *et al.* 1966; Pratt 1966; Droop 1968; Sieburth and Mac 1968; Bell and Mitchell 1972; Kroes 1972; Bell *et al.* 1974; Elbrachter 1976; Keating 1976; Kayser 1979; Kogure *et al.* 1979; Sharp *et al.* 1979; Chan *et al.* 1980; Wium-Andersen *et al.* 1982; Bakus *et al.* 1986; Cooksey and Cooksey 1988; Kellam *et al.* 1988; Kellam and Walker 1989; Shaw *et al.* 1995a; Shaw *et al.* 1995b; Hay 1996; Garson 2001; Steinke *et al.* 2002). However, very few studies were able to unequivocally link the interaction to a specific compound. More recent studies have tended to use a more applied approach where the active compounds are isolated, characterised and biotechnological potential studied (Aubert *et al.* 1979; Reichelt and Borowitzka 1984; Cooper *et al.* 1985; Cohen 1986; Metting and Pyne 1986; review by Boney 1989; Gerwick *et al.* 1994; Direkbusarakom *et al.* 1997; Tringali 1997; Naviner *et al.* 1999; Skulberg 2000).

1.5 Antimicrobial peptides: A likely candidate produced by microalgae

As stated above, the general consensus is that eukaryotic microalgae do contain compounds with antimicrobial activity and that these compounds may have a role in chemical defence (Steeman-Nielsen 1955; Sieburth and Mac 1959; Conover and McN. Sieburth 1964; Jorgensen and Nielsen 1961; Droop and Elson 1966; Duff *et al.* 1966; Sieburth and Mac 1968; Bell *et al.* 1972, 1974; Provasoli and Pintner 1980; Wium-Andersen *et al.* 1982; Kellam and Walker 1989). However, no universal group of chemical compounds has been identified. As yet, antimicrobial activity by microalgae seems to be highly strain specific and governed by environmental conditions. This fundamental gap in our knowledge raises the questions; does chemical defence by eukaryotic microalgae play a significant role in controlling community structure? If so, what are the compound(s) responsible? It might be useful to step back and approach the question from a different perspective by asking if eukaryotic microalgae have acquired, through evolution, an active chemical defence, that include compounds that are antimicrobial and of potential biotechnology value?

There is one group of antimicrobial agents that eukaryotic microalgae might reasonably be expected to produce and these are the antimicrobial peptides. Recently with the aid of improved biochemical techniques, over 500 different antimicrobial peptides have been identified from living organisms, and new forms are continually being discovered, reviewed by Zasloff (2002). They have been isolated from vertebrates (Lehrer *et al.* 1991a; Nicolas and Mor 1995), invertebrates (Cocianich *et al.* 1994; Humphreys and Reinherz 1994), fungi (Christophersen *et al.* 1999; Malmstrom 1999), bacteria (McCarthy *et al.* 1994; Kleerebezem *et al.* 1997),

cyanobacteria (Patterson *et al.* 1994; Skulberg 2000) and higher plants (Cammue *et al.* 1994; Broekaert *et al.* 1997). Antimicrobial peptides are therefore considered to be widespread throughout the kingdoms as a ubiquitous defence strategy that combats attack by microorganisms.

It also now appears that a single species may produce a cocktail of antimicrobial peptides of different structural classes (reviews by Epand and Vogel 1999; Zasloff 2002). This diversity of antimicrobial peptides produced by a single species has been suggested to explain why microbes have not been successful in resisting the activity of antimicrobial peptides throughout evolution as, although induced resistance has been reported for some individual antimicrobial peptides, it is unlikely that a microbe is able to be instantaneously resistant to multiple antimicrobial peptides of different modes of action before becoming susceptible (Zasloff 2002). Although there is great diversity in the sequences of antimicrobial peptides, even in a single or closely related species, there is significant conservation of amino acid sequences in the preproregion of the precursor molecules or in secondary structures of some peptides from different groups within a kingdom; indicating that they have been conserved throughout evolution (reviews by Medzhitov and Janeway 1998; Simmaco *et al.* 1998; Broekaert *et al.* 1997).

So far there has only been one report in the literature of a eukaryotic microalga (*Stichochrysis immobilis*) producing a peptide with antibacterial activity (Berland *et al.* 1972). The molecular mass of the peptide was deduced by amino acid analysis to be approximately 3.4 kDa (Berland *et al.* 1972), but no sequence information was reported or has been published since.

With the current occurrence of antibiotic-resistant pathogens (Cooksey 1991; Lohner and Staudegger 2001) there is an increasing demand for new antimicrobial agents (Lancini *et al.* 1995; Mann and Crabbe 1996; Lohner and Staudegger 2001). The isolation and characterisation of an antimicrobial peptide from a microalga could therefore have great potential from a pharmaceutical point of view. In addition, with the aid of molecular techniques, the information gained from the amino acid sequence of an antimicrobial peptide could be used to design molecular probes, which could then be used to study the effects of environmental parameters on expression. The implications of this would be very interesting, as it would go some way to elucidate the significance of microalgal chemical ecology within the environment.

1.6 Aims and objectives of study

The broad objective of this study was to investigate the production of antibacterial activity by microalgae and to isolate the compound(s) responsible for the activity observed. Upon the isolation of an antibacterial compound, the chemical nature was to be characterised and its potential value as a novel antimicrobial agent assessed.

It is hypothesised that microalgae might express one or more peptides with antimicrobial properties. Specifically, the study was aimed at isolating and characterising at least one antimicrobial peptide from a model species of microalgae. This information would facilitate studies of antimicrobial peptide expression under a range of environmental and biochemical conditions. To this end, an empirical approach of protein purification techniques was to be used to fractionate extracts from a model species of microalgae whilst retaining biological activity of the sample.

1.7 Model species for investigation

The marine diatom, *Phaeodactylum tricornutum* (Bohlin), was chosen as the model species to search for antimicrobial agents. Since it's original isolation, numerous strains have been isolated from temperate waters (Allen and Nelson 1910; Craggs 1994). It is pleomorphic in that it can be observed as ovoid, fusiform, triradiate and cruciform morphotypes (Figure 1.1) and can be found in different form in freshwater (Yongmanitchai and Ward 1991) marine littoral, planktonic, and benthic habitats (reviewed by Round *et al.* 1990). However, it is considered a relatively rare component of most marine phytoplankton assemblages (Guillard and Kilham 1977; Nelson and Guillard 1979; Rushforth *et al.* 1988). The taxonomic position of *P. tricornutum* is uncertain, but in light of its unique characteristics it has been placed in a new suborder, Phaeodactylineae, of which it is the only member (Lewin 1958; Round *et al.* 1990). A recent molecular phylogenetic study of six naviculoid diatoms based on 18S rDNA sequences revealed that *P. tricornutum* showed close affinity to *Eolimna minima* (Beszteri *et al.* 2001).

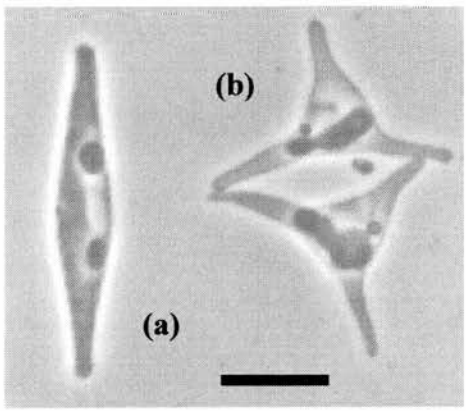


Figure 1.1 The fusiform (a) and triradiate (b) morphotypes of *Phaeodactylum tricornutum*. Scale bar = 10 μm, (× 50 objective, × 12.5 periplan).

1.7.1 Pleomorphism in *P. tricornutum*

The unique ability of this diatom to be pleomorphic has stimulated much interest and discussion in the early research (Wilson 1946; Lewin *et al.* 1958; Cooksey and Cooksey 1974). Lewin *et al.* (1958) reported that the triradiate morphotype is rare and arose as atypical forms of the fusiform morphotype. The oval morphotypes were observed to arise as endospores within a fusiform morphotype (Lewin *et al.* 1958). These observations were later confirmed by Gutenbrunner *et al.* (1994). Lewin *et al.* (1958) also suggested that the oval and fusiform morphotypes are the typical prevalent stages in the life cycle, although the complete life cycle was not understood. Interestingly, the oval morphotype was found to predominate when grown on an agar surface, whilst the fusiform morphotype predominated in liquid culture indicating that the oval morphotype has the ability to 'spread over' an agar surface due to its unique ability to produce mucilage compared to the other morphotypes (Lewin *et al.* 1958). By contrast, Wilson (1946) showed that growth in contact with a surface did not favour oval morphotypes, whereas conditions of "good" illumination did. However, Cooksey and Cooksey (1974) found that oval morphotypes are able to grow in liquid culture if the calcium content of the medium is below 15 mg l^{-1}. When the calcium concentration was increased, the fusiform morphotype then developed (Cooksey and Cooksey 1974). Interestingly, no triradiate morphotypes were observed during the experiments of Cooksey and Cooksey (1974), whereas Lewin *et al.* (1958) and Wilson (1946) commonly observed this cell type. Both of these authors used seawater based media with much higher calcium concentration than that used by Cooksey and Cooksey (1974). Furthermore, the calcium content used by Cooksey and Cooksey (1974) is

significantly lower than the expected calcium content of natural seawater at 35.325 ‰ salinity, 25°C of 400 mg l^{-1} (Sverdrup *et al.* 1970). In addition, production of the triradiate morphotype was found to be promoted by the addition of tryptone (Lewin *et al.* 1958). These findings indicate that dominance of a cell morphotype in liquid culture appears to be in response to specific nutrient availability.

Each morphotype differs in terms of silica organisation (Lewin *et al.*, 1958; Borowitzka, 1977; Borowitzka and Volcani 1978; Round, 1990; Lewin *et al.* 1958) and production of mucilage (Borowitzka and Volcani 1978; Reimann and Volcani 1968). In addition, Gutenbrunner *et al.* (1994) found that the polypeptide composition of the oval and fusiform morphotypes were slightly different when comparing protein extracts resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Furthermore, a phenotype-restoration experiment showed that the oval type readily re-established to the fusiform morphotype and also the characteristic protein pattern (Gutenbrunner *et al.* 1994). These results indicate that the pleomorphic phenotypes of *P. tricornutum* are not only the consequence of specific gene expression, but also the result of significant, general post-translational modifications (Gutenbrunner *et al.* 1994).

1.7.2 *P. tricornutum* as a model species in other studies

P. tricornutum has often been used as an experimental model species for fundamental research in laboratory-based microalgal phycology (Lewin *et al.* 1958; Beardall and Morris 1976; Fabregas *et al.* 1997; Alwyn and Rees 2001; Scala *et al.* 2002), and in mass culture studies (Raymont and Adams 1958; Ansell *et al.* 1963a; Ansell *et al.* 1963b; Ansell *et al.* 1964; Goldman and Stanley 1974; Goldman and

Ryther 1976; Goldman 1979; Goldman *et al.* 1982a; Goldman *et al.* 1982b; Parrish and Wangersky 1987). The biochemistry and environmental requirements of *P. tricornutum* have been widely studied by many workers (Ford and Percival 1965a, b; Hayward 1968; Griffiths 1973; Beardall and Morris 1975; Glover *et al.* 1975; Holdsworth and Colbeck 1976; Styron *et al.* 1976; Glover 1977; D'Elia *et al.* 1979; Nelson and Guillard 1979; Fanuko 1981; Terry *et al.* 1983; Fawley 1984; Geider *et al.* 1985; Fawley and Grossman 1986; Geider *et al.* 1986; Owens 1986; Owens and Wold 1986; Brown 1991; Behrenfield *et al.* 1992; Yongmanitchai and Ward 1992; Geider *et al.* 1993; Yongmanitchai and Ward 1993; Larson and Rees 1994; Spooner *et al.* 1994; Ting and Owens 1994; Johnston and Raven 1996; Larson *et al.* 1996; Kudo *et al.* 2000). This extensive use of *P. tricornutum* is mainly due to its ease of culture under laboratory culture conditions (Lewin *et al.* 1958). Apart from the already high level of knowledge about the biochemistry of this alga, there is also some limited evidence, for the production of compounds with antimicrobial activity, making it a strong candidate for the search for antibacterial compounds.

1.7.3 Allelopathy by *P. tricornutum*

The early research focused on how this species was able to dominate enriched cultures (Ansell *et al.* 1963a; D'Elia *et al.* 1977), where it was often considered an undesirable "weed" species (Goldman and Stanley 1974; Goldman and Ryther 1976; D'Elia *et al.* 1979). Goldman and Stanley (1974) suggested that the dominance of this species over other phytoplankton was due to allopathic interactions. Later, Goldman and Ryther (1976) found that temperature influenced the dominance of *P. tricornutum* in mass culture of marine phytoplankton, although these authors

suggested that temperature might have influenced the excretion of metabolites that were toxic to other phytoplankton. D'Elia *et al.* (1979) and Nelson and Guillard (1979) observed no allelopathic interaction between *P. tricornutum* and the diatom *Thalassiosira pseudonana* in mixed culture. Instead, they found the dominance of *P. tricornutum* was attributed to its ability to maintain productivity in silica-limited cultures or at low light intensity. By contrast, Sharp *et al.* (1979) found that cell-free filtrate from a batch culture of *P. tricornutum*, caused an initial lag phase and reduced terminal population density for the alga *T. pseudonana*. Sharp *et al.* (1979) suggested that this may have been due to an allelopathic interaction. However, Goldman *et al.* (1981) found no allelopathic interaction between *P. tricornutum* and *Dunaliella tertiolecta* when filtrates of either microalga were mixed with whole cultures of the other species. Instead, the unique ability of *P. tricornutum* to tolerate pH levels above 9.5 may be the leading factor for the dominance of *P. tricornutum* in intensive phytoplankton cultures (Goldman *et al.* 1981), as confirmed later by Goldman *et al.* (1982a,b).

Pinter and Altmeyer (1979) demonstrated that *P. tricornutum* releases a protein or protein complex into the culture medium that binds free vitamin B_{12}. This was shown to have inhibitory activity against other microalgal species, although was described as competitive, being reversed by adding B_{12} (Pinter and Altmeyer 1979). However, in some cases, the inhibition was not overcome by adding B_{12}, indicating that other inhibitory factors were also present (Pinter and Altmeyer 1979). These other inhibitory factors were shown to be nonpolar, heat-stable, with a molecular weight of less than 10 kDa, but were not isolated nor characterised further (Pinter and Altmeyer 1979).

1.7.4 Antibacterial activity by *P. tricornutum*

P. tricornutum has occasionally been used in studies where microalgae have been screened for antibacterial activity. Duff *et al.* (1966) examined antibacterial activity of *P. tricornutum* and reported strong activity against 8 of 9 marine bacterial strains and 1 of 14 terrestrial bacterial strains using organic solvent extracts of *P. tricornutum* cells grown to late logarithmic phase. The compound responsible for the antibacterial activity observed was not isolated.

Brown *et al.* (1977) reported strong antibacterial activity from a cell suspension and cell-free culture supernatant of *P. tricornutum* using *Escherichia coli* strain M13 as test bacterium. The cleavage of dimethyl-β-propiothetin upon cell death of the alga, liberating acrylic acid to the culture medium was suggested as the active agent (Brown *et al.* 1977). However, acrylic acid was not isolated from the cell-free culture supernatant and tested separately at the concentration at which it occurs in the culture medium. In addition, the fact that the anticoliform agent was able to pass through Visking dialysis tubing to affect the bacterium, indicated that the active factor was of low molecular weight (Brown *et al.* 1977).

Cooper *et al.* (1983) subsequently prepared aqueous and organic solvent phases of an organic solvent cell extract of *P. tricornutum* grown to early logarithmic phase, late logarithmic phase or stationary phase. Each of the extract phases was tested for antibacterial activity against 4 terrestrial and 6 marine strains of bacteria. In addition, an extract of the cell-free culture media at each growth phase of *P. tricornutum* were also tested for antibacterial activity. The aqueous phase of the cell extract from *P. tricornutum* in log phase of growth was more active against marine

bacteria than the aqueous phase of the cell extract from *P. tricornutum* at stationary growth (Cooper *et al.* 1983). By contrast, the lipophilic phase of the extract was mostly active when *P. tricornutum* was at stationary growth phase (Cooper *et al.* 1983). For the cell-free culture media of *P. tricornutum* at stationary growth phase, strong activity was detected against *Pseudomonas aeruginosa* (Cooper *et al.* 1983). These findings indicate that the spectrum of activity of *P. tricornutum* is related to the time of harvest and nature of the extract preparation (Cooper *et al.* 1983). The active component was later reported to be a mixture of fatty acids (Cooper *et al.* 1985). However, the extraction method used for these studies excluded components less than 10 kDa, so may not have included low weight antimicrobial agents such as peptides.

Kellam and Walker (1989) studied the antibacterial activity of methanol and hexane cell extracts of two strains of *P. tricornutum* grown to stationary phase against six strains of bacteria. They also tested extracts of the cell-free culture media after the cells were harvested for antibacterial activity. Strong antibacterial activity was found against *Staphylococcus aureus* and *Bacillus subtilis* with hexane extracts of both strains of *P. tricornutum* (Kellam and Walker 1989). Slight antibacterial activity was also observed against *S. aureus* using a methanol extract of one strain of *P. tricornutum*, although no antibacterial activity was detected for the other *P. tricornutum* strain tested; indicating that the production of antibacterial compounds might be strain specific (Kellam and Walker 1989). No antibacterial activity was observed with the cell-free culture media for any of the other bacteria tested, or with the cell extracts tested against *Escherichia coli*, *Streptococcus faecalis*, *Klebsiella pneumoniae* or *P. aeruginosa.* Again, active compounds were not isolated.

1.7.5 Antifungal activity by *P. tricornutum*

P. tricornutum has also been screened for antifungal activity. Pesando *et al.* (1979) found that an aqueous cell extract of *P. tricornutum* had strong antifungal activity against the fungus *Epidermophyton floccosum* (Dermatophyte), although the active compound was not isolated. Later, Kellam *et al.* (1988) demonstrated that a hexane cell extract of *P. tricornutum* showed antifungal activity against a known spoilage fungus, *Mucor mucedo* (Zygomycotina). However, no antifungal activity was detected for cell-free culture filtrates or for methanol cell extracts of *P. tricornutum* (Kellam *et al.* 1988). As with most other studies of this type the active component was neither isolated nor characterised.

1.7.6 Other biologically active compounds from *P. tricornutum*

Other biologically active compounds produced by *P. tricornutum* include long-chain, polyunsaturated fatty acids, such as the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Yongmanitchai and Ward 1991, 1992, 1993; Chrismadha and Borowitzka 1994). EPA is widely used in aquaculture feeds or for pharmaceutical use (reviewed by Borowitzka 1988; Dunstan *et al.* 1993). At present, the commercial source of EPA and DHA is marine fish and their oil although these fatty acids are not synthesised by fish, but originate from their diet of phytoplankton (Yongmanitchai and Ward 1993). Yongmanitchai and Ward (1991) reported that a freshwater strain of *P. tricornutum* is considered a promising source of EPA, as it contains EPA as ca. 40 % of it's total fatty acids. A summary of the biological activities reported for *P. tricornutum* is shown in Table 1.2.

Table 1.2 Biological activity by *P. tricornutum*.

Bioactivity	Compound isolated	Citation(s)
antialgal	glycoprotein (Vitamin B_{12}-binder)	Pinter and Altmeyer 1979.
antifeeding (copepod)	apo-carotenoids 1-4.	Shaw *et al.* 1995a, b.
antibacterial	not isolated	Duff *et al.* 1966.
antibacterial	fatty acid; trimethylsilyl esters	Cooper *et al.* 1983; Cooper *et al.* 1985.
antibacterial	not isolated, metal complexing ligands suggested	Vasconcelos *et al.* 2002.
antibacterial	not isolated, acrylic acid suggested	Brown *et al.* 1977.
nicotinic block	not isolated	Borowitzka 1995.
central nervous system depressant, muscle relaxant	not isolated	Villar *et al.* 1992.
antiaggregatory, antithrobolytic, antiinflammatory	omega-3 fatty acids	Yongmanitchai and Ward 1991.
antifungal	not isolated	Pesando *et al.* 1979.
antiviral	not isolated, sulphated polysaccharides suggested	Fabregas *et al.* 1999.
anti-inflammatory, analgesic, free radical scavenging	not isolated	Guzman *et al.* 2001.
peroxidase	not isolated	Murphy *et al.* 2000.
metal chelation	not isolated	Morelli and Scarano 1995.

1.8 Factors to consider when investigating antimicrobial activity by microalgae

From the above review, it is clear that the existing knowledge about the ecology, biochemistry and biological activity of *P. tricornutum* makes it an ideal candidate for further investigation to isolate and characterise compounds responsible for antimicrobial activity. However, there are several aspects that must be considered when studying antimicrobial activity by microalgae. Many of the studies, highlighted above, were conducted using laboratory maintained uni-algal cultures, and can therefore be open to the argument that such studies do not necessarily reflect what could be happening in the environment. The phase of growth in which the microalga is harvested for the isolation of antimicrobial compounds is important. If antimicrobial compounds are sought from 'healthy' actively growing microalgae, cells in logarithmic phase should be used so as not to include compounds released by the microalga during stationary phase or subsequent cell decomposition. It should be appreciated however, that many of the bioactive compounds produced by microalgae have been reported to mainly occur during late exponential / stationary phase (Pratt *et al.* 1945; Pinter and Altmeyer 1979; Harrison *et al.* 1990; Morton and Bomber 1994; Borowitzka 1995; Skulberg 2000). In addition, the algal strain (Okami 1986; Kellam and Walker 1989), growth media (Cannell *et al.* 1988) and culture conditions (Skulberg 2000; Carmichael, 1986) are also known to affect the production of antimicrobial agents by microalgae.

There is conflicting discussion regarding the use of axenic microalgal cultures for studying antimicrobial activity. Some authors suggest that axenic cultures must be used otherwise it is unknown if the active substance is produced by the alga or by

other organisms present in the culture (Borowitzka, 1995). By contrast, other authors suggest that the production of antimicrobial agents by microalgae may be in response to the presence of other microbes (Hay 1996).

Another aspect for consideration is that if the active compound is not identified and characterised it cannot be certain that the compound is produced by the microalga in its active form or whether the compound has been altered by the extraction method in such a way as to become antimicrobial. For chemical ecologists, the main consideration is that although a substance isolated from a concentrated cell extract or culture filtrate is found to be antimicrobial, its original cellular concentration may be so low that it is ecologically ineffective (Duff *et al.* 1966).

This isolation and characterisation of the antibacterial compounds present in cells of *P. tricornutum* is vital. With this information the cellular concentration of an antibacterial compound can be calculated and antibacterial activity at this concentration determined to elucidate its potential in chemical defence from an ecological perspective. Moreover, if the chemical nature of an antibacterial compound is known, the cell-free culture media of *P. tricornutum* in logarithmic growth phase can be studied to ascertain if it is being released by actively growing cells into their surroundings. This could then confirm whether *P. tricornutum* is able to influence microbial populations when grown in culture. This may have far reaching implications for the ecological significance of microalgae in the environment.

1.9 Specific aims of study

1. Determine antibacterial activity of *P. tricornutum* cells using a range of extraction solvents/buffers.
2. Use an empirical approach of protein purification techniques to fractionate extracts of *P. tricornutum* whilst retaining biological activity.
3. Isolate antibacterial agents in fractionated extracts of *P. tricornutum*. In particular, isolate at least one antimicrobial peptide from this alga.
4. Characterise the chemical and biological nature of isolated antibacterial compounds.

Chapter 2

Standardisation of culture conditions and selection of extraction methods for antibacterial activity in *Phaeodactylum tricornutum*

2.1 Introduction

This chapter describes the standardisation of experimental methods for a reliable protocol to routinely culture *P. tricornutum* for stock cultures and larger batch culture volumes required for gaining high biomass yields for the screening of antibacterial compounds (see subsequent chapters). Standardisation of culture conditions is required so that subsequent screening of antibacterial compounds can be compared with regard to the starting material. The analytical methods selected for growth measurements of *P. tricornutum* were chosen for their accuracy, reliability, reproducibility, cost, and speed (Craggs 1994; Whiston 1996) and were used throughout this study.

The aim of this chapter is to determine suitable methods of extraction and assay for antibacterial activity in *P. tricornutum* cells for further investigation. The choice of extraction buffer is very important. The ideal extraction buffer would solubilise all components of the cell lysate whilst the sample retains biological activity. However, no single buffer is capable of this for all biological material due to the wide range of chemistry within a microalgal cell. So a screening of the most appropriate extraction buffer was required.

The types of extraction buffer used include acidic, alkaline, neutral, detergents, and organic solvents. In addition, when the cellular material is solubilised degradation often occurs, particularly with respect to the presence of phenolics and enzymes. Inhibitors can be included to reduce the risk of degradation in addition to maintaining the sample at low temperature during experimental procedures.

A wide range of bacterial species is required to test against as some antimicrobial agents can have a narrow range of activity, such as only being active against Gram positive or negative bacteria. The most susceptible bacterial species would then be used for future antibacterial tests after each step of purification due to the small volumes gained.

2.2 Methods

2.2.1 Sterilisation of culture equipment and solutions

To reduce the risk of contamination the culture vessels, cotton wool plugs, aeration tubing, glassware and all non-volatile buffers were sterilised by autoclaving at 121 °C, 1.5 atm for 15 min before use. Unless specified, chemicals were acquired from Sigma-Aldrich, Poole, Dorset, UK. All solutions were prepared with deionised or ultra pure deionised water (Option 3, Maxima; Elga, High Wycombe, Buckinghamshire, UK), except when specified. Aseptic technique was also used throughout this study.

2.2.2 Source of *Phaeodactylum tricornutum*

Pipette isolation (Hoshaw and Rosowski 1979) was used to gain single isolates from a laboratory maintained stock culture of *P. tricornutum*, donated by previous workers (Craggs 1994; Whiston 1996), for the formation of a pure initial stock culture. One millilitre of the laboratory maintained stock culture in Erd-Schreiber soil extract media (ES - Appendix 1) (Foyn 1934) was centrifuged at 4,000 × g for 5 min at room temperature, resuspended in 1 ml of sterile 3.2 % (w/v) NaCl and serially diluted with sterile 3.2 % (w/v) NaCl to isolate individual cells. Five cells were separated using fire-drawn and polished Pasteur pipettes under a dissecting microscope and inoculated into individual sterile test-tubes, each containing 5 ml sterile ES, and incubated in a refrigerated illuminated incubator at 15 °C, 20 μmol $s^{-1}m^{-2}$ PPFD (LI-189; LI-COR Biosciences, Cambridge, Cambridgeshire, UK), automatically controlled to a regime of 12 hr light and 12 hr dark per day (12:12 LD), for 3 weeks. After incubation, samples from 3 test-tubes showing growth were observed for

contamination and cell condition under a phase contrast Leitz Dialux microscope (×50 objective). The remaining contents of one test-tube were used as the inoculum in a 250 ml sterile glass round bottom flask containing 150 ml sterile ES, plugged with non-absorbent cotton wool and incubated in a light chamber, as described in section 2.2.4.3. This was regarded as the starting stock culture.

2.2.3 Maintenance of stock culture

The stock culture was gently swirled by hand at weekly intervals to ensure mixing of nutrients and prevent cells settling. It was sub-cultured monthly in 150 ml sterile ES in a 250 ml sterile glass round bottom flask plugged with sterile non-absorbent cotton wool. In every case, the volume of the initial inoculum was 10 % of the total volume of the culture (Liao *et al.* 1983).

2.2.4 Batch culture and cell preparation of *P. tricornutum* for high biomass yields required for the screening of antibacterial compounds

2.2.4.1 Preparation of culture medium/vessels

Enriched artificial seawater medium (ESAW), based on that devised by Harrison *et al.* (1980), except that the vitamins and organic pH buffer were omitted to reduce the amount of substrates available for bacterial metabolism (Appendix 1), was used for the culture of *P. tricornutum*. The pH of the culture medium was measured with an electrode pH meter before inoculation (pH 7.8) and at the time when cells were harvested (pH 8.0). The culture vessels used were 10 or 20 l sterile Nalgene™ polycarbonate carboys. Prior to first use, the carboys were acid rinsed with 10 % (v/v) HCl. After extensive washing in de-ionised water, ESAW was added to the carboys and sterilised as described in section 2.2.1.

2.2.4.2 Inoculation and sub-culture

Initially, 100 ml of the starting stock culture, grown to mid-exponential phase, was used as inoculum in 10 litres sterile ESAW. The inoculated culture was then plugged with sterile non-absorbent cotton wool wound round a sterile aeration tube and wedged into the opening of the carboys before incubating until the culture reached mid-exponential phase (typically 10-14 days for 10 l, 0.5 OD_{570}; 6 $\times 10^6$ cells ml^{-1}) under the culture conditions described below. All subsequent batch cultures were sub-cultured and, in every case, the volume used was 10 % of the total volume of the culture to be inoculated. To minimise the volume of the inoculum, the cells were harvested from the culture and washed, as described in section 2.2.4.4, before resuspension in 50 ml of sterile 3.2 % (w/v) NaCl. A high cell concentration was used to promote high growth rates and yield by maintaining the cells in exponential phase.

2.2.4.3 Culture conditions

The culture(s) were illuminated by four cool white fluorescent tubes (F18W/33) automatically controlled to a regime of 14:10 LD. The fluorescent tubes were fixed to two sides of a cubic chamber that had white reflective walls (internal dimensions; length; 90 cm, height; 60 cm, depth; 44 cm). The PPFD was measured with a digital quantum light meter at the side of the culture vessel(s) to be 105 µmol s^{-1} m^{-2} (LI-189; LI-COR). The batch cultures were agitated by a continuous flow of laboratory air passing through a sterile Pyrex® glass sintered thimble (sparger) positioned at the centre of the culture vessel. The air was filtered through a sterile in-line filter (0.3 µm; Hepa-vent™, Whatman, Maidstone, Kent, UK) before entering the culture. The

air temperature of the culture room was thermatically controlled by a refrigerated air unit to 20 ± 0.5 °C (Daikin, Woking, Surrey, UK). The air temperature of the culture chamber was checked daily (at different times of the day) with a mercury-in-glass thermometer.

2.2.4.4 Harvesting cells

The desired volume of cells (see subsequent chapters) was harvested from the culture and centrifuged at 3580 ×g for 11 min at 4°C to remove supernatant. To remove possible microbial contaminants and flocculates the cell pellet(s) were resuspended to 50 ml with sterile 3.2 % (w/v) NaCl and centrifuged at 900 ×g for 15 min at 4°C. This washing step was repeated once more before finally centrifuging at 3000 ×g for 15 min at 4°C. For each preparation the final cell pellet, after discarding the supernatant, was stored at –80 °C until use for no longer than 4 weeks unless otherwise stated. The wet cell volume of a cell pellet from 9 l of culture medium in mid exponential phase was approximately 5 ml.

2.2.4.5 Cell disruption/lysis

Ultrasonication was selected as the most appropriate method for the disruption of *P. tricornutum* cells throughout this study. Cell suspensions were ultrasonicated with 0.5-second pulses at maximum power for the probe tip used (Status US 200; Philip Harris Scientific, Macclesfield, Cheshire, UK) until complete / near complete cell lysis as determined by observation under a Leitz Dialux microscope (× 50 objective). An ice bath surrounded the vessel containing the resuspended cells to prevent temperature increase of the cells/lysate during ultrasonication.

2.2.5 Growth characteristics of *P. tricornutum* in batch culture

The growth characteristics of cultures of *P. tricornutum* in 10 litres ESAW were determined by daily measurements of growth over 17 days. In order to record standing crop within a culture, turbidity and direct microscopic counting were measured. Sampling and measurements were made at the same time of each day to reduce any errors due to possible synchronous differences. The samples were not returned to the culture after growth measurements to prevent risk of contaminating the on-going culture.

2.2.5.1 Turbidity

Mean optical density (OD) at 570 nm was calculated from three replicate 1 ml samples taken aseptically with sterile glass Pasteur pipettes from the culture. Measurements were made directly from each 1 ml sample in disposable polystyrene cuvettes at 570 nm against a blank of sterile ESAW medium.

2.2.5.2 Direct microscopic counting

Mean cell densities were calculated from ten replicate counts of 1 ml culture with a New Improved Neubauer haemocytometer (Stein 1973; Vonshak 1986) using a phase contrast Leitz Dialux microscope (×25 objective). When cell densities in the culture sample were deemed too high to count accurately ($>2 \times 10^6$ ml^{-1}) the sample was diluted with 3.2 % (w/v) NaCl and calculations made accordingly.

2.2.6 Screening of extraction solvents and buffers

P. tricornutum grown in batch culture to late exponential phase in 10 litres sterile ESAW and harvested as described in section 2.2.4. The cell pellet was resuspended and mixed to homogeneity in 30 ml sterile 3.2 % (w/v) NaCl. One millilitre aliquots were pipetted into 1.5 ml sterile Eppendorfs and centrifuged at 13,000 × g for 2 min at room temperature and supernatants discarded (100 µl wet cell volume). The cell pellets were stored at ¯80 °C until use. After thawing, each cell pellet was resuspended with 0.5 ml of a different extraction solvent (Table 2.2) and ultrasonicated as described in section 2.2.4.5. The cell lysates were kept on ice and mixed on an orbital shaker for 60 minutes (60 rev. min^{-1}) to aid extraction before centrifuging at 15,000 × g for 60 min at 4 °C to remove cellular debris. Each supernatant was transferred by pipette into a 1.5 ml sterile Eppendorf and lyophilised (Modulyo; BOC Edwards, Crawley, West Sussex, UK) before reconstituting in 150 µl sterile 50 mM HEPES at pH 7.8.

2.2.6.1 Total protein concentration

The total protein concentration of each test solution (above) was determined by the Bradford assay (Bradford 1976). Triplicate 5 µl aliquots of each sample were mixed with 250 µl of Coomassie Brilliant Blue reagent (G-250, Pierce, Rockford, Illinois, USA) in 96-well microtitre plates. Loaded plates were incubated at room temperature for 30 minutes and absorbance read at 595 nm against sample diluent blanks in triplicate on a micro-titre plate reader (MR5000; Dynatech, Chantilly, VA, USA). To determine total protein concentration the absorbance values were compared to a standard curve generated in triplicate from serially diluted bovine serum albumin (0.1-1.0 mg ml^{-1}; Sigma)

2.2.6.2 Test bacteria

The test bacteria stains were the marine Gram-positive, *Planococcus citreus* (NCIMB 1493), the marine Gram-positive isolate, designated as Y# (isolated from a previous culture of *P. tricornutum*), the marine Gram-positive, *Aerococcus viridens* (NCIMB 1120), the marine Gram-negative, *Psychrobacter immobilis* (NCIMB 308), and the marine Gram-negative, *Listonella (Vibrio) anguillarum* (NCIMB 1291). Each bacterium was maintained at 4 °C on sterile polystyrene round petri dishes (9 cm diameter) of Marine Agar (2216E; Difco, West Molesey, Surrey, UK) made to the manufacturer's specifications. When required for antibacterial assays, a loop full (ca 15 µl) was picked from an individual colony and grown at 20 °C in Marine Broth (Difco) to exponential phase for 16-18 hr. The bacteria were then harvested from the broth by centrifugation at 1,900 × g for 10 min at 4 °C. Each bacterium was washed once in sterile 3.2 % (w/v) NaCl, and resuspended in sterile 3.2 % (w/v) NaCl to an absorbance of 0.3 at 570 nm using diluent as blank, giving an approximate bacterial concentration of ca 1×10^7 cfu ml^{-1}.

2.2.6.3 Assaying for antibacterial activity using radial diffusion assay

The antibacterial activity of each test sample was assessed semi-quantitatively by a modification of the two-layer radial diffusion (RDA) method of Lehrer *et al.* (1991). Fifteen millilitres of sterile base agar (Appendix 1) was heated until molten, allowed to cool to approximately 40 °C before seeding with 50 µl of a washed bacterial suspension as described above. The seeded agar solution was quickly poured into a 12 × 12 cm sterile polystyrene square petri dish (Greiner, Nűrtingen, Germany) on a level surface (checked with a spirit level). Once the base agar was set, wells (2 mm diameter) were made with a 2 ml sterile plastic transfer pipette (Sarstedt, Beaumont

Leys, Leicester, UK) using a template to ensure the required number of evenly spaced wells. Three microlitres of each solution to test was then pipetted into individual wells. Two wells were used for controls; the negative control well contained 3 µl of sample diluent; the positive control well contained 3 µl of 25 or 50 µg ml^{-1} penicillin-G. The plate was incubated at 4 °C for 3 hr to allow the samples to diffuse from the wells to the surrounding base layer before adding 15 ml of molten (40 °C) sterile top agar (Appendix 1). The plate was then incubated for 12-24 hr at the required conditions for each test bacteria as described in section 2.2.6.3. The diameter of clear zones of bacterial growth inhibition surrounding wells containing a test sample were then measured and the value converted to area minus the area of the well (mm^2).

2.3 Results

2.3.1 Growth of *P. tricornutum* in batch culture

An increase in cell concentration occurred until approximately day 14 (exponential phase), then no further significant increase in cell concentration was observed (stationary phase) (Figure 2.1). A similar trend was found for absorbance readings (Figure 2.1).

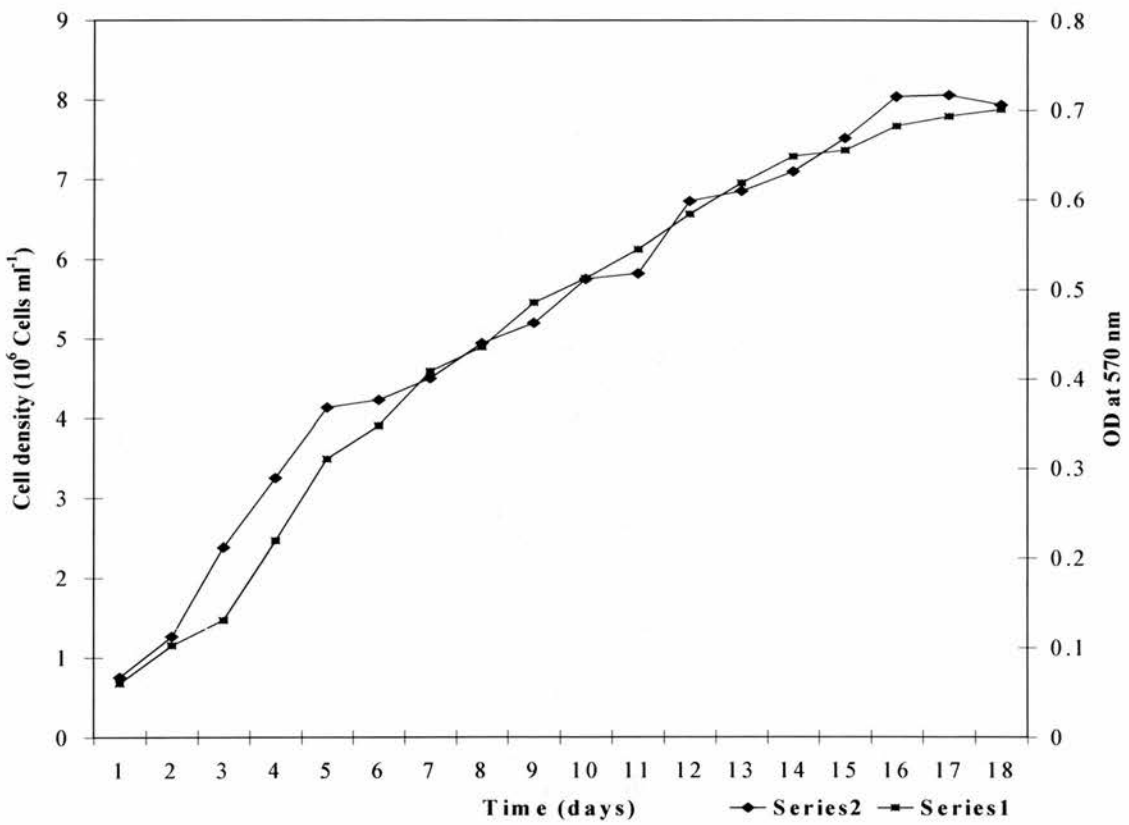


Figure 2.1 Growth of *P. tricornutum* in batch culture. Mean cell density (n = 10) (Series 1) and mean optical density at 570 nm (n = 3) (Series 2) of *P. tricornutum* in batch culture over 17 days. Measurements at time of inoculation are shown at day 1.

2.3.2 Antibacterial activity of extracts

No antibacterial activity was observed for experimental wells on RDA plates seeded with *Planococcus citreus* or *Listonella (Vibrio) anguillarum*, although positive controls showed strong activity. No antibacterial activity was also observed for any of the negative controls. With *Psychrobacter immobilis,* antibacterial activity was observed with those wells corresponding to the 60 % ethanol and 100 % methanol extracts, with the 60 % ethanol extract showing the highest activity index value (Table 2.1).

Table 2.1 Total protein concentration and antibacterial activity against *Psychrobacter immobilis* for cell extracts of *P. tricornutum.*

Extraction buffer / solvent	Total protein conc. ($\mu g\ ml^{-1}$)	Antibacterial activity. Clear zone area (mm^2)	Activity index (Clear zone area / protein conc. × 100)
60 % (v/v) ethanol	306	9	2.9
100 % (v/v) methanol	313	4	1.3

Antibacterial activity was observed against Y#, for solutions corresponding to extraction in acidic, alkaline and neutral pH buffers except for 3.2 % NaCl, 10 % acetic acid and 1 % trifluoracetic acid (TFA) (Table 2.2). The lack of antibacterial activity from the 3.2 % NaCl extract indicates that neutral pH was not the factor responsible for extraction of antibacterial compounds as antibacterial activity was observed for the phosphate buffer extract at neutral pH. In addition, no antibacterial activity was observed against *Aerococcus viridens* for any of the acidic extractions (Table 2.3). This, combined with the data for Y#, indicates that antibacterial compounds were not extracted in strong acids or that biological activity was destroyed by the low pH.

Antibacterial activity was detected against Y# and *Aerococcus viridens* for extracts of *P. tricornutum* with Triton X 100 concentrations 0.1 and 1.0 %, although the area of clear zones were generally less than those produced from other extraction solvents/buffers (Tables 2.2 and 2.3).

Strong antibacterial activity was observed against Y# and *Aerococcus viridens* from all types of organic solvent extraction, although not for all of the concentrations used (Tables 2.2 and 2.3). Consistently the 60 % concentration of ethanol, acetonitrile or chloroform produced the strongest antibacterial activity for organic solvent extracts against Y# or *Aerococcus viridens,* whereas 100 % concentration of methanol or hexane produced the strongest antibacterial activity for these bacteria (Tables 2.2 and 2.3).

The extracts with the strongest antibacterial activity against Y# was obtained by extraction in 100 % methanol, phosphate buffer, 60 % ethanol and 0.1 % TFA (Table 2.2). In addition, the 100 % methanol and 60 % ethanol extracts had the highest activity index values against Y# (Table 2.2). By contrast, the strongest antibacterial activity against *Aerococcus viridens* was obtained in the 60 % acetonitrile and phosphate buffer extracts (Table 2.3). When expressed as activity index, extraction with 30 and 60 % ethanol and 100 % methanol showed the highest values. No antibacterial activity was detected in extracts obtained with 3.2 % NaCl, 10 % acetic acid, 1 % trifluoroacetic acid, 100 % ethanol or 30 % chloroform for any of the bacteria tested (Tables 2.1, 2.2 and 2.3).

There was no clear correlation between the antibacterial activity and total protein concentration, as extraction with 3.2 % NaCl was able to solubilise one of the highest concentration of protein (> 1 mg ml^{-1}; Tables 2.2 and 2.3), whereas no antibacterial activity was observed for any of the bacteria tested. This was confirmed by the antibacterial activity expressed as activity index, as although the phosphate buffer extract showed strong antibacterial activity against Y# or *Aerococcus viridens,* it's activity index value was low due to the high protein concentration (Tables 2.2 and 2.3).

Table 2.2 Total protein concentration and antibacterial activity against Y# for cell extracts of *P. tricornutum*

Extraction buffer / solvent (v/v)	Total protein conc. ($\mu g\ ml^{-1}$)	Antibacterial activity. Clear zone area (mm^2)	Activity index. Clear zone area / protein conc. × 100
3.2 % (w/v) NaCl, 1 % inhibitor mix*	1036	0	0.0
phosphate buffer**, 1 % inhibitor mix*	1339	75	5.6
10 % acetic acid	25	0	0.0
0.1 % trifluoracetic acid	1017	60	5.8
1.0 % trifluoracetic acid	<1***	0	0.0
50 mM NaOH	874	16	1.8
0.1 % Triton X 100	945	25	2.6
0.5 % Triton X 100	933	25	2.7
30 % ethanol	852	25	2.9
60 % ethanol	306	75	24.5
100 % ethanol	9	0	0.0
30 % methanol	1139	35	3.1
60 % methanol	258	25	9.7
100 % methanol	313	198	63.2
30 % acetonitrile	907	0	0.0
60 % acetonitrile	517	47	9.1
100 % acetonitrile	<1***	4	<4.0
30 % chloroform	615	0	0.0
60 % chloroform	368	25	6.8
100 % chloroform	91	9	9.9
30 % hexane	1418	4	0.3
60 % hexane	1337	16	12.0
100 % hexane	<1***	25	<25.0
25 $\mu g\ ml^{-1}$ penicillin G	-	47	-

* Protease inhibitor mix, prepared as manufacturers instructions (Sigma)

** Phosphate buffer; 10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 2 mM EDTA (pH 7.0)

*** Below detectable limit

Table 2.3 Total protein concentration and antibacterial activity against *Aerococcus viridens* for cell extracts of *P. tricornutum*

Extraction buffer / solvent (v/v)	Total protein conc. ($\mu g\ ml^{-1}$)	Antibacterial activity. Clear zone area (mm^2)	Activity index. Clear zone area / protein conc. × 100
3.2 % (w/v) NaCl + 1 % inhibitor mix*	1036	0	0.0
phosphate buffer** + 1 % inhibitor mix*	1339	60	4.5
10 % acetic acid	25	0	0.0
0.1 % trifluoracetic acid	1017	0	0.0
1.0 % trifluoracetic acid	<1***	0	0.0
50 mM NaOH	874	47	5.4
0.1 % Triton X 100	945	16	1.7
0.5 % Triton X 100	933	16	1.7
30 % ethanol	852	35	15.3
60 % ethanol	306	47	15.4
100 % ethanol	9	0	0.0
30 % methanol	1139	60	5.3
60 % methanol	258	25	9.7
100 % methanol	313	47	15.0
30 % acetonitrile	907	35	3.8
60 % acetonitrile	517	75	14.5
100 % acetonitrile	<1***	35	<35.0
30 % chloroform	615	0	0.0
60 % chloroform	368	47	12.8
100 % chloroform	91	35	2.5
30 % hexane	1418	35	2.5
60 % hexane	1337	47	3.5
100 % hexane	<1***	0	0.0
50 $\mu g\ ml^{-1}$ penicillin G (Sigma)	-	35	-

* Protease inhibitor mix, prepared as manufacturers instructions (Sigma)

** Phosphate buffer; 10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 2 mM EDTA (pH 7.0)

*** Below detectable limit

2.4 Discussion

2.4.1 Batch culture conditions for *P. tricornutum*

The seawater base used for the present study needed to be artificial to standardise the medium for comparisons of antimicrobial compound screening from different cultures. The artificial seawater based medium ESAW was chosen for the culture of *P. tricornutum* as it has the practical benefits of being easy to prepare and completely autoclavable without the formation of precipitates, whilst able to maintain a wide range of microalgae in culture (Harrison *et al.* 1980). Since it's development, ESAW has been extensively used in phycological research laboratories worldwide; being regarded as a reliable standardised culture medium for the culture of microalgae (Fawley 1984) and has been recently reviewed and improvements recommended (Berges *et al.* 2001). In addition, ESAW maintained the pH of the culture at 8.0, this compares to the optimum pH (8.0 to 8.2) for maximum biomass production of *P. tricornutum* (Goldman *et al.* 1982). A vitamin solution was not added to the culture medium as is has been reported that the growth of *P. tricornutum* may be inhibited by the addition of vitamins B_{12}, biotin and thiamine (Fanuko 1981). In addition, many microalgal species are known to produce vitamins (Borowitzka 1988).

Fluorescent lamps were used as the illumination source for the culture of *P. tricornutum* as these were favoured due to their efficiency and lower heat generation when compared to conventional light bulbs. The intensity and quality of the illumination source affects numerous aspects of the growth of microalgae, such as growth rates, biochemical composition, pigment concentrations and the partitioning of cellular carbon (Terry *et al.* 1983; Fawley 1984; Geider *et al.* 1985). The

irradiance that the cells receive within a culture vessel will vary due to the type of the culture vessel and the increased self-shading by the cells as the culture develops. Unlike some microalgae, *P. tricornutum* can grow well under constant illumination (Glover *et al.* 1975; Geider *et al.* 1985, 1986; Owens 1986; Owens and Wold 1986), however, in an attempt to resemble diurnal rhythm in the environment a light/dark cycle was used.

The physical mixing of microalgal cultures may have multifarious effects on microalgal growth. Not only does mixing ensure that the cells are kept in suspension, but also decreases the nutritional and gaseous gradients that surround the cells in actively growing cultures, thereby increasing the rate of provision of nutrients including carbon dioxide (Richmond 1986). Another benefit of mixing is the intermittent positioning of the cells close to the sides of the culture vessel; thereby experiencing the benefits of flashing light effects by being temporarily exposed to intense illumination (Richmond 1986). For this study, the cultures were agitated by bubbling filtered air through them.

Although *P. tricornutum* can survive when grown from 18°C to 32°C it's optimum temperature has been reported to be 20°C (Fanuko 1981). By contrast, Fawley (1984) reported maximum growth at 23°C at high light intensities for *P. tricornutum*, although at low light intensities the optimum temperature decreased. Since the light intensity used for this study was considered low, 20 °C was chosen as the incubation temperature for the culture of *P. tricornutum.*

2.4.2 Antibacterial activity and total protein concentration of extracts

With the culture conditions used, *P. tricornutum* cells, extracted in a wide range of solvents / buffers, were shown to display antibacterial activity against three out of the five bacteria tested by RDA. Antibacterial activity was detected in 18 of 23 extracts using Y# and *Aerococcus viridens.* However it is possible that antibacterial compounds extracted in organic solvents or in strong acid / alkaline conditions may not have been soluble in the aqueous HEPES buffer (pH 7.8) used to reconstitute the lyophilised extracts. In addition, it is also possible that the extraction solvents / buffers may have affected the chemical nature of *P. tricornutum* cell constituents so that they became antibacterial, but were not in their original form.

Organic solvent extracts of microalgae have often been used in screening studies for antimicrobial activity (Duff *et al.* 1966; Cannell *et al.* 1988; Kellam and Walker 1989). Cannell *et al.* (1988) tested methanol, acetone or hexane cell extracts of 300 freshwater eukaryotic microalgae for antibacterial activity. Methanol was found to be the most successful solvent for extraction of compounds with antibacterial activity and that Gram-positive bacteria appeared to be most susceptible (Cannell *et al.* 1988). Kellam and Walker (1989) also found that none of the methanol extracts of 132 marine microalgae displayed antibacterial activity against the Gram-negative bacteria tested. However, hexane extracts were shown to be strongly antibacterial against both Gram-positive and Gram-negative bacteria (Kellam and Walker 1989). By contrast, hexane extracts were not found to be antibacterial against the Gram-negative bacteria tested. The 60 % ethanol and 100 % methanol extracts used in this study were the only solutions active against both Gram-negative and Gram-positive

bacteria, indicating that more than one type of antibacterial compound was present in *P. tricornutum* cells.

Although no negative control for extraction solvents / buffers without *P. tricornutum* cells were assayed for antibacterial activity, the absence of antibacterial activity against *Planococcus citreus* or *Listonella (Vibrio) anguillarum* for all extracts indicate that the contents of the solvents / buffers that may have remained after lyophilisation were not antibacterial.

Although it appears there was no correlation between protein concentration and antibacterial activity, caution must be used when interpreting the protein concentration values due to the presence of chromophoric material in the extracts interacting with the Coomassie Brilliant Blue Reagent, possibly producing erroneous values (Smith *et al.* 1985).

To isolate and characterise the active agents responsible for the antibacterial activity, larger volumes of extract are necessary using the extraction solvents / buffers that solubilised compounds with the highest antibacterial activity.

Chapter 3

Fractionation of a phosphate buffer cellular extract of *P. tricornutum*

3.1 Intoduction

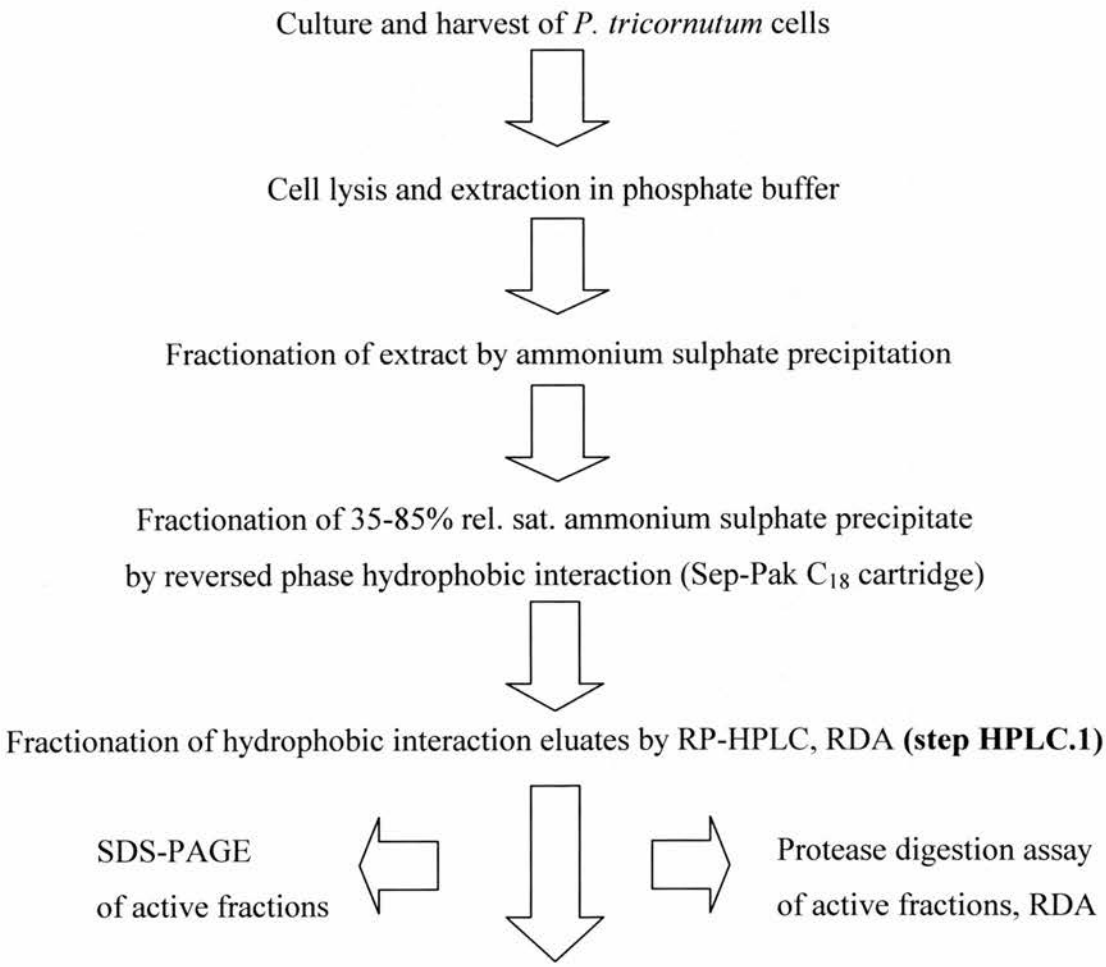
This Chapter describes the fractionation of a phosphate buffer cellular extract of *P. tricornutum* with the aim of isolating and characterising antibacterial agents responsible for the strong antibacterial activity of a phosphate buffer extract reported in Chapter 2.

The preparation of the extract was modified from previous studies where antimicrobial peptides were isolated from plant material (Cammue *et al.* 1992; Terras *et al.* 1992; Moreno *et al.* 1994). The procedures chosen for fractionation of the extract were ammonium sulphate precipitation and reversed-phase high performance liquid chromatography. For this case, antibacterial activity of fractions, after each stage of fractionation, was to be determined by RDA.

3.2 Methods

An aqueous extract of *P. tricornutum* cells was fractionated by ammonium sulphate precipitation then by reversed phase hydrophobic interaction, followed by two steps of reversed phase high performance liquid chromatography (RP-HPLC), as summarised in Figure 3.1. Antibacterial activity of fractions, after RP-HPLC, were determined by RDA against a Gram-positive marine bacterium. The detailed methodology of each step is described in the accompanying Sections.

Figure 3.1 Flowchart of the fractionation of *P. tricornutum* phosphate buffer cell extract



Unless specified, chemicals were acquired from Sigma-Aldrich. All solutions are prepared with deionised or ultra pure deionised water (Elga), except when specified.

3.2.1 Preparation of *P. tricornutum* cells and extraction in phosphate buffer

P. tricornutum was grown to exponential phase (6.8×10^6 cells ml^{-1}) in 20 l ESAW and cells harvested from 18 l culture as described in Section 2.2.4. After thawing, the cell pellet was resuspended 1:3 (v/v) with sterile phosphate buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 2 mM Na_2EDTA, pH 7.0) containing 1.5 % (w/v) PVPP and 1 % (v/v) reconstituted stock protease inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride, Na_2EDTA, leupeptin, trans-epoxysuccinyl-L-leucylamino(4-guanidino)butane, bestatin and aprotinin, prepared to manufacturers instructions) (Sigma) then snap-frozen in liquid nitrogen. After thawing, the cell suspension was ultrasonicated on ice for 3 cycles of 5 minutes at 100 % power with ½ second pulses (Status US 200; Philip Harris Scientific), stirred overnight at 4 °C and then centrifuged at 3,000 × g for 30 min at 4 °C. The supernatant was decanted and stored at 4 °C until use. The pellet was extracted once more in 50 ml of sterile phosphate buffer (as above) and stirred for 2 hr at 4 °C before centrifuging at 3,000 × g for 30 min at 4 °C. The supernatant was decanted, combined with the previous supernatant and clarified by centrifugation at 51,000 × g for 60 min at 4 °C. The decanted supernatant was designated the phosphate extract.

3.2.2 Fractionation of phosphate extract by ammonium sulphate precipitation

To remove chromophore-containing material and to fractionate for low molecular weight proteins, ammonium sulphate was dissolved in the phosphate extract to 35 % relative saturation and left to stand for 60 min at 4 °C before centrifuging at 3,000 ×

g for 35 min at 4 °C. The supernatant was decanted and filtered (GF/C; Whatman, UK) before adding ammonium sulphate to 85 % relative saturation, stirred and left to stand for 60 min at 4 °C before centrifuging at 3,000 × g for 35 min at 4 °C. The supernatant was discarded and the pellet resuspended with 40 ml of deionised water.

3.2.3 Fractionation of 35-85 % relative saturation ammonium sulphate precipitate with reversed phase hydrophobic interaction using a C_{18} Sep-Pak cartridge.

The reconstituted pellet was acidified with TFA to 0.1 % and loaded onto a 5 g Sep-Pak C_{18} cartridge (Waters, Watford, Hertfordshire, U.K.), previously equilibrated in 0.1 % (v/v) TFA. Stepwise elution under gravity (c. 1 ml min^{-1}) followed with sequential steps of 20, 40, 60 and 90 % (v/v) acetonitrile (BDH, Poole, Dorset, UK), 0.1 % (v/v) TFA. Each elution was collected as 20 ml fractions, lyophilised and reconstituted in 1 ml ultra pure deionised water.

3.2.4 Fractionation of Sep-Pak fractions by RP-HPLC (step HPLC.1)

The 20% Sep-Pak fraction was acidified to 0.1 % (v/v) TFA, filtered through a 0.2 μm cartridge filter (Anatop 10LC, Whatman) and loaded on a C_{18} column (250 mm × 10 mm; ODS2, Capital HPLC, Broxburn, West Lothian, UK) previously equilibrated in ultra pure deionised water, 0.1 % (v/v) TFA. The column was eluted at 1 ml min^{-1} with the following gradients (solvent B is HPLC-grade acetonitrile (BDH), 0.1 % (v/v) TFA, 0-20 min, 0 % (v/v) B; 20-80 min, 0-30 % (v/v) B; 80-110 min, 30-100 % (v/v) B; 110-120 min, 100 % (v/v) B. The eluate was monitored using a photo diode array detector (PDA) (Waters) at 210 to 400 nm. One millilitre fractions of the eluate were collected, solvent removed by lyophilisation and each fraction

reconstituted in 60 µl of 50 mM HEPES at pH 7.0 before assaying 3 µl of each fraction for antibacterial activity by RDA using *P. immobilis* (NCIMB 308) or Y# as test bacteria as described in Section 2.2.6.4.

The above procedure was repeated using the 40 and 60 % acetonitrile Sep-Pak elution fractions and eluted with the following gradients: for the 40 % acetonitrile Sep-Pak fraction, 0-20 min, 0 % (v/v) B; 20-80 min, 0-60 % (v/v) B; 80-90 min, 60 % (v/v) B; 90-110 min, 60-100 % (v/v) B; 110-120 min, 100 % (v/v) B, the 90 % acetonitrile elution fraction: 0-20 min, 0 % (v/v) B; 20-40 min, 0-50 % (v/v) B; 40-50 min, 50 % (v/v) B; 50-100 min, 50-100 % (v/v) B; 100-120 min, 100 % (v/v) B.

3.2.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of active fractions from RP-HLPC of 40 and 60% acetonitrile Sep-Pak elution fractions.

The protein content of fraction 76 from RP-HPLC of the 40 % Sep-Pak eluate and fractions 73, 78, 89 and 110 from RP-HPLC of the 60 % Sep-Pak eluate were visualised by high resolution SDS-PAGE under reducing conditions using a 16 % separating gel, 14 % spacer gel and 5 % stacking gel as described by Schägger and von Jagow (1987 – Appendix 2). Low molecular weight markers (Promega, Madison, WI, USA) of 3.5 to 31 kDa were used. Following electrophoresis the gel was stained with nondiamine chemical development silver staining (Appendix 2).

3.2.6 Protease digestion of active fractions from RP-HPLC of 60 % acetonitrile Sep-Pak eluate

To determine the proteinaceous nature of active fractions from RP-HPLC of 60 % acetonitrile Sep-Pak eluate, the fractions 88 to 90 were pooled and subjected to digestion with Proteinase K. Three solutions, A-C, were prepared as follows; solution A contained 5 µl test solution plus 1.2 µl 0.3 mg ml-1 Proteinase K; solution B contained 1.2 µl selected protease plus 5 µl ultra pure water (negative control); solution C contained 5 µl test solution plus 1.2 µl ultra pure deionised water (control). The three solutions were incubated at 35 °C for 4 hr before assaying 4 µl of each test solution for antibacterial activity by RDA using Y# as test bacterium as described in Section 2.2.6.4.

3.2.7 Fractionation by RP-HPLC of active fractions from HP-HPLC of 60 % acetonitrile Sep-Pak eluate (step HPLC.2)

Fractions 77-80 from HP-HPLC of 60 % acetonitrile Sep-Pak eluate (step HPLC.1) were pooled and resuspended to 1 ml ultra pure deionised water, acidified to 0.1 % TFA, filtered through a 0.2 µm cartridge filter (Anatop 10LC, Whatman) and loaded on a C_{18} column (250 × 4.6 mm; Rsil HL, BioRad, Hemel Hempstead, Hertfordshire, UK) previously equilibrated in ultra pure deionised water, 0.1 % (v/v) TFA. The column was eluted at 1 ml min^{-1} with the following gradients (solvent B is HPLC-grade acetonitrile (BDH), 0.1 % (v/v) TFA, 0-10 min, 0 % (v/v) B; 10-20 min, 0-30 % (v/v) B; 20-30 min, 30 % (v/v) B; 30-90 min, 30-60 % (v/v) B; 90-100 min, 60-100 % (v/v) B; 100-110 min, 100 % (v/v) B. The eluate was monitored using the PDA and collected as 1 ml fractions, solvent removed by lyophilisation and reconstituted in 60 µl of 50 mM HEPES at pH 7.0 before assaying 3 µl of each

fraction for antibacterial activity by RDA using Y# as test bacterium as described in Section 2.2.6.4.

The above chromatography was repeated with pooled fractions 88 to 90 and 110 to 113 from HP-HPLC of 60 % acetonitrile Sep-Pak eluate (step HPLC.1) using the following different elution gradients (solvent B is HPLC-grade acetonitrile (BDH), 0.1 % (v/v) TFA, pooled fractions 88 to 90: 0-10 min, 0 % (v/v) B; 10-30 min, 0-40 % (v/v) B; 30-40 min, 40 % (v/v) B; 40-80 min, 40-60 % (v/v) B; 80-90 min, 60 % (v/v) B; 90-110 min, 60-100 % (v/v) B; 110-120 min, 100 % (v/v) B, pooled fractions 110 to 113: 0-60 min, 0-60 % (v/v) B; 60-80 min, 60-100 % (v/v) B; 80-90 min, 100 % (v/v) B. The eluted fractions were monitored, collected, concentrated by lyophilisation and reconstitutes were assayed for antibacterial activity as described above.

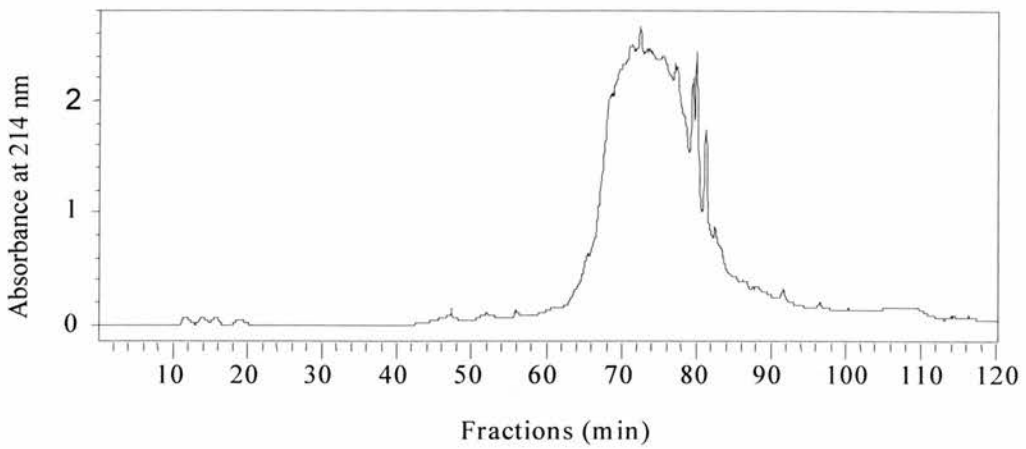
3.3 Results

No antibacterial activity was detected against *P. immobilis* for any of the fractions from RP-HPLC of the 20, 40, 60 or 90 % acetonitrile elution Sep-Pak fractions (step HPLC.1), although the positive controls showed strong activity (not shown).

Whilst no antibacterial activity was detected against Y# for any of the fractions from RP-HPLC of the 20 % acetonitrile elution Sep-Pak fraction (not shown), antibacterial activity was detected in fractions 71-80 from the RP-HPLC of 40 % acetonitrile elution Sep-Pak fraction, corresponding to elution at 50-60 % acetonitrile (Figure 3.2).

Antibacterial activity was also detected against Y# in fractions 73-80, 88-96 and 110-113 from the RP-HPLC of 60 % acetonitrile elution Sep-Pak fraction (Figure 3.3). For the RP-HPLC of 90 % acetonitrile elution Sep-Pak fraction (step HPLC.1), weak antibacterial activity was detected against Y# in fractions 52, 53 and 58, eluted at 50-60 % acetonitrile (Figure 3.4).

(a)



(b)

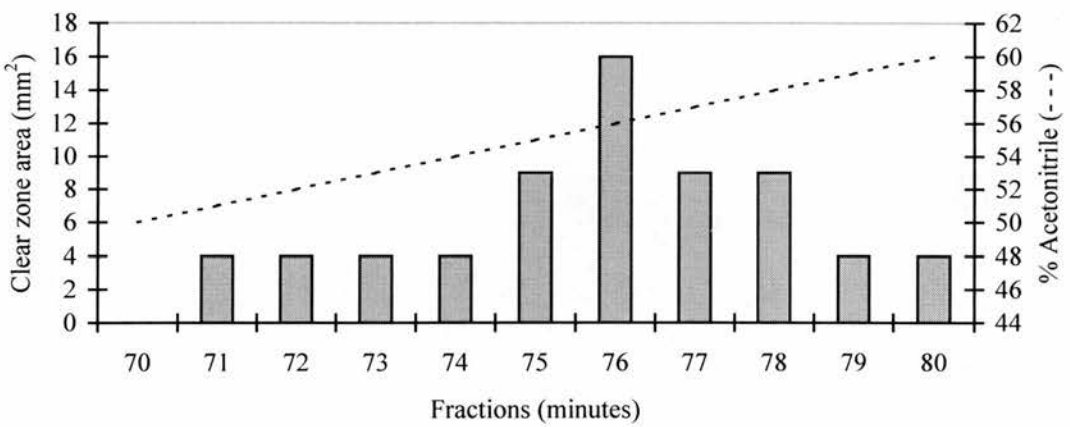
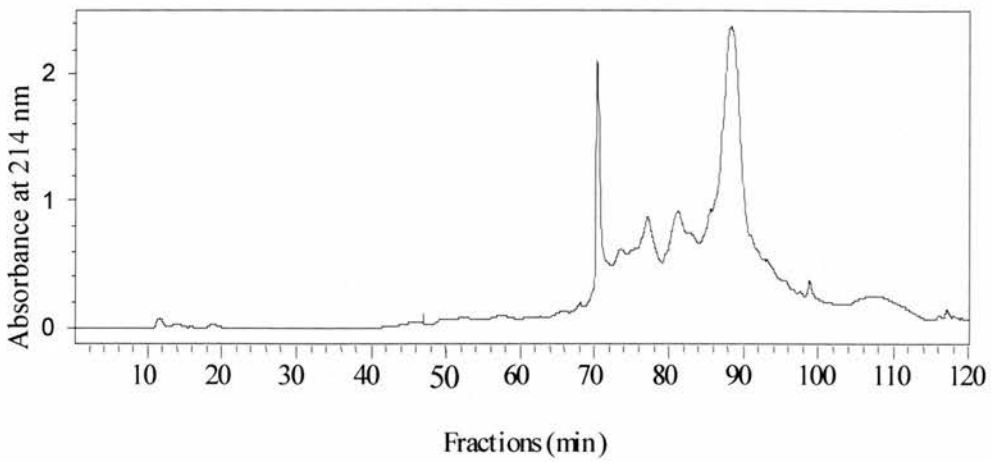


Figure 3.2 Chromatographic analyses of antibacterial activity against *Y#* of 40 % acetonitrile Sep-Pak elution fraction. (a) C_{18} RP-HPLC of 40 % acetonitrile Sep-Pak elution fraction eluted with 0.1 % TFA to 100 % ACN/0.1 % TFA gradient, as described in Section 3.2.4. The eluant was monitored at 214 nm and 1 ml fractions were collected at 1 ml min^{-1}. (b) Antibacterial activity in the fractions after lyophilisation, reconstitution in 60 µl of 50 mM HEPES (pH 7.0) and 3 µl of each reconstituted fraction assayed against Y# with RDA. Y-axis variables on the left refer to the histogram as the clear zone area minus the area of the well (mm^2). Antibacterial activity can be seen for fractions 71 to 80. No antibacterial activity was detected for fractions 1 to 70 or 81 to 120.

(a)



(b)

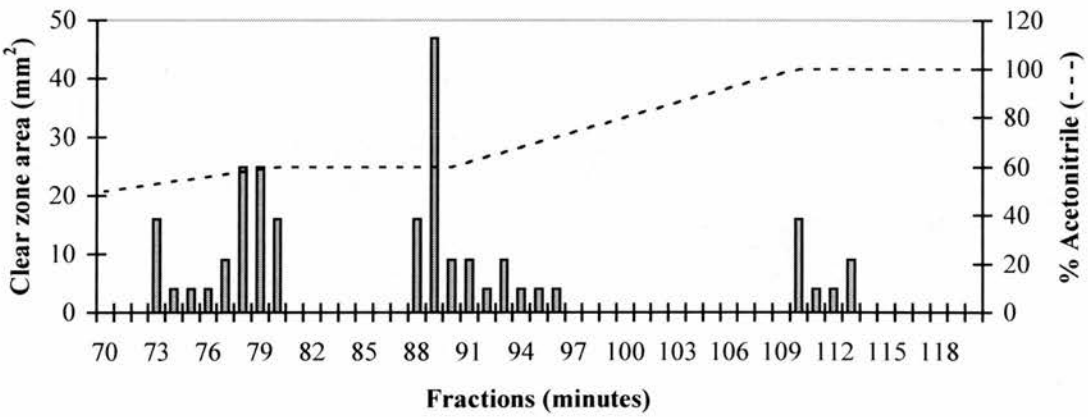
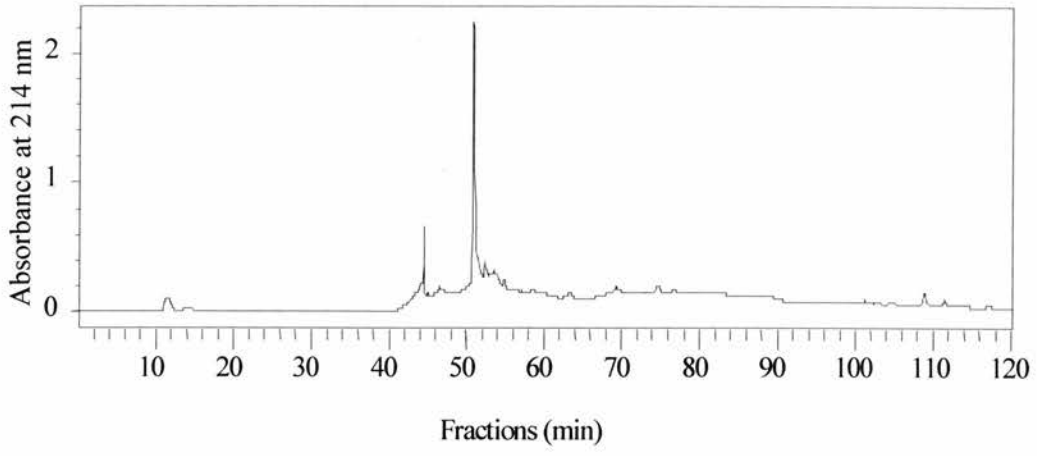


Figure 3.3 Chromatographic analyses of antibacterial activity against *Y#* of 60 % acetonitrile Sep-Pak elution fraction. (a) C_{18} RP-HPLC of 60 % acetonitrile Sep-Pak elution fraction eluted with 0.1 % TFA to 100 % ACN/0.1 % TFA gradient, as described in Section 3.2.7. The eluant was monitored at 214 nm and 1 ml fractions were collected at 1 ml min^{-1}. (b) Antibacterial activity in the fractions after lyophilisation, reconstitution in 60 µl of 50 mM HEPES (pH 7.0) and 3 µl of each reconstituted fraction assayed against Y# with RDA. Y-axis variables on the left refer to the histogram as the clear zone area minus the area of the well (mm^2). Strong antibacterial activity can be seen for fractions 73 to 80, 88 to 96 and 110 to 113. No antibacterial activity was detected for fractions 1 to 70.

(a)



(b)

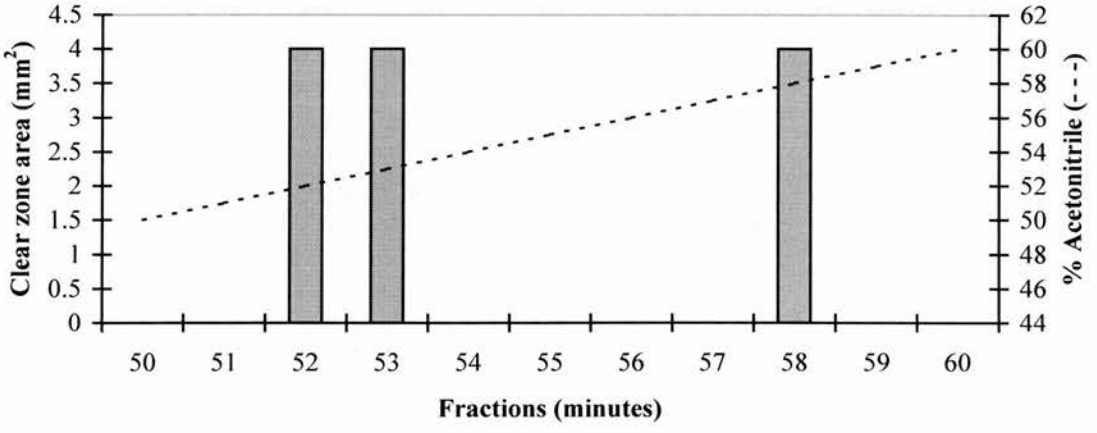


Figure 3.4 Chromatographic analyses of antibacterial activity against *Y#* of 90 % acetonitrile Sep-Pak elution fraction. (a) C_{18} RP-HPLC of 90 % acetonitrile Sep-Pak elution fraction eluted with 0.1 % TFA to 100 % ACN/0.1 % TFA gradient, as described in Section 3.2.7. The eluant was monitored at 214 nm and 1 ml fractions were collected at 1 ml min^{-1}. (b) Antibacterial activity in the fractions after lyophilisation, reconstitution in 60 µl of 50 mM HEPES (pH 7.0), 3 µl of each reconstituted fraction assayed against Y# with RDA. Y-axis variables on the left refer to the histogram as the clear zone area minus the area of the well (mm^2). Antibacterial activity can be seen for fractions 52, 53 and 58. No antibacterial activity was detected for fractions 1 to 50 or 60 to 120.

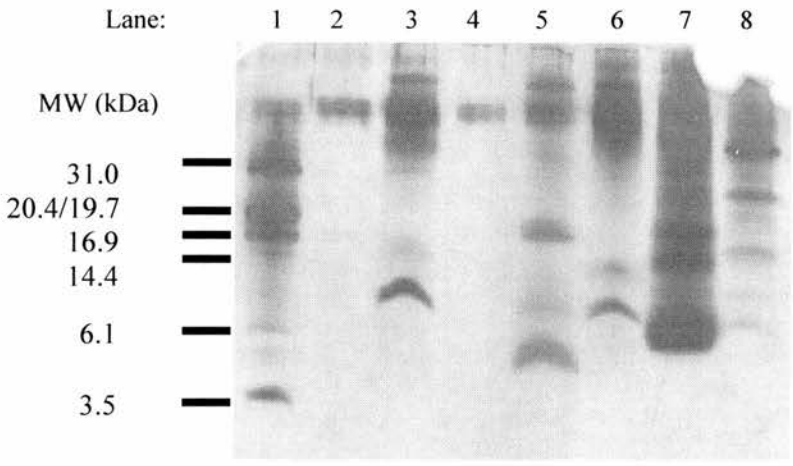


Figure 3.5 Tris-Tricine SDS-PAGE profile (silver stained) of active fractions from RP-HLPC of 40 and 60% acetonitrile Sep-Pak elution fractions. Lane 1: low weight molecular markers; lane 2: sample buffer; lane 3: fraction 76 from RP-HPLC of 40 % Sep-Pak eluate; lane 4: sample buffer; lane 5: fraction 73 from RP-HPLC of 60 % Sep-Pak eluate; lane 6: fraction 78 from RP-HPLC of 60 % Sep-Pak eluate; lane 7: fraction 89 from RP-HPLC of 60 % Sep-Pak eluate; lane 8: fraction 110 from RP-HPLC of 60 % Sep-Pak eluate. Each lane contains 10 µl of sample. The numbers on the left are the molecular mass of the markers in kDa.

A similar protein band, with an apparent molecular weight between 6.1 to 14.4 kDa was observed on an SDS-PA gel stained with silver for fraction 76 from the RP-HPLC of the 40 % acetonitrile Sep-Pak eluate and for fraction 78 from the RP-HPLC of the 60 % acetonitrile Sep-Pak eluate (Figure 3.5), indicating that this may be the factor responsible for the strong antibacterial activity of each of these fractions.

Antibacterial activity against Y# of pooled fractions (88 to 90) from RP-HPLC of 60% acetonitrile Sep-Pak eluate after incubation with Proteinase K was weaker than that for the pooled fractions incubated without the addition of Proteinase K (Figure 3.6), but did not abolish it altogether, indicating that the antibacterial activity may be of proteinaceous nature. No antibacterial activity was observed for the negative control, solution B, (Figure 3.6).

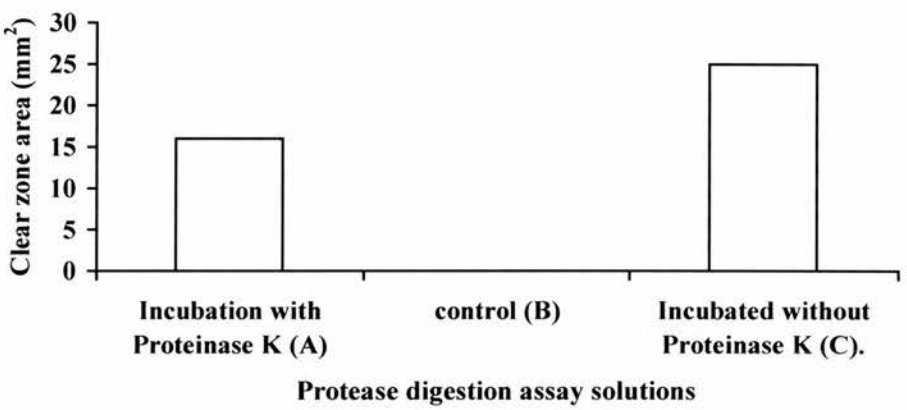


Figure 3.6 Antibacterial activity of protease digestion assay solutions. Antibacterial activity of pooled active fractions 88-90, from C_{18} RP-HPLC of 60% acetonitrile Sep-Pak eluate (step HPLC.1) after incubation with (A) and without (C) the addition of Proteinase K, 4 µl of each solution assayed against Y# by RDA.Y-axis variables are the clear zone area minus the area of the well (mm^2). Stronger antibacterial activity was detected for the test sample without addition of Proteinase K (C), whilst no antibacterial activity was observed for Proteinase K at the concentration used (B).

No antibacterial activity was detected against Y# for any of the fractions from RP-HPLC of pooled active fractions 77 to 80, 88 to 90 or 110 to 113 (step HPLC.2) from RP-HPLC of 60 % acetonitrile elution fraction (step HPLC.1), although the positive controls showed strong activity (not shown).

3.4 Discussion

The present study shows that fractionation by hydrophobic interaction was successful in separating the extract as no antibacterial activity was found in any of the fractions from RP-HPLC of the 20 % Sep-Pak elution fraction. By contrast, antibacterial activity was detected in fractions from RP-HPLC of the 40, 60 and 90 % acetonitrile Sep-Pak elutates. Interestingly, for each RP-HPLC of the 40, 60 and 90 % Sep-Pak fractions, antibacterial activity was found for those fractions corresponding to elution with 50 to 60 % acetonitrile. Although it could be coincidental, it is possible that the same factor could have been present in each of the Sep-Pak eluates. SDS-PAGE of active fractions corresponding to elution at 50 to 60 % acetonitrile from RP-HPLC of the 40 and 60 % Sep-Pak eluates also indicate that the same protein might have been present in both the 40 and 60 % Sep-Pak fractions. If this is the case, then elution of the Sep-Pak with 60 % acetonitrile is optimal as this eluate contained the greatest number of HPLC fractions with the strongest antibacterial activity eluted at 50 to 60 % acetonitrile.

The 60 % Sep-Pak eluate was also the only fraction that contained an antibacterial factor that eluted from the HPLC column at a high concentration of acetonitrile (100 %), showing that more than one antibacterial factor was present in the phosphate buffer extract. It is also possible that there may have been other antibacterial factors present in the extract. The techniques used for the fractionation of the extract may not have been applicable for such factors. In particular, it is possible that other antibacterial factors may have precipitated during the first stage of ammonium sulphate precipitation, i.e. 35 % relative saturation of ammonium sulphate. However, this stage is vital due to the inherent difficulties of the presence of

carbohydrates and chromophore-containing material during subsequent stages of fractionation, by such components competing for binding sites on chromatographic matrixes and also becoming irreversibly bound due to the hydrophobic nature of the material (Benevides *et al.* 1998; Sampaio *et al.* 1998).

The lack of antibacterial activity against the Gram-negative bacterium, *P. immobilis,* for any of the fractions from RP-HLPC of the Sep-Pak eluants was expected as the phosphate buffer extract, reported in Chapter 2, was also found not to have antibacterial activity against this bacterium. The culmination of these results indicate that the antibacterial factors present in the phosphate buffer extract were only active against Gram-positive bacteria, although further assays of susceptibility against a greater number of Gram-negative strains is required before this could be ascertained.

The protease digestion assay of fractions 88 to 90 (the fractions with the strongest antibacterial activity) from RP-HPLC of the 60 % Sep-Pak fraction indicated that the factor responsible for the antibacterial activity observed for these fractions appeared to be proteinaceous. However, as antibacterial activity was present after incubation with the protease there may have been one or more non-proteinaceous factor(s) present in the sample. Alternatively, it may be possible that the protease cleaved protein into active fragments. Repeating the assay using an alternative protease with different cleavage sites than that for Proteinase K, such as α-chymotrypsin, may have elucidated this. However, by SDS-PAGE, it was clear that there were numerous proteins present in fraction 89, so further fractionation of the sample was performed using a narrower-bore C_{18} RP-HPLC column to achieve improved resolution of the eluting fractions, with the aim that the resulting fractions with antibacterial activity

could be subjected to the protease digestion assay using α-chymotrypsin. Unfortunately, no antibacterial activity was found in any of the fractions gained from this second step. It is unlikely that the conditions and reagents used for the second HPLC caused the complete loss of activity as similar conditions were used for the first chromatography. Most probable is the consequence of dilution of the sample prior to, during and after the second HPLC. In addition to the accepted losses during lyophilisation, unavoidable dilution of the sample could have occurred by the antibacterial factor adhering by hydrophobic interactions to any surface it is in exposed to, such as the fraction microtubes, pipette tips, HPLC glass injection syringe and the HPLC system pipes (Scope 1993). To account for such losses, a greater yield of starting material could be used so that the antibacterial factor(s) could be isolated before dilution results in the factor(s) becoming undetectable by RDA.

Chapter 4

Fractionation of ethanol and methanol cellular extracts of *Phaeodactylum tricornutum*

4.1 Introduction

This chapter describes the fractionation of ethanol and methanol cellular extracts of *P. tricornutum* with the aim of isolating and characterising antibacterial agents responsible for the antibacterial activity of the extracts. Extraction of *P. tricornutum* cells in 60% ethanol or 100% methanol was chosen for fractionation as extracts using these solvents were found in Chapter 2 to have antibacterial activity against Gram-positive and Gram-negative bacteria.

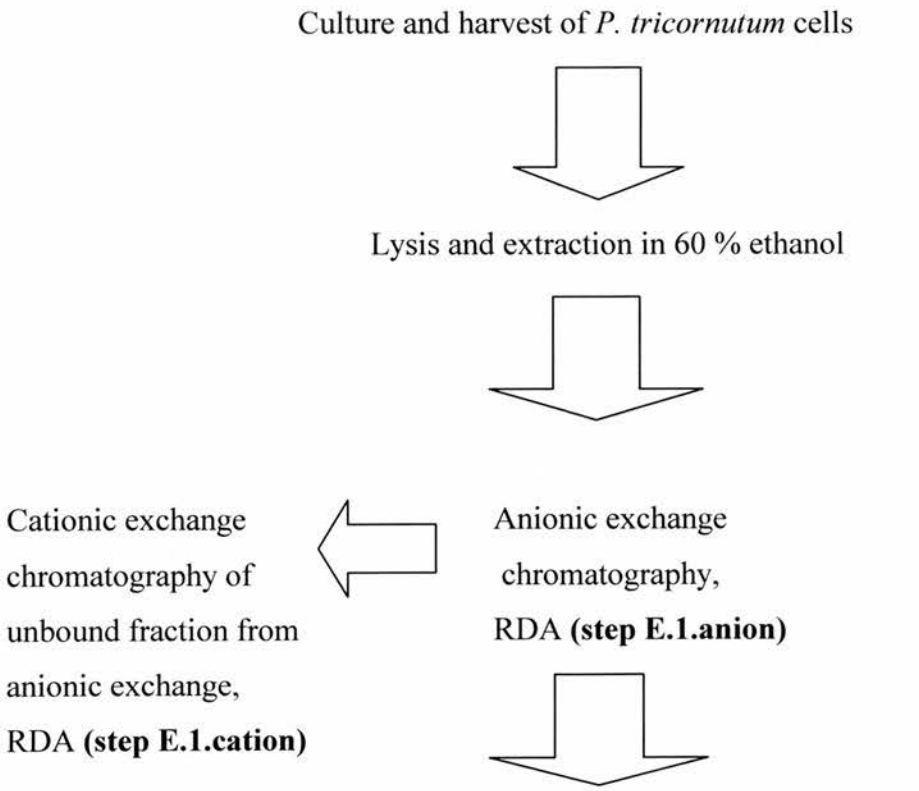
The techniques employed for the separation of the extracts were ion exchange chromatography and reversed phase-high performance liquid chromatography. These techniques are typically used for protein purification as the biological activity of proteinaceous material can be retained for subsequent assays (Scope 1993).

4.2 Methods

4.2.1 Fractionation of *P. tricornutum* ethanol extract

A 60 % ethanol extract of *P. tricornutum* cells was fractionated by anionic and cationic exchange chromatography, followed by RP-HPLC, as summarised in Figure 4.1. Antibacterial activity of fractions, after each stage of fractionation, were determined with RDA. The detailed methodology of each step is described in the accompanying Sections.

Figure 4.1 Flowchart of the fractionation of *P. tricornutum* ethanol extract



Unless specified, chemicals were acquired from Sigma-Aldrich. All solutions are prepared with deionised or ultra pure deionised water (Elga), except when specified.

4.2.1.1 Preparation and ethanol extraction of *P. tricornutum* cells

P. tricornutum was grown to late exponential phase (7.5×10^6 cells ml^{-1}) in 20 l ESAW and cells harvested from 18 l culture as described in Section 2.2.4. After thawing, the cell pellet was resuspended to 80 ml with 60 % (v/v) ethanol and cells lysed by ultrasonication (4 cycles of 5 minutes at 100 % power with ½ second pulses). The lysate was mixed on an orbital shaker for 60 minutes (60 rev. min^{-1}) before centrifuging at 4,000 × g for 15 min at 4 °C. The decanted supernatant was snap-frozen in liquid nitrogen and solvent removed by lyophilisation.

4.2.1.2 Anionic exchange chromatography of extract (step E.1.anion)

The lyophilised extract was reconstituted in 20 ml of 20 mM HEPES (pH 8.0) then centrifuged at 4,000 × g for 10 min at 4 °C. The supernatant was loaded on an 8 × 1 cm DEAE Sepharose Fast Flow column previously equilibrated in 20 mM HEPES (pH 8.0). The unbound fraction was collected for subsequent cationic exchange chromatography (step E.1.cation). The column was eluted at 1 ml min^{-1} with a linear gradient of 0-1 M NaCl in 20 mM HEPES (pH 8.0) over 100 minutes. The eluate was monitored at an absorbance of 280 nm using a flow cell (set to 1 absorbance unit, AU, full scale deflection, FSD, Econo System; BioRad) and collected as 1 ml fractions. Each fraction was concentrated by lyophilisation, reconstituted in 100 µl 50 mM HEPES (pH 8.0) and 4 µl of each fraction assayed for antibacterial activity using *Psychrobacter immobilis* (NCIMB 308) or Y# as test bacteria as described in Section 2.2.6.4.

4.2.1.3 Cationic exchange chromatography of unbound fraction from anionic exchange chromatography (step E.1.cation)

The unbound fraction from the anionic exchange chromatography (step E.1.anion) was adjusted to pH 6.0 with HCl, then combined 1:2 (v/v) with 20 mM NH_4 Ac. (pH 6.0) and loaded on an 8 × 1 cm CM Sepharose Fast Flow column previously equilibrated to 20 mM NH_4 Ac. (pH 6.0). The column was eluted at 1 ml min^{-1} with a linear gradient of 0-1 M NaCl in 20 mM NH_4 Ac. (pH 6.0) over 80 minutes. The eluate was monitored at 280 nm (set to 1 AU FSD, Econo System; BioRad) and collected as 1 ml fractions. Following chromatography, the fractions were concentrated by lyophilisation, reconstituted in 100 µl of 50 mM HEPES (pH 8.0) and 4 µl from each fraction assayed for antibacterial activity using *Psychrobacter immobilis* (NCIMB 308) or Y# as test bacteria as described in Section 2.2.6.4.

4.2.1.4 Separation by RP-HPLC of anionic exchange fractions (step E.2)

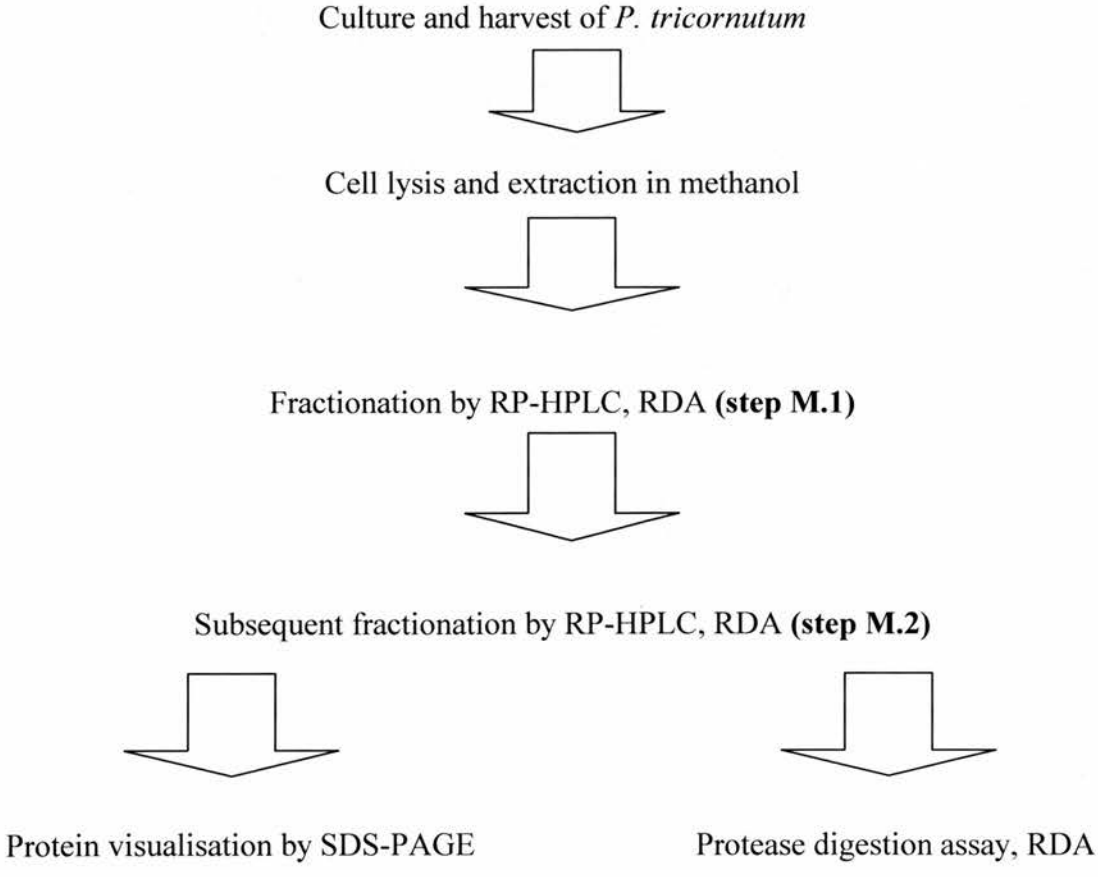
The remainder of fractions, 45 to 49 from anionic exchange chromatography (step E.1.anion) were pooled, lyophilised and reconstituted in 1 ml of 5 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA. The sample was filtered through a 0.2 µm cartridge filter (Anatop 10LC, Whatman) and applied to a C_{18} column (250 × 4.6 mm; Rsil HL, BioRad) previously equilibrated in 5 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA. The column was eluted at 1 ml min^{-1} with the following gradients (solvent B is 95 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA), 0-20 min, 0 % (v/v) B; 20-80 min, 0-60 % (v/v) B; 80-100 min, 60-100 % (v/v) B; 100-120 min, 100 % (v/v) B. The eluate was monitored using the PDA at 210 to 400 nm. One millilitre fractions of the eluate were collected, solvent removed by lyophilisation and reconstituted in 60 µl of ultra pure deionised water before

assaying 4 µl of each fraction for antibacterial activity by RDA using *P. immobilis* (NCIMB 308) as described in Section 2.2.6.4.

4.2.2 Fractionation of *P. tricornutum* methanol extract

A methanol extract of *P. tricornutum* cells was prepared then fractionated by RP-HPLC, as summarised in Figure 4.2. The antibacterial activity of fractions was determined by RDA after each chromatography. The protein profile of antibacterial fractions were visualised by SDS-PAGE and subjected to protease digestion. The detailed methodology of each step is described in the accompanying Sections.

Figure 4.2 Flowchart of the fractionation of *P. tricornutum* methanol extract



4.2.2.1 Preparation and methanol extraction of *P. tricornutum* cells

P. tricornutum was grown to late exponential phase (6.8 $\times 10^6$ cells ml^{-1}) in 10 l ESAW and harvested from 9 l culture as described in Section 2.2.4. After thawing, the cell pellet was resuspended 1:4 (v/v) in methanol and ultrasonicated (5 cycles of 5 minutes at 100% power with ½ second pulses). The cell lysate was kept on ice and mixed on an orbital shaker for 60 minutes (60 rev. min^{-1}) before centrifuging at 4,000 × g for 30 minutes at 4 °C. The decanted supernatant was snap-frozen in liquid nitrogen and solvent removed by lyophilisation. The contents remaining after lyophilisation were reconstituted in 2 ml of 70 % (v/v) HPLC-grade methanol (BDH) and centrifuged at 15,300 × g for 10 min at 4 °C before filtering the supernatant through a 0.2 μm cartridge filter (Anatop 10LC, Whatman).

4.2.2.2 Fractionation of methanol extract by RP-HPLC (step M.1)

The filtrate (1 ml) was loaded on a C_{18} column (250 mm × 10 mm; ODS2, Capital HPLC) previously equilibrated in 70 % HPLC-grade methanol (BDH). The column was eluted at 2 ml min^{-1} with the following gradients (solvent B is 95 % (v/v) HPLC-grade methanol; BDH), 0-10 min, 0-90 % (v/v) B; 10-15 min, 90-100 % (v/v) B; 15-25 min, 100 % (v/v) B. The eluate was monitored using the PDA and 1 ml fractions were collected. The solvent was removed by lyophilisation and the sample in each fraction reconstituted in 60 μl of 50 mM HEPES (pH 8.0). Three microlitres from each fraction were assayed for antibacterial activity by RDA using *P. immobilis* (NCIMB 308) or Y# as test bacteria as described in Section 2.2.6.4.

4.2.2.3 Subsequent separation by RP-HPLC of fractions from initial HPLC (step M.2)

Fractions 30 to 43 from the initial separation (step M.1) were pooled, lyophilised and reconstituted in 1 ml of 5 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA, filtered through a 0.2 μm cartridge filter (Anatop 10LC, Whatman), and applied to a C_{18} column (250 × 4.6 mm; Rsil HL, BioRad) previously equilibrated in 5 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA. The column was eluted at 1 ml min^{-1} with the following gradients (solvent B is 95 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA), 0-5 min, 0 % (v/v) B; 5-25 min, 0-60 % (v/v) B; 25-65 min, 60-100 % (v/v) B; 65-85 min, 100 % (v/v) B. The eluate was monitored using a PDA (Waters) and 1 ml fractions collected. The solvent was removed by lyophilisation and each sample reconstituted in 60 μl of 20 mM HEPES (pH 7.8) before assaying 4 μl from each fraction for antibacterial activity by RDA using Y# as test bacterium as described in Section 2.2.6.4.

Pooled fractions, 45 to 66 from the initial HPLC (step M.1), were also subjected to further separation by RP-HPLC using the protocol described above with the following different elution gradients, 0-10 min, 0 % (v/v) B; 10-70 min, 0-60 % (v/v) B; 70-110 min, 60-100 % (v/v) B; 110-120 min, 100 % (v/v) B. The eluate was monitored, collected and tested for antibacterial activity as described above.

4.2.2.4 Protease digestion and SDS-PAGE of active fractions from step M.2

Fractions 29 and 34 from the RP-HPLC (step M.2) of pooled fractions 45 to 66 from the initial HPLC (step M.1) were subjected to digestion with Proteinase K as described in Section 3.2.6. Five microlitres of each test solution, after incubation, was assayed for antibacterial activity by RDA using Y# as test bacterium as described in Section 2.2.6.4.

The protein content of fractions 29 and 34 were visualised by high resolution SDS-PAGE and the gel stained with silver, as described in Section 3.2.5.

4.3 Results

4.3.1 Fractionation of ethanol extract

No antibacterial activity was observed on RDA plates seeded with *P. immobilis* or Y# using fractions from cationic exchange chromatography (step E.1.cation), although positive controls showed strong activity (not shown). Antibacterial activity was also not observed against Y# using fractions from anionic exchange chromatography (step E.1.anion) (not shown). However, antibacterial activity was detected for fractions 45 to 69 from step E.1.anion against *P. immobilis* (Figure 4.3).

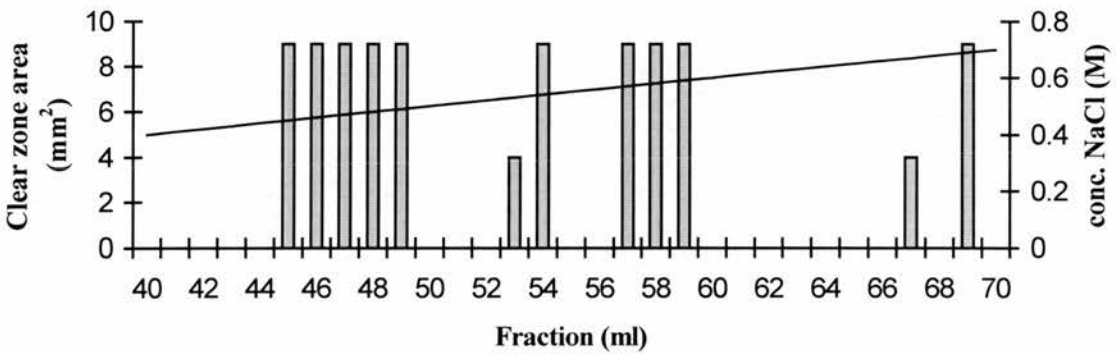


Figure 4.3 Antibacterial activity against *P. immobilis* of fractions from anionic exchange of ethanol extract. The histograms refer to the clear zone area and the line graph to the NaCl concentration. Four distinct groups of fractions with antibacterial activity can be observed, eluted at 0.45 to 0.69 M NaCl. No antibacterial activity was detected in fractions 1 to 44 or 70 to 100. The chromatograph is not displayed as no peaks of absorbance were detected with the settings used.

No antibacterial activity was detected against *P. immobilis* or Y# for any of the fractions from RP-HPLC of anionic exchange pooled fractions 44 to 49 (step E.2), although the positive controls showed strong activity (not shown).

4.3.2 Fractionation of methanol extract

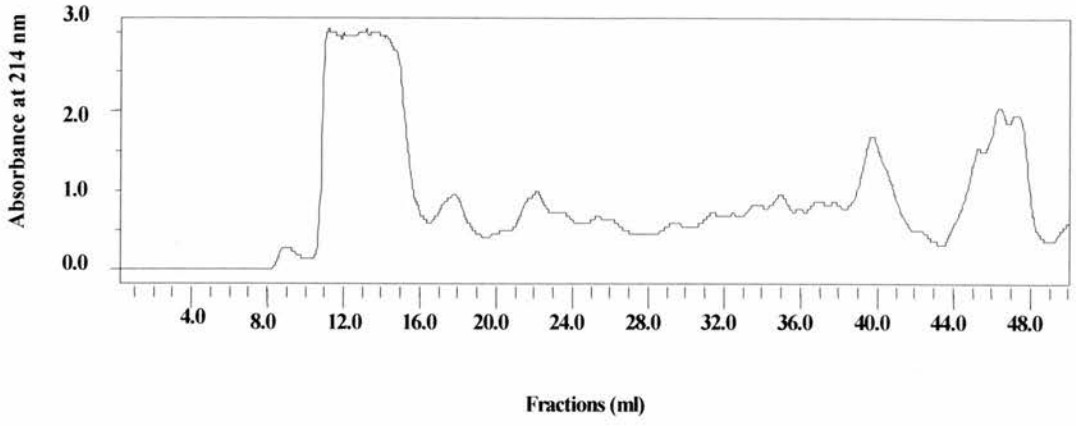
No antibacterial activity was detected against *P. immobilis* for any of the fractions from RP-HPLC of the methanol extract (step M.1), although the positive controls showed strong activity (not shown). However, antibacterial activity was detected against Y# in fractions 21 to 66, eluted at 90-100 % methanol (Figure 4.4)

Similarly, antibacterial activity was not detected against Y# for any of the fractions from RP-HPLC (step M.2) of pooled fractions 30-43 from the initial RP-HPLC of the methanol extract (step M.1), although again positive control showed strong activity (not shown).

By contrast, strong antibacterial activity was detected against Y# for fractions 29 and 34 eluted at 85-90 % acetonitrile from RP-HPLC (step M.2) of pooled fractions 45 to 66 from step M.1 (Figure 4.5). Fraction 29 corresponds to an absorbance peak at 28 to 29 minutes (Figure 4.5).

There was no difference in the antibacterial activities of fractions 29 (60 mm^2) or 34 (16 mm^2) incubated with and without Proteinase K, indicating that the factor(s) responsible for the antibacterial activity in these fractions is/are not of proteinaceous nature. No antibacterial activity was observed for the negative control, solution B.

(a)



(b)

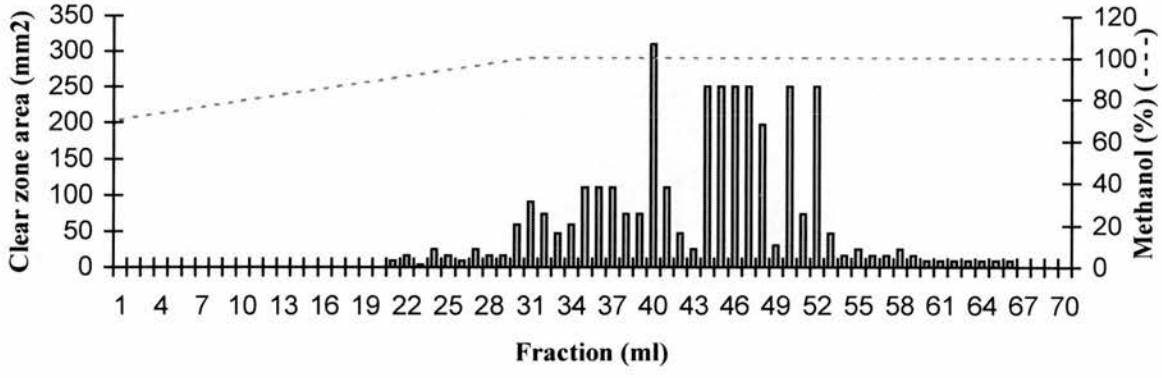
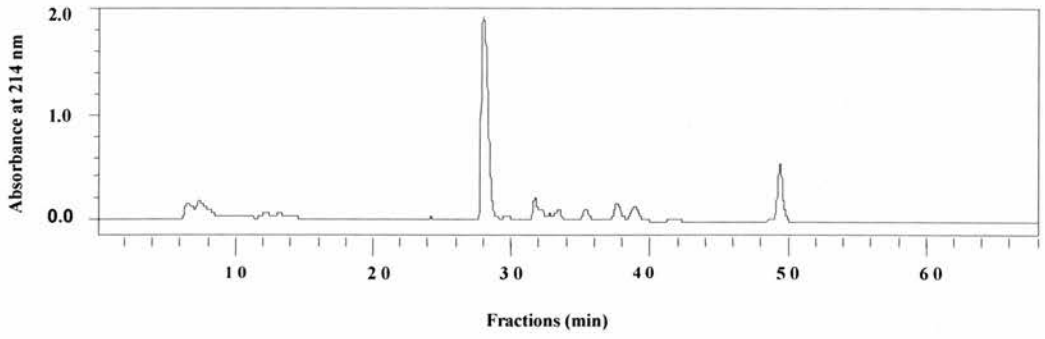


Figure 4.4 Chromatographic analyses of antibacterial activity of methanol extract (step M.1.). (a) C_{18} RP-HPLC of methanol extract eluted with 70-100% methanol at 2 ml min^{-1} and 1 ml fractions collected. The eluant was monitored at 214 nm for a pre-set duration of 25 min, 1 ml fractions were then collected for a further 10 min, as absorbance peaks continued to be observed, but no chromatograph was recorded.
(b) Antibacterial activity in the fractions after lyophilisation, reconstitution in 60 µl of 50 mM HEPES (pH 8.0) and assay against Y# with RDA. Y-axis variables on the left refer to the histogram as the clear zone area minus the area of the well (mm^2). Strong antibacterial activity can be observed for numerous fractions eluted at 100% methanol, whilst no antibacterial activity is evident in fractions 1 to 20 or 67 to 70.

(a)



(b)

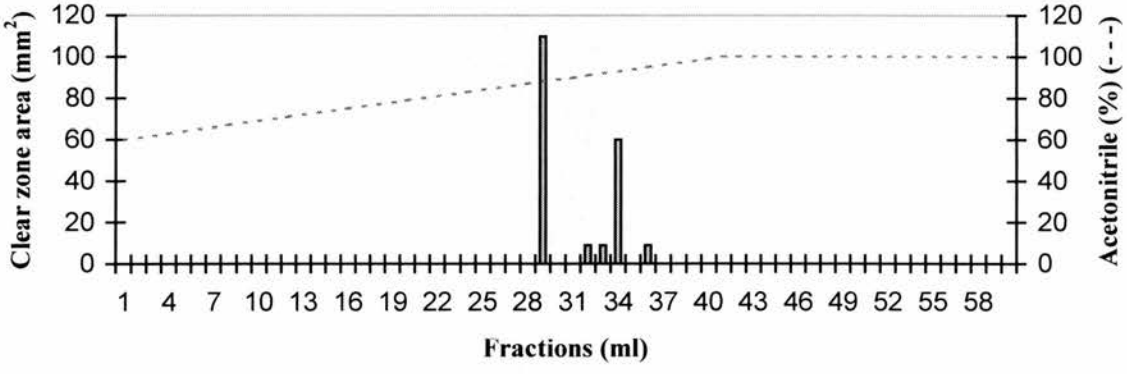


Figure 4.5 Chromatographic analyses of antibacterial activity in fractions 45 to 66 from step M.1 (step M.2.) (a) C_{18} RP-HPLC of pooled fractions 45 to 66 eluted with 5 % ACN/0.07 % TFA to 95% ACN/0.07 % TFA gradient, as described in Section 4.2.2.3. The eluant was monitored at 214 nm and 1 ml fractions were collected at 1 ml min^{-1}. (b) Antibacterial activity in the fractions after lyophilisation, reconstitution in 60 µl of 20 mM HEPES (pH 8.0) and assay against Y# with RDA. Y-axis variables on the left refer to the histogram as the clear zone area minus the area of the well (mm^2). Strong antibacterial activity can be seen for fractions 29 and 34. No antibacterial activity was detected for fractions 1 to 28 or 37 to 60.

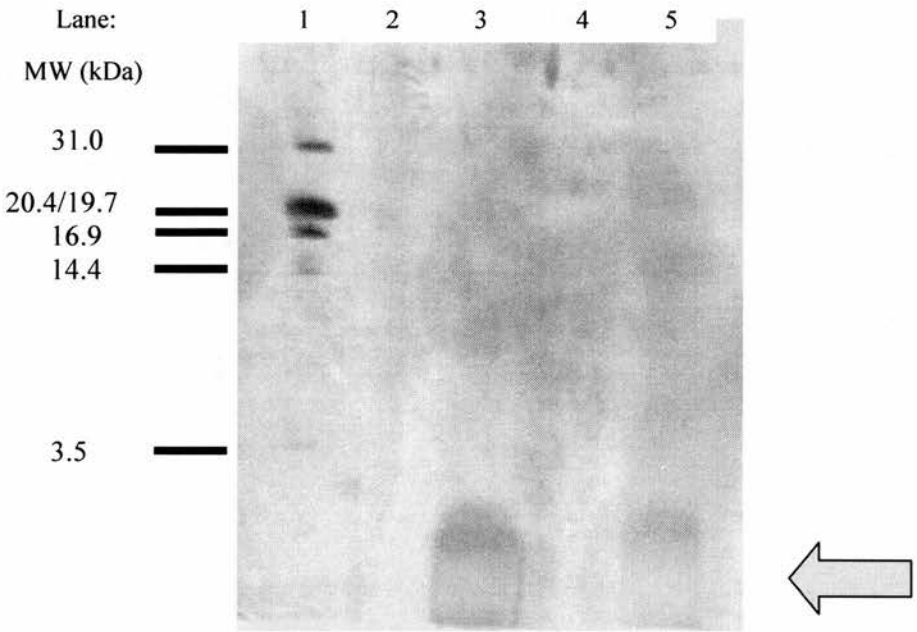


Figure 4.6 SDS-PAGE profile of active fractions 29 and 34 from step M.2. Lane 1: low-range markers; Lane 2: sample buffer; lane 3: fraction 29; lane 4: sample buffer; lane 5: fraction 34. 10 µl of sample was applied per lane. Over-developed silver staining revealed a 'smudge' with an apparent molecular weight of <3.5 kDa in the lanes containing fraction 29 and 34, indicated by the arrow.

4.4 Discussion

Although antibacterial activity of the 60 % ethanol extract of *P. tricornutum* cells was successfully fractionated by anionic exchange chromatography at pH 9.0, no activity was found in any of the fractions from the subsequent cationic exchange chromatography at pH 6.0, indicating that the factor or factors responsible for the activity are anionic. Alternatively, antibacterial cationic agents were present in the extract but did not bind to the ion exchange columns under the conditions used.

As the fractions from the anion exchange column were active against the Gram-negative *P. immobilis* but not the Gram-positive Y#, it is likely that the bioactive factor(s) are active against only Gram-negative bacteria. However, a wider range of bacteria needs to be screened for susceptibility before conclusions can be made. Notwithstanding, *P. immobilis* was used as the "indicator microorganism" for the subsequent RP-HPLC fractionation.

Unfortunately, even with this bacterium, no antibacterial activity was detected in any of the fractions collected after RP-HPLC. However, as the antibacterial activity of the fractions from anionic exchange was very weak, the antibacterial factor or factors might have been diluted or biological activity affected, as sample losses are expected due to the harsh conditions employed for HPLC (Scope 1993). It is for this reason that the other antibacterial fractions from anionic exchange were not subjected to HPLC. Possibly a larger volume of *P. tricornutum* cells in the starting material might be appropriate to gain a higher yield of antibacterial factor(s), but the yield of cells from culture was always limited in the present study.

With the methanol extract, antibacterial activity was detected against Y# but not *P. immobilis* in fractions 21 to 59 from the first RP-HPLC (M.1). This indicates that the antibacterial factor or factors present in the methanol extract may only be active against Gram-positive bacteria. Although again, a greater number of Gram-negative bacteria need to be tested before this can be properly assessed. Cannell *et al.* (1988) and Kellam and Walker (1989) have also found that methanol extracts of a range of microalgae species appeared to be solely active against Gram-positive bacteria.

Surprisingly, the strong antibacterial activity against Y# was lost upon the second RP-HPLC of fractions 30 to 43 from the first HPLC (M.1). Since the nature of the bio-active factor(s) at this stage is unknown, it is possible that the reagents, acetonitrile and TFA, used for this second chromatography could have had a detrimental effect on the biological activity of the sample through conformational or charge changes. As these reagents are commonly used as a volatile buffer system for the separation of proteins or peptides by HPLC (Mant and Hodges 1991) it is unlikely that the use of these reagents would cause the complete loss of biological activity of proteinaceous samples. It therefore follows that the factor(s) responsible for the strong antibacterial activity in fractions 30 to 43 from the initial HPLC may not be proteinaceous.

By contrast, second step RP-HPLC was successful in fractionating the antibacterial activity observed in the later fractions (45 to 66) from the first HPLC (M.1). As three groups of fractions with strong antibacterial activity against Y# were eluted from 85 to 100 % acetonitrile, three separate hydrophobic factors may be present.

The two fractions with the strongest activity (29 and 34), were resistant to protease digestion with Proteinase K indicating that they were not proteinaceous in nature. The hydrophobic nature of the active factors could account for their antibacterial activity, as this would permit binding to the cytoplasmic membrane of bacteria thereby causing major disorganisation and loss of function of the membrane (Mann and Crabbe 1996; Piddock 1998; Epand and Vogel 1999). Detergents, phenolic compounds or long-chain fatty acids are examples of this type of antimicrobial agent. Alternatively, a hydrophobic molecule may insert into the membrane, forming a pore, which may affect the membrane's permeability to ions (Mann and Crabbe 1996; Piddock 1998).

Importantly, the appearance of a silver stained 'smudge' in the SDS-PAGE gel run with fractions 29 and 34 from the second stage chromatography (M.2) is indicative that the factor(s) responsible for activity could be a fatty acid (Dzandu *et al.* 1984). Certainly, many microalgae are known to produce fatty acids with antibacterial activity (Pratt *et al.* 1944; Findlay and Patil 1984; Cooper *et al.* 1985; Pesando 1990). Cooper *et al.* (1985) identified an antibacterial factor from sequential organic solvent extraction of *P. tricornutum* cells to be a mixture of fatty acids. Mass spectrometry of this fatty acid mixture revealed 6 trimethylsilyl esters (TMS) derivatives of tetradecanoic, hexadecatetraenoic, hexadecadienoic, octadecatetraenoic and eicosapentaenoic fatty acids (Cooper *et al.* 1985). The relative proportions of these fatty acids were shown to vary quite considerably with the growth phase of *P. tricornutum* (Cooper *et al.* 1985). To determine the antibacterial activity of the fatty acids, model fatty acid standards, which chemically resembled those identified, were tested against *Bacillus subtilis* or *Vibrio parahaemolyticus* (Cooper *et al.* 1985).

Interestingly, only the longer carbon skeleton linolenic and arachidonic acids were found to be active against the bacteria tested (Cooper *et al.* 1985). In this study, no further characterisation was done with the remaining contents of the antibacterial fractions after SDS-PAGE due to the minute quantity remaining.

Chapter 5

Isolation and characterisation of an antibacterial ammonium phenolate from *P. tricornutum* cells

5.1 Introduction

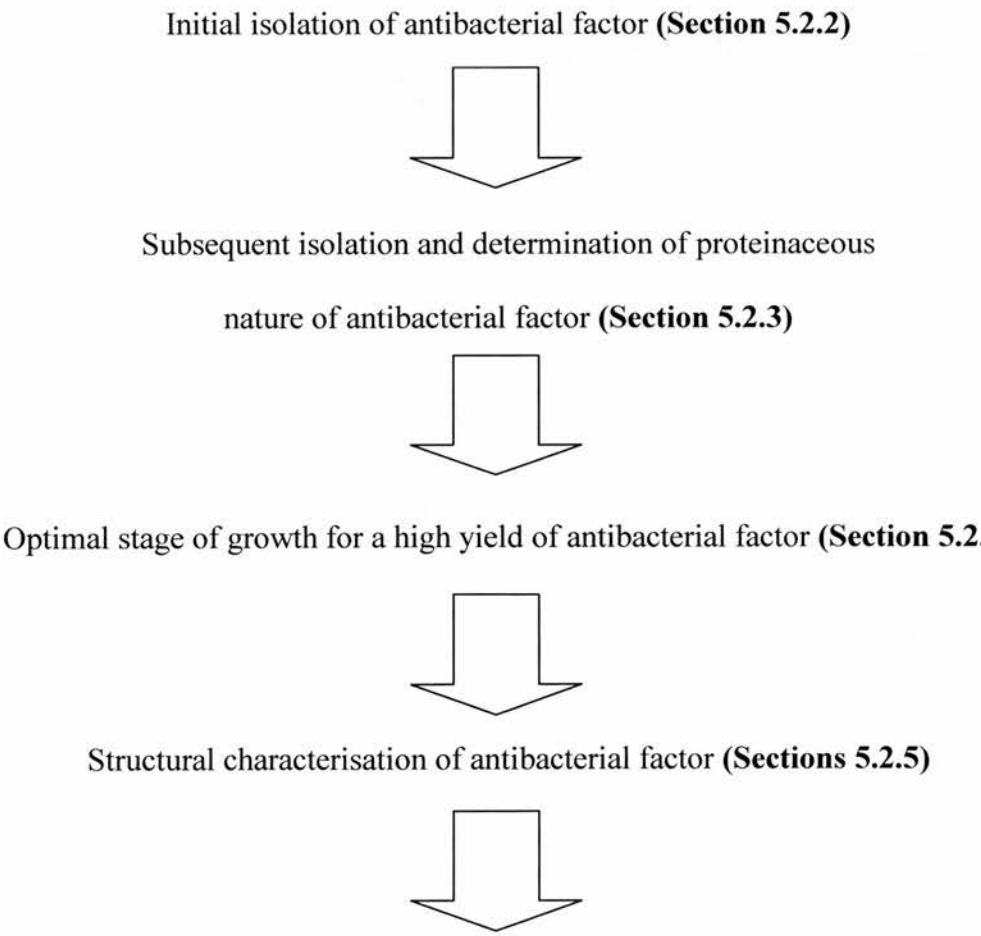
This Chapter describes the fractionation of a methanol cellular extract of *P. tricornutum*, with the aim of isolating and characterising antibacterial agents responsible for the antibacterial activity of the extract. Extraction of *P. tricornutum* cells in methanol was chosen for further investigation as extracts using this methanolic solvent were found in Chapters 2 to have antibacterial activity against Gram-positive and Gram-negative marine bacteria, yet in Chapter 4, the factor with activity against Gram-negative bacteria was not isolated.

High performance liquid chromatography was used for the fractionation of the extracts as this technique was successfully used in Chapter 4 for isolating an antibacterial factor from a methanol extract. A series of biochemical techniques were to be employed to determine the proteinaceous nature of any antibacterial factors gained, and nuclear magnetic resonance spectroscopy for structural characterisation.

5.2 Methods

Initially, a methanol extract of *P. tricornutum* cells was fractionated for antibacterial activity. The fractionation was then repeated for a subsequent extract to determine the proteinaceous nature of an antibacterial factor. The optimal stage of growth of *P. tricornutum* for high yield of the antibacterial factor was determined before characterising the factor by it's structure and assaying for spectrum of antibacterial and haemolysis activity as summarised in Figure 5.1. The detailed methodologies for each experiment are described in the accompanying sections.

Figure 5.1 Flowchart of methanol extract fractionation experiments



Unless specified, chemicals / reagents were acquired from Sigma-Aldrich. All solutions are prepared with deionised or ultra pure deionised water (Elga), except where specified.

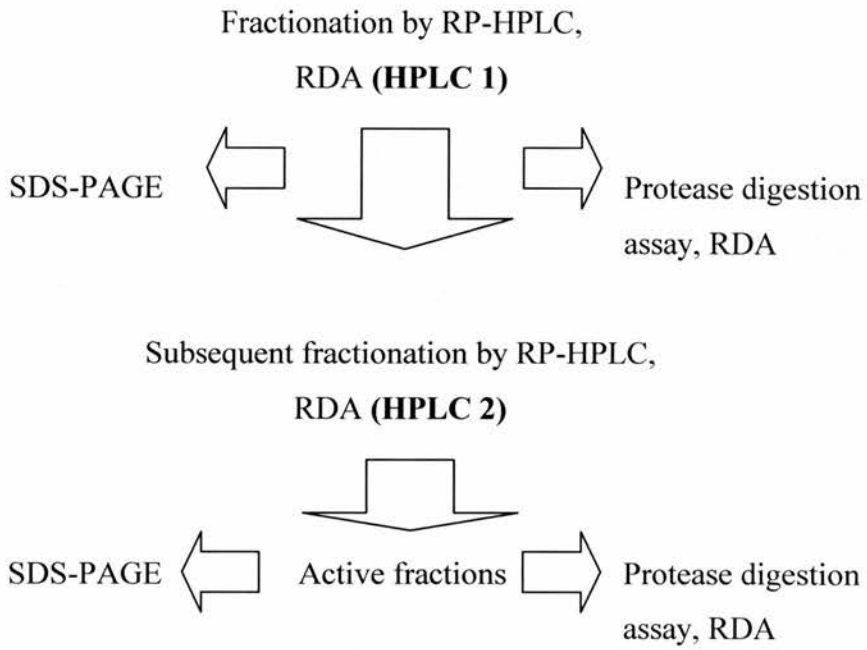
5.2.1 Preparation of *P. tricornutum* cellular methanol extract

P. tricornutum was grown to exponential phase (3.6×10^6 cells ml^{-1}) in 20 l ESAW and cells harvested from 18 l culture as described in Section 2.2.4. After thawing, the cell pellet was resuspended 1:4 (v/v) with methanol (BDH) and cells lysed by ultrasonication for 5 cycles of 5 minutes at 60% power with ½ second pulses before mixing on an orbital shaker for 60 minutes (60 rev. min^{-1}) and centrifuged at 4,000 × g for 10 min at 4 °C. The decanted supernatant was snap-frozen in liquid nitrogen and solvent removed by lyophilisation. The contents remaining after lyophilisation were reconstituted in 2 ml of 70 % (v/v) HPLC-grade methanol and centrifuged at 21,460 × g for 10 min at 4 °C to remove insoluble precipitates before filtering the supernatant through a 0.2 μm cartridge filter (Anatop 10LC, Whatman). The filtrate was designated, the methanol extract.

5.2.2 Fractionation of methanol soluble *P. tricornutum* cellular extract

A methanol extract of *P. tricornutum* cells was prepared then fractionated by RP-HPLC, as summarised in Figure 5.2. The antibacterial activity of fractions was determined by RDA after each chromatography. Antibacterial fractions were visualised by SDS-PAGE and subjected to protease digestion. The detailed methodology of each step is described in the accompanying sections.

Figure 5.2 Flowchart of the initial fractionation of methanol extract



5.2.2.1 Fractionation of methanol extract by RP-HPLC (HPLC 1)

The methanol extract (1 ml), prepared as described in Section 5.2.1 was loaded on a C_{18} column (250 mm × 10 mm; ODS2, Capital) previously equilibrated in 70 % (v/v) HPLC-grade methanol (BDH). The column was eluted at 1 ml min^{-1} with the following gradients (solvent B is 95 % (v/v) HPLC-grade methanol; BDH), 0-10 min, 0 % (v/v) B; 10-20 min, 0-60 % (v/v) B; 20-30 min, 60 % (v/v) B; 30-60 min, 60-90 % (v/v) B; 60-70 min, 90-100 % (v/v) B; 70-110 min, 100 % (v/v) B. The eluate was monitored using the PDA at 210 to 400 nm. One millilitre fractions of the eluate were collected, solvent removed by lyophilisation and reconstituted in 100 µl of ultra pure deionised water before assaying 3 µl of each fraction for antibacterial activity by RDA against Y# as described in Section 2.2.6.4.

5.2.2.2 Protease digestion and SDS-PAGE of active fractions

Fractions 27 was subjected to digestion with Proteinase K as described in Section 3.2.6. Five microlitres of each test solution was assayed for antibacterial activity by RDA using Y# as test bacterium as described in Section 2.2.6.4. The protein content of fractions 27 and 28 were visualised by high resolution SDS-PAGE and the gel stained with silver as described in Section 3.2.5.

5.2.2.3 Subsequent separation by RP-HPLC (HPLC 2)

The remainder of active fractions from the initial separation were pooled, lyophilised and reconstituted in 1 ml of 5 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA, filtered through a 0.2 μm cartridge filter (Anatop 10LC, Whatman) and applied to a C_{18} column (250 × 4.6 mm; Rsil HL; BioRad) previously equilibrated in 5 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA. The column was eluted at 1 ml min^{-1} with the following gradients (solvent B is 95 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % TFA), 0-10 min, 0 % (v/v) B; 10-70 min, 0-60 % (v/v) B; 70-90 min, 60-100 % (v/v) B; 90-100 min, 100 % (v/v) B. The eluate was monitored using the PDA and 1 ml fractions were collected. The solvent was removed by lyophilisation, and each sample was reconstituted in 60 μl of ultra pure deionised water before assaying 4 μl of each fraction for antibacterial activity by RDA using Y# as test bacterium as described in Section 2.2.6.4.

5.2.2.4 Protease digestion of antibacterial fractions from HPLC 2

The active fraction 59 from the second RP-HPLC was subjected to digestion with proteinase K, as described in Section 3.2.6. Five microlitres of each test solution was

assayed for antibacterial activity with RDA using Y# as test bacterium as described in Section 2.2.6.4.

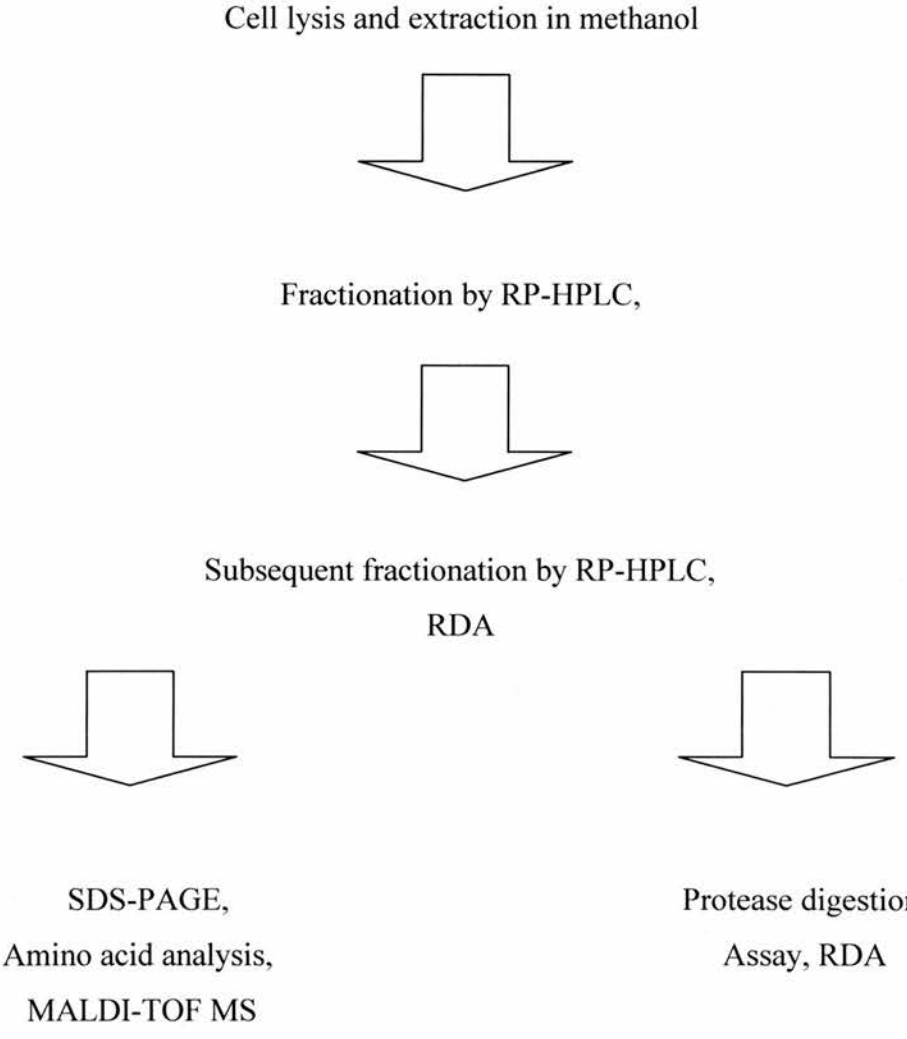
5.2.2.5 High resolution (SDS-PAGE) of active fractions from HPLC 2

Tris-tricine SDS-PAGE of fraction 59 under reducing conditions was performed and the gel stained with silver as described in Section 3.2.5.

5.2.3 Subsequent fractionation and determination of proteinaceous nature of the antibacterial factor

A methanol extract of *P. tricornutum* cells was prepared then fractionated by RP-HPLC using the outlined protocol described above, but with the main exceptions that an optimised elution gradient was used for RP-HPLC and to conserve the sample, only those fractions gained from the second chromatography were assayed for antibacterial activity. This repeated fractionation was performed to gain more sample for further biochemical characterisation of the antibacterial factor, summarised in Figure 5.3. The detailed methodology of each step is described in the accompanying Sections.

Figure 5.3 Flowchart of the subsequent fractionation of methanol extract



5.2.3.1 Preparation and methanol extraction of *P. tricornutum* cells

P. tricornutum was grown, harvested (6.5 $\times 10^6$ cells ml^{-1}) and methanol extract prepared as described in Section 5.2.1, except that the supernatant was transferred by pipette into glass test tubes and solvent removed by centrifugal evaporation under vacuum for 4 hr at 30 °C. The contents remaining after evaporation were reconstituted in 4 ml of 70 % (v/v) HPLC-grade methanol (BDH) and centrifuged at 21,460 × g for 10 min at 4 °C to remove insoluble precipitates before filtering the supernatant through a 0.2 μm cartridge filter (Anatop 10LC; Whatman).

5.2.3.2 Purification of antibacterial factor by 2 steps of RP-HPLC

One millilitre of the filtrate (4 ml) was loaded on a C_{18} column (250 mm × 10 mm; ODS2, Capital) previously equilibrated in 70 % (v/v) HPLC-grade methanol (BDH). Isocratic elution followed for 20 minutes at 70 % (v/v) HPLC-grade methanol (BDH), with a flow rate of 2 ml min^{-1}. The eluate was monitored using the PDA. The eluate corresponding to the characteristic absorbance peak observed during the previous isolation (above) was collected. This fractionation procedure was repeated a further three times for the remaining filtrate. The four eluates collected were pooled and solvent removed by centrifugal evaporation under vacuum for 3 hr at 30 °C. The concentrated eluate was not assayed for antibacterial activity at this stage to conserve sample.

The concentrated sample was reconstituted in 1 ml of 5 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA, filtered through a 0.2 μm cartridge filter (Anatop 10LC, Whatman), and applied to a C_{18} column (250 × 4.6 mm; Rsil HL, BioRad) previously equilibrated in 5 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA. The column was eluted at 1 ml min^{-1} with the following gradients (solvent B is 95 % (v/v) HPLC-grade acetonitrile (BDH), 0.07 % (v/v) TFA), 0-10 min, 0-30 % (v/v) B; 10-20 min, 30 % (v/v) B; 20-40 min, 30-50 % (v/v) B; 40-50 min, 50-100 % (v/v) B; 50-60 min, 100 % (v/v) B. The eluate was monitored using the PDA and 1 ml fractions were collected. The solvent was removed by centrifugal concentration under vacuum at 30 °C for 3 hr, and each sample reconstituted in 70 μl of ultra pure deionised water before assaying 4 μl of each fraction for antibacterial activity with RDA using *Psychrobacter immobilis* (NCIMB 308), or Y# as the test bacterium as described in Section 2.2.6.4.

5.2.3.3 Protease digestion of active fractions

Fraction 40 from the second RP-HPLC (above) was subjected to digestion with α-chymotrypsin before assaying 5 µl of each test solution for antibacterial activity with RDA using Y# as test bacterium as described in Section 3.2.6, substituting proteinase K for α-chymotrypsin.

5.2.3.4 High resolution (SDS-PAGE) of antibacterial fractions

Active fractions from the second RP-HPLC were subjected to tris-tricine SDS-PAGE and the gel stained with silver as described in Section 3.2.5. Each well received 15µl of each fraction.

5.2.3.5 Amino acid analysis and Matrix-Assisted Laser Desorption / Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS)

The remainder of fraction 40 from the second HPLC, was subjected to amino acid analysis (a), Accutag analysis (b) and MALDI-TOF analysis (c) by Peter Sharratt at the Department of Biochemistry, University of Cambridge, UK.

(a)

For the ninhydrin method of amino acid analysis, an internal standard, norleucine, was added to the protein solution and the mixture transferred to a pyrolysed tube. The tube was placed in a centrifugal evaporator and the mixture was concentrated to dryness. Gas phase acid (hydrochloric) hydrolysis was performed in a hydrolysis vial at 115 degrees for 22 hours. After removing traces of acid, sodium citrate

loading buffer, at pH 2.2, was added to dissolve the residue. The resulting solution was filtered under centrifugation through a 0.2 micron filter. An aliquot of the filtrate was then injected into a loading capsule, placed in an amino acid analyser (Alpha plus series II; Pharmacia, Little Chalfont, Buckinghamshire, UK) and chromatography was performed on an ion exchange resin (sodium system) eluting with a series of buffers over the pH range 3.2 to 6.45. Peak detection was achieved by mixing the eluate with ninhydrin and measuring the absorbance at 570 and 440nm, analysed with Chromeleon software (version 4.2). All reagents used were obtained from Biochrom Ltd, Cambridge, Cambridgeshire, UK.

(b)

The protocol used for sample hydrolysis for the Accutag method of amino acid analysis was as described above. The dried hydrolysate was dissolved in the appropriate amount of hydrochloric acid (20 mM) and a 20 ul aliquot treated with 60 µl of borate buffer and mixed. Twenty microlitres of AccQ.Fluor reagent was then added and mixed. The tube was then kept at room temperature for 1 min followed by incubation at 55 °C for 10 min. The sample was then taken to dryness and the residue reconstituted in eluate A. After centrifugation, 100 µl of the supernatant was injected in an amino acid analyser (AccQ.Tag system, Waters) and chromatography performed on a reverse phase C_{18} column (Nova-Pak; Waters) eluting with eluant A, followed by an increasing percentage of acetonitrile. Peak detection was detected by fluorescence (250nm excitation, 395nm emission). All reagents used were obtained from Waters. The spectra was analysed using Millenium32 software (Waters).

(c)

MALDI-TOF analysis was performed by depositing 0.5 µl of sample solution onto the sample slide followed by 0.5µl of matrix. The sample-matrix mixture was then allowed to dry and was washed to remove inorganic salts. Mass spectra were acquired on a Kompact MALDI IV (Kratos, Manchester, U.K.) time-of-flight mass spectrometer in the positive-ion linear mode, equipped with a 337 nm nitrogen laser and a 20 kV extraction voltage with time-delayed extraction. Each spectrum was the average of 50 laser shots.

5.2.4 Determination of optimal stage of harvest of *P. tricornutum* for gaining a high yield of the antibacterial factor

P. tricornutum was grown in 20 l ESAW for 21 days and cells harvested from 2 l of culture at day 3 (early exponential phase, 2.3 $\times 10^6$ cells ml^{-1}), day 14 (early exponential phase, 5.7 $\times 10^6$ cells ml^{-1}) or day 21 (stationary phase, 6.2 $\times 10^6$ cells ml^{-1}) as described in Section 2.2.4. For each cell pellet, a methanol extract was prepared and the antibacterial factor 'purified' by two stages of RP-HPLC as described in Section 5.2.3, before assaying the final respective fractions for antibacterial activity against Y# by RDA as described in Section 2.2.6.4. As a measure of the concentration of the antibacterial factor in each sample, the absorbance peak area at 280 nm was determined with the HPLC system analysis software (Millennium 32, Waters) and calculations relating peak area to cell number for each extract made accordingly.

5.2.5 Subsequent isolation and structural characterisation of antibacterial factor by nuclear magnetic resonance spectroscopy

P. tricornutum was grown to early exponential phase in 20 l ESAW and cells harvested as described in Section 2.2.4. The culture and harvesting of *P. tricornutum* cells were repeated a further nine times in order to gain a high yield starting material (range of cell density at harvest, 1.8 - 4.3 $\times 10^6$ cells ml^{-1}). The cell pellets were pooled, and subjected to the fractionation procedures described in Section 5.5.3. The final 'purified' eluates from the second RP-HPLC of fractionation were pooled and lyophilised. This was used for structural characterisation by nuclear magnetic resonance spectroscopy (NMR). All structural characterisation procedures and analyses were kindly performed by Dr Philp, Centre for Biomolecular Sciences, University of St Andrews, UK. NMR spectra were collected on a Varian UNITY plus spectrometer (Varian Inc., Walton on Thames, Surrey, UK). A series of one and two-dimensional NMR experiments (^1H, ^{13}C, ^1H-^1H COSY, ^1H-^{13}C HSQC) were performed on the purified sample from Section 5.2.3.1. Briefly, the sample was reconstituted in 0.7 ml of CD_3OD and introduced into the probe in a Wilmad 528PP NMR sample tube. ^1H spectra were recorded at 25 °C, 500.13 MHz and for ^{13}C, 75.5 MHz, using standard Varian software and pulse sequences (VNMR 6.1C; Varian). Two-dimensional ^1H-^{13}C correlation experiments (HSQC) were run using gradient-enhanced pulse sequences to reduce experimental acquisition times. For the determination of molecular mass, the NMR sample was diluted 100:1 (v/v) in methanol and subjected to electrospray mass spectrometry. Spectra were obtained on a Micromass LCT instrument (Waters) in positive ion mode at 20 Volts. The determination of accurate masses (± 5 ppm) was achieved using the Lock Spray Protocol.

5.2.6 Spectrum of antibacterial activity of factor

The antibacterial factor was purified from four 20 l cultures of *P. tricornutum* grown to early exponential phase. The culture conditions and purification procedure for the antibacterial factor was as described in Sections 2.2.4 and 5.2.3 respectively. The final purified eluates from the second RP-HPLC of fractionation were pooled with the remaining sample after structural characterisation (in ca. 2 ml methanol) and solvent removed by centrifugal concentration at 45°C for 3 hr. The concentrated sample was reconstituted in 60 µl of ultra pure deionised water to make a stock solution for antibacterial tests and two-fold and four-fold dilutions made with ultra pure deionised water. Antibacterial activity was determined with RDA for each solution as described in Section 2.2.6.4. The test bacterial strains were the marine Gram-positive, *Planococcus citreus* (NCIMB 1493), the marine Gram-negative, *Psychrobacter immobilis* (NCIMB 308), a clinical isolate of the non-marine Gram-positive, methicillin-resistant *Staphylococcus aureus* (MRSA) (kindly gifted by Dr Andrew Spragg), and the non-marine Gram-negative, *Escherichia coli* (NCIMB 12210). The non-marine strains were maintained and prepared as described in Section 2.2.6.3 with the exceptions that growth media were nutrient agar or broth (Oxoid, Basingstoke, Hampshire, UK), incubation temperature was 35 °C and sterile 0.8 % (w/v) NaCl was used for washing the bacterial cells and for resuspension. The constituents of the RDA base and top agar used for the non-marine strains are described in Appendix 1. The positive control for each bacterium was 200, 100 or 50 µg ml-1 melittin and sample diluent as negative control. Three microlitres of each solution to test was applied into individual wells in duplicate.

5.2.7 Haemolysis activity of antibacterial factor

The erythrocyte lysis assay was modified from the method described by Eschbach *et al.* (2001). The erythrocytes were prepared from 3 ml of heparinised whole blood sample from a Harbour seal, *Phoca vitulina*, kindly supplied by the Sea Mammal Research Unit, University of St Andrews, UK. The whole blood sample was centrifuged, at approximately 4 hr after the blood was taken, at 2,600 × g for 10 min at 25 °C. The plasma and leucocytes were removed with a 2 ml sterile transfer pipette (Sarstedt) and the remaining erythrocyte cell pellet washed in sterile 1.05 % (w/v) NaCl before centrifuging as described above. The supernant was decanted and final erythrocyte cell pellet resuspended with fresh sterile 1.05 % (w/v) NaCl and adjusted for the lysis assay to 10^7 cells ml^{-1}, determined by use of a New Improved Neubauer Haemocytometer (Sigma) using a phase contrast Leitz Dialux microscope (×25 objective). The lysis incubation step was performed in a 96-well sterile polypropylene microtitre plate with U-shaped wells (Corning, High Wycombe, Buckinghamshire, UK) with 150 µl of erythrocyte suspension (10^7 cells ml^{-1}) and 10 µl of the remaining purified antibacterial factor stock (solution A) described in Section 5.2.6. Erythrocyte suspension incubated with 10 µl of ultra pure deionised water served as negative control (solution B). For the positive control representing 100 % lysed erythrocyte suspension, an aliquot of the erythrocyte suspension (10^7 cells ml^{-1}) was ultrasonicated on ice with 0.5-second pulses at maximum power for the probe tip used until complete cell lysis as determined by observation under a Leitz Dialux microscope (× 50 objective). The cell lysate was incubated with 10 µl of ultra pure deionised water (solution C). Each set of samples were pipetted in triplicate and the contents of each well mixed with a micro pipette before wrapping in foil and incubated at 25 °C for 30 min. Following incubation the microtitre plate

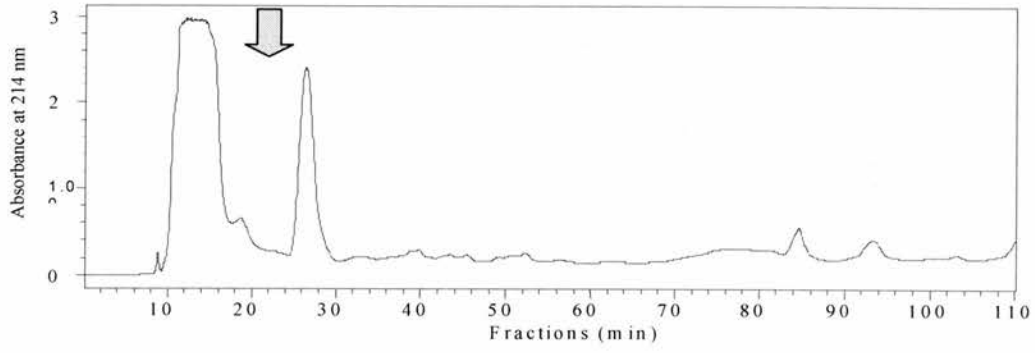
was centrifuged at 2000 × g for 5 min at 25 °C. The optimum wavelength (414 nm) used for the measurement of absorption was selected by a photometric scan with the erythrocyte lysate used for positive control. Absorption was scanned from 360 to 600 nm with a UV/Visible photometer (Ultrospec 3300 pro, Biochrom Ltd) in a 1 ml disposable polystyrene cuvette (10 mm effective light path). As the microtitre plate reader did not support absorbance reading at the optimal wavelength of 414 nm, 100 µl of supernatant from each triplicate sample well was transferred to a 1.5 ml Eppendorf and diluted to 1 ml with sterile 1.05 % (w/v) NaCl before transferring to 1 ml disposable polystyrene cuvettes. Absorption was read at 414 nm and at 650 nm for reference, this reference value was subtracted from the absorption values obtained at 414 nm and adjustment for dilution made accordingly.

5.3 Results

5.3.1 Fractionation of *P. tricornutum* cellular methanol extract

After the first stage of separation by RP-HPLC, strong antibacterial activity against Y# was found for fractions 26 to 28, corresponding to a peak of absorbance at 214 nm (Figure 5.4).

(a)



(b)

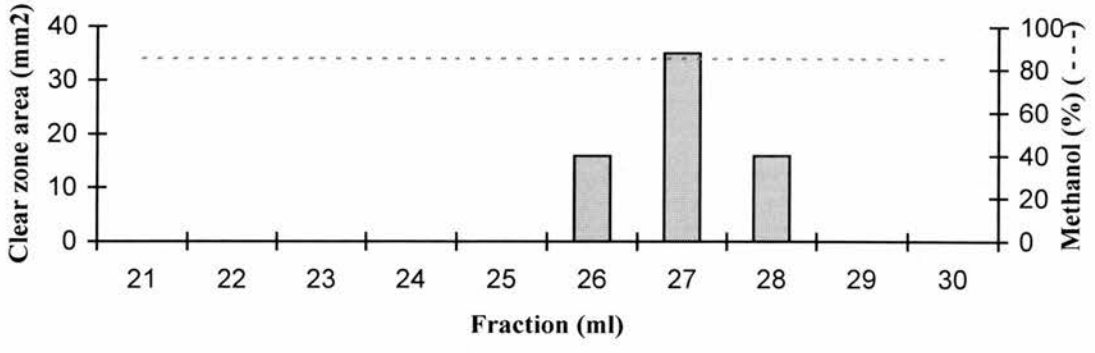


Figure 5.4 Chromatographic analyses of antibacterial activity of methanol extract (HPLC 1). (a) C_{18} RP-HPLC of methanol extract eluted with 70 to 95 % methanol at 1 ml min^{-1}. The eluant was monitored at 214 nm and 1 ml fractions collected. (b) Antibacterial activity in the fractions after lyophilisation, reconstitution in 100 µl of ultra pure deionised water and assay against Y# with RDA. Y-axis variables on the left refer to the histogram as the clear zone area minus the area of the well (mm^2). Antibacterial activity can be observed for fractions 26 to 28, eluted at 85 %

methanol, corresponding to a peak of absorbance (indicated by the arrow), whilst no antibacterial activity was detected in fractions 1 to 20 or 31 to 110.

SDS-PAGE of fractions 27 and 28 revealed a single protein band stained, for each sample, which resolved within the spacer Section of the gel, indicating that it was a high molecular mass protein (Figure 5.5).

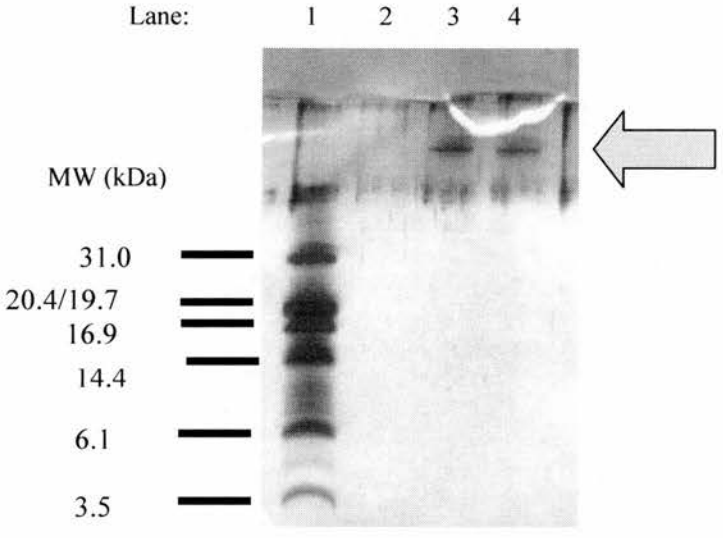
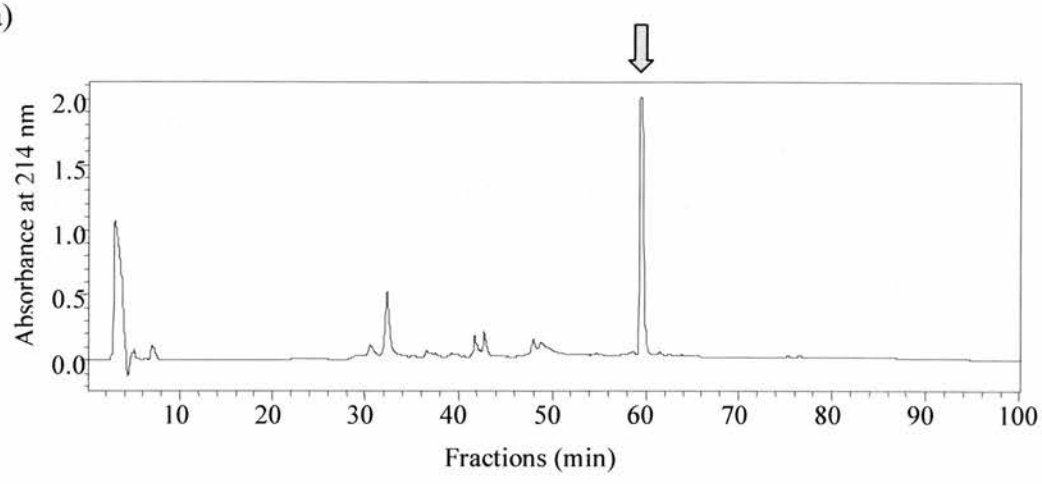


Figure 5.5 Tris-tricine SDS-PA gel (silver stained) of active fractions from RP-HPLC of *P. tricornutum* methanol extract. Lane 1: low molecular weight markers; lane 2: sample buffer; lane 3: fraction 27 from first RP-HPLC of methanol extract; lane 4: fraction 28 from first RP-HPLC of methanol extract. 10 µl of sample was applied per lane. The numbers of the left correspond to the molecular mass of the markers in kDa. The bands of interest are indicated by an arrow.

However, there were no differences in the antibacterial activities of fraction 27 (strongest antibacterial activity) incubated with or without Proteinase K, indicating that the factor(s) responsible for the antibacterial activity in this fraction is/are not of proteinaceous nature. No antibacterial activity was observed for the negative control, solution B (not shown).

After the second fractionation step by RP-HPLC of fractions 26 to 29 from the first HPLC, antibacterial activity was detected for fractions 58 to 60, corresponding to a discrete peak of absorbance at 214 nm (Figure 5.6).

(a)



(b)

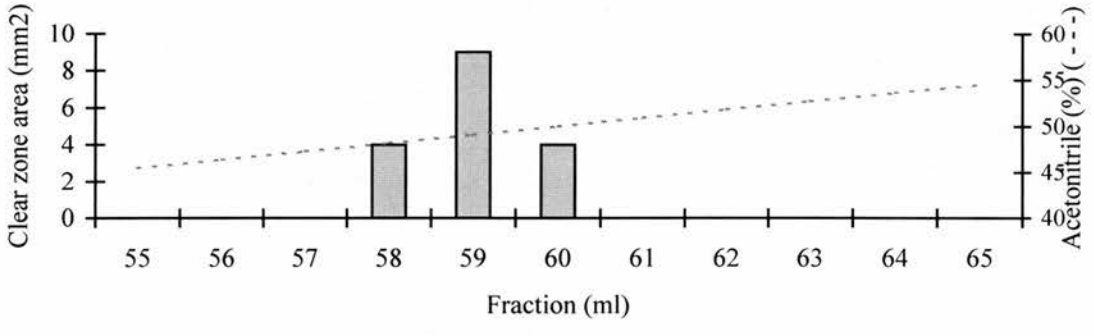


Figure 5.6 Chromatographic analyses of antibacterial activity of active fractions from HPLC 1 (HPLC 2). (a) C_{18} RP-HPLC of pooled fractions 26 to 29 from HPLC 1, eluted with 5 to 95 % acetonitrile over 100 min at 1 ml min^{-1}. The eluant was monitored at 214 nm and 1 ml fractions collected. (b) Antibacterial activity in the fractions after lyophilisation, reconstitution in 60 µl of ultra pure deionised water and assayed against Y# with RDA. Y-axis variables on the left refer to the histogram as the clear zone area minus the area of the well (mm^2). Antibacterial activity observed in fractions 58 to 60, eluted at 48 to 50 % acetonitrile, corresponding to a peak of absorbance (indicated by the arrow). No antibacterial activity was detected in fractions 1 to 54 or 65 to 100.

Again, there was no difference in the antibacterial activity of fraction 59 (strongest antibacterial activity) incubated with or without Proteinase K, indicating that the factor(s) responsible for the antibacterial activity in this fraction may not be proteinaceous. No antibacterial activity was observed for the negative control, solution B (not shown). However, two silver stained bands were observed following SDS-PAGE of fraction 59. One band resolved within the spacer section of the gel and the other resolved within the separating section, corresponding to between the 6.1 and 14.4 kDa molecular markers (Figure 5.7).

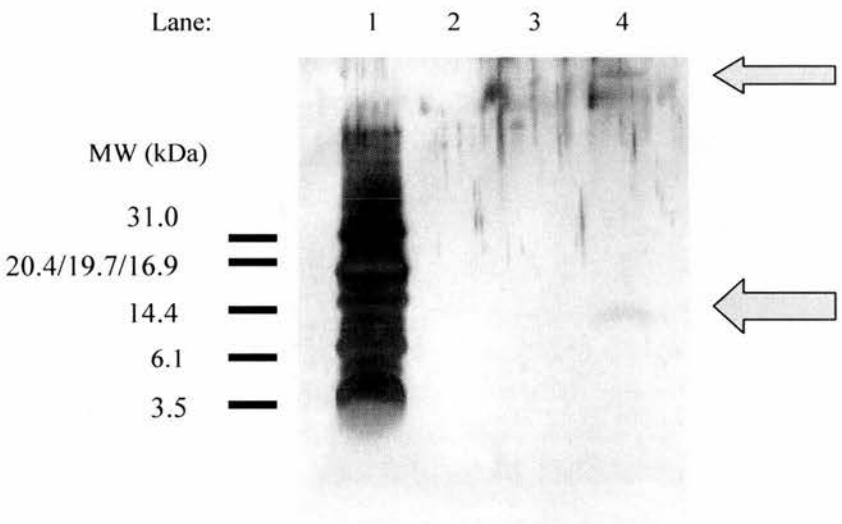


Figure 5.7 Tris-tricine SDS-PA gel (silver stained) of fractions 59 from the second RP-HPLC of *P. tricornutum* methanol extract. Lane 1: low molecular weight markers; lane 2 and 3: sample buffer; lane 4: 20 µl of fraction 59 from second RP-HPLC of methanol extract. The numbers of the left correspond to the molecular mass of the markers in kDa. The bands of interest are indicated by arrows.

5.3.2 Subsequent fractionation and determination of proteinaceous nature of the antibacterial substance

Antibacterial activity against Y # was detected for fraction 40 from the second RP-HPLC, corresponding to the peak of absorbance indicated by the arrow in Figure 5.8. For fraction 40, absorbance maxima are shown to be at 278 nm and at 226 nm, indicating the presence of an aromatic compound/molecule (Figure 5.9).

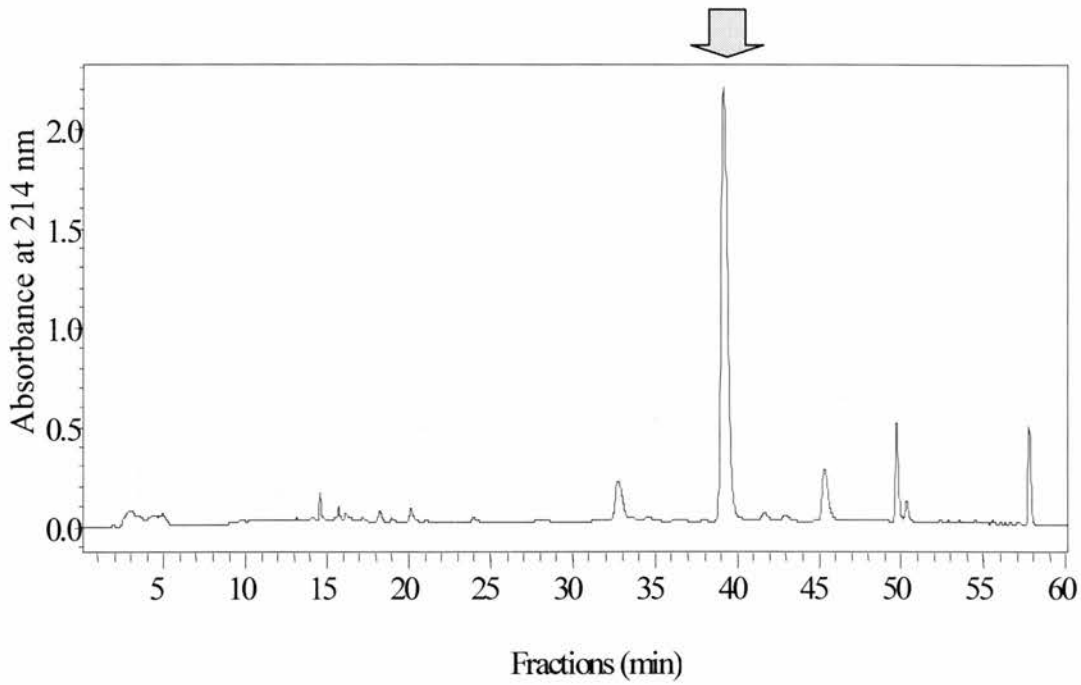
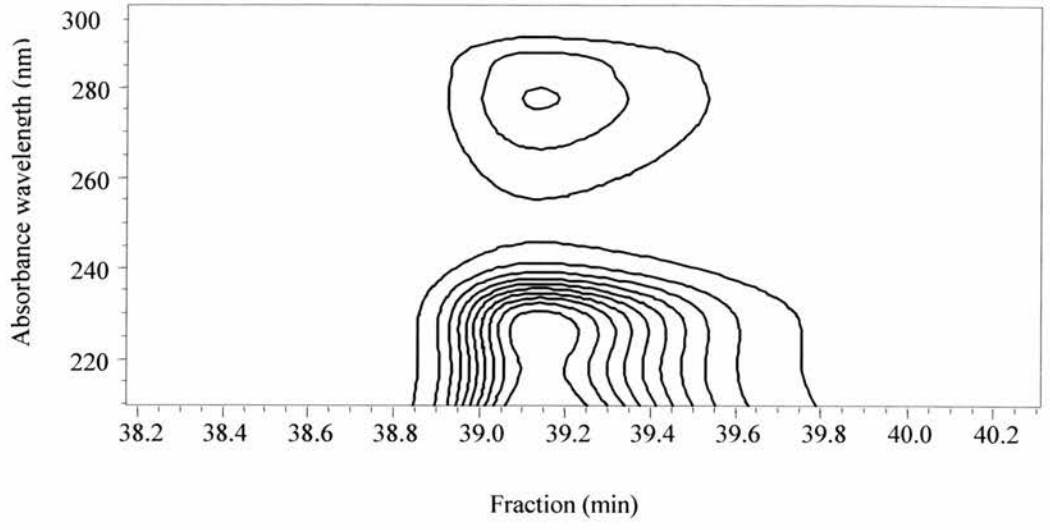


Figure 5.8 C_{18} RP-HPLC Chromatograph of pooled fractions corresponding to characteristic peak of absorbance from first RP-HPLC. Elution gradient: 5 to 95 % acetonitrile over 60 min at 1 ml min^{-1} (solvent B is 95 % HPLC-grade acetonitrile, 0.07 % TFA), 0-10 min, 0-30 % B; 10-20 min, 30 % B; 20-40 min, 30-50 % B; 40-50 min, 50-100 % B; 50-60 min, 100 % B. The eluant was monitored at 214 nm and fractions 38 to 42 were collected to include the characteristic peak of absorbance, indicated by the arrow and tested for antibacterial activity against Y # with RDA. Antibacterial activity was detected in fraction 40 (4 mm^2 clear zone area; not shown).

(a)



(b)

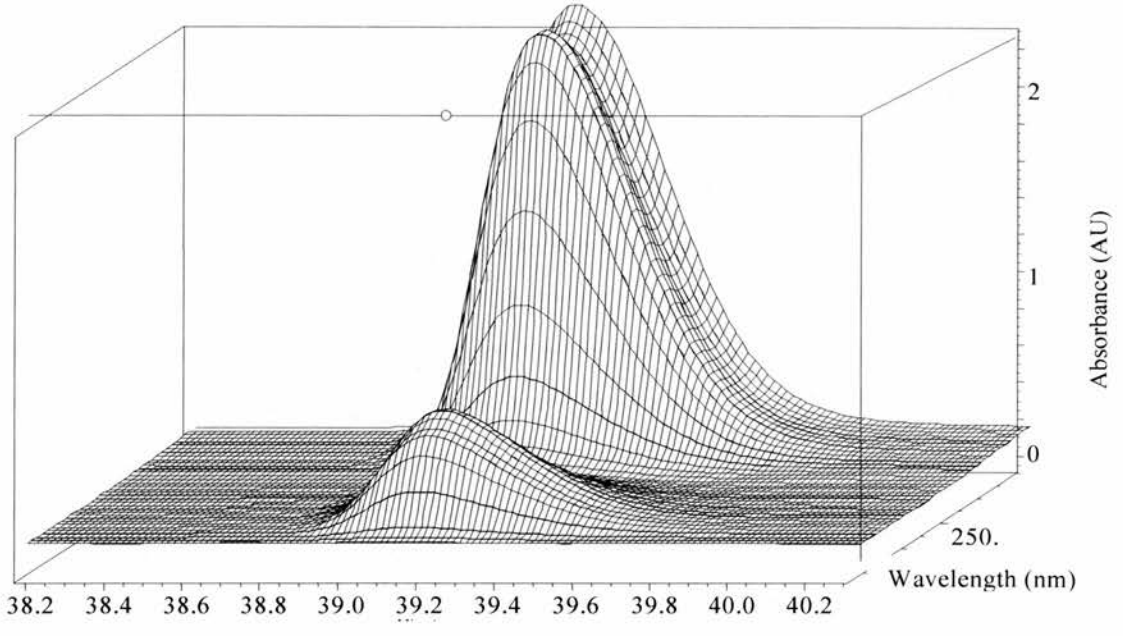


Figure 5.9 Chromatographic analyses of fraction 40 from C_{18} RP-HPLC of pooled fractions corresponding to characteristic peak of absorbance from first RP-HPLC. (a) Contour absorbance chromatograph of fraction 40 from 210 nm to 300 nm. Peak absorbance is at 278 and 226 nm. (b) Three-dimensional chromatograph of fraction 40 from 210 nm to 300 nm. Absorbance maximum are at 226 nm and 278 nm, the later has a lower peak height than the former.

No protein bands were detected by Tris-tricine SDS-PAGE for fractions 39 to 42 (not shown). In addition, there was no significant difference in the antibacterial activity of fraction 40 incubated with or without α-chymotrypsin (not shown). These results indicate that the factor(s) responsible for the antibacterial activity in this fraction is/are not proteinaceous. No antibacterial activity was observed for the negative control (not shown).

For the remaining sample, MALDI-TOF MS failed to provide any signal for the sample with the parameters used. Amino acid analysis detected the presence of a range of amino acids consistent with the presence of a protein, equivalent to 90 nmol residues ml^{-1} (not shown), but the level of amino acid detection was at the limit of sensitivity for the method so cannot be considered meaningful. By the Accutag method, information on the amino acid residues of the sample is also not reliable due to the presence of a large ammonia peak not seen using the ninhydrin method, possibly due to contamination (not shown).

5.3.3 Determination of optimal stage of harvest of *P. tricornutum* for gaining a high yield of the antibacterial factor

Although definite clear zones of antibacterial activity against Y# were not observed for any of the final fractions containing the 'purified' antibacterial factor, a similar diameter (5 mm) of growth inhibition, containing individual colonies of bacteria, were observed for each test solution. As at this stage, the chemical nature of the compound is unknown and cannot be assumed to be a protein, the absorbance peak area was used as a measure of antibacterial factor concentration. The *P. tricornutum*

culture harvested at early exponential phase was found to contain the highest concentration of antibacterial factor per cell when compared to harvesting cells at late exponential or stationary phase (Figure 5.10).

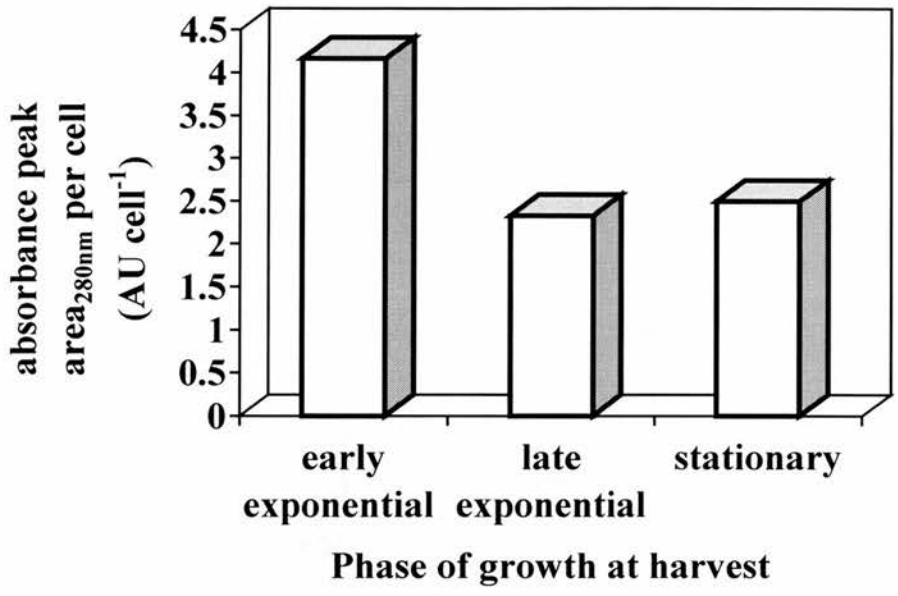


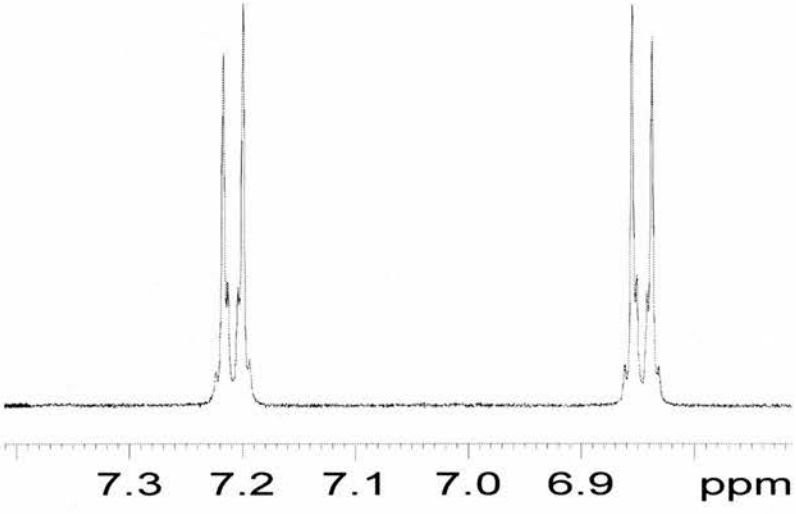
Figure 5.10 Analysis of antibacterial factor concentration with growth phase of *P. tricornutum*. Concentration of antibacterial factor was determined by measurement of respective absorbance peak area at 280 nm divided by the total number of *P. tricornutum* cells harvested from culture. Early exponential, late exponential and stationary phase corresponds to the harvest of *P. tricornutum* cells at day 3, 14 or 21 respectively.

5.3.4 Structural characterisation of the antibacterial factor

NMR experiments revealed the presence of a tetrabutyl-ammonium phenolate (TBAP) within the sample (Figure 5.11). The constituent ions are 4-substituted phenolate anion and a tetrabutyl-ammonium cation (Figure 5.12). There remains ambiguity regarding the functional group at position 4 (* Figure 5.12) for the phenolate anion. Possible candidates include a halogen or methyl group. The

molecular mass of the alkyl substituted ammonium cation was determined by electrospray mass spectrometry to be 242.4 Da. The molecular mass of the phenolate anion was not obtained due to the negative charge on the phenolate and the conditions used for electrospray mass spectrometry.

(a)



(b)

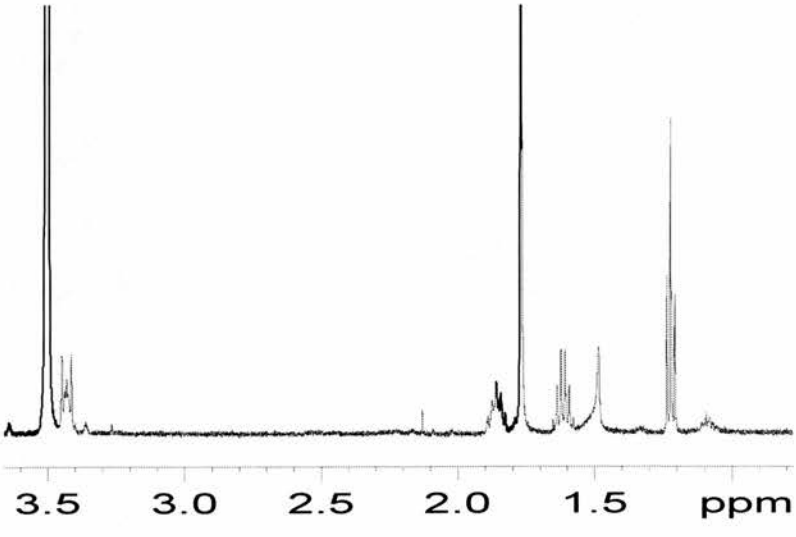
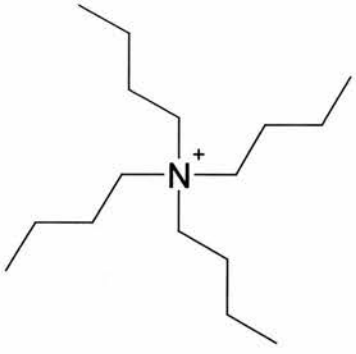


Figure 5.11 Spectrum obtained with ^{1}H NMR for antibacterial factor purified from *P. tricornutum*. (a) Chemical shift spectra for phenolate anion (ppm). (b) Chemical shift spectra for tetrabutyl-ammonium cation (ppm).

(a)



Tetrabutyl-ammonium ion

Molecular weight: 242.47

Molecular Formula: $C_{16}H_{36}N$

Molecular composition: C 79.26 %; H 14.97 %; N 5.78 %

(b)

Phenolate ion

Molecular weight: 92.10

Molecular Formula: C_6H_4O

Molecular composition: C 78.25 %; H 4.38 %; N 17.37 %

Figure 5.12 Schematic representation of constituent ions of tetrabutyl-ammonium phenolate. (a) Tetrabutyl-ammonium cation. (b) Phenolate anion. Calculation of molecular mass, formula and composition accompany each ion (Isis Draw 2.4, MDL Information Systems, Inc., US). The calculations relating to the phenolate ion do not take into account the unknown functional group indicated by the asterisk.

5.3.5 Spectrum of activity of purified Tetrabutyl-ammonium phenolate

The purified TBAP was found to be active, at the original stock concentration, against all four strains tested, *P. citreus*, *P. immobilis*, *E.coli* and MRSA (Table 5.1). For *P. citreus* or MRSA, the antibacterial activity for the stock TBAP was equal to that obtained for 50 μg ml-1 melittin (positive control) (Table 5.1). Comparison cannot be made with melittin for *P. immobilis* or *E.coli* as the smallest clear zone area for melittin was larger than that gained for TBAP stock (Table 5.1). However, at 2-fold dilution of the antibacterial factor stock solution, only the marine strains, *P. citreus* and *P. immobilis*, were susceptible (Table 5.1). No antibacterial activity was detected against any of the strains for the 4-fold diluted stock solution (Table 5.1).

Table 5.1 Spectrum of antibacterial activity of purified TBAP from *P. tricornutum*. Numerical values are mean clear zone area minus area of well in mm^2 (n=2). Positive controls were melittin and sample diluent (water) as negative control.

Test sample	**Antibacterial activity (mean clear zone area in mm^2)**			
	P. citreus	***P. immobilis***	***E.coli***	**MRSA**
Stock TBAP	16 ± 0	16 ± 0	16 ± 0	9 ± 0
2-fold diluted stock	4 ± 1	4 ± 0	0 ± 0	0 ± 0
4-fold diluted stock	0 ± 0	0 ± 0	0 ± 0	0 ± 0
melittin (pos.control)				
200 μg ml^{-1}	35 ± 0	75 ± 0	130 ± 0	25 ± 0
100 μg ml^{-1}	25 ± 0	47 ± 0	75 ± 0	16 ± 0
50 μg ml^{-1}	16 ± 0	35 ± 0	47 ± 0	9 ± 0
Ultra pure deionised water (negative control)	0 ± 0	0 ± 0	0 ± 0	0 ± 0

5.3.6 Haemolysis assay

The antibacterial factor did not to lyse *P. vitulina* seal erythrocytes at the concentration used (Figure 5.13).

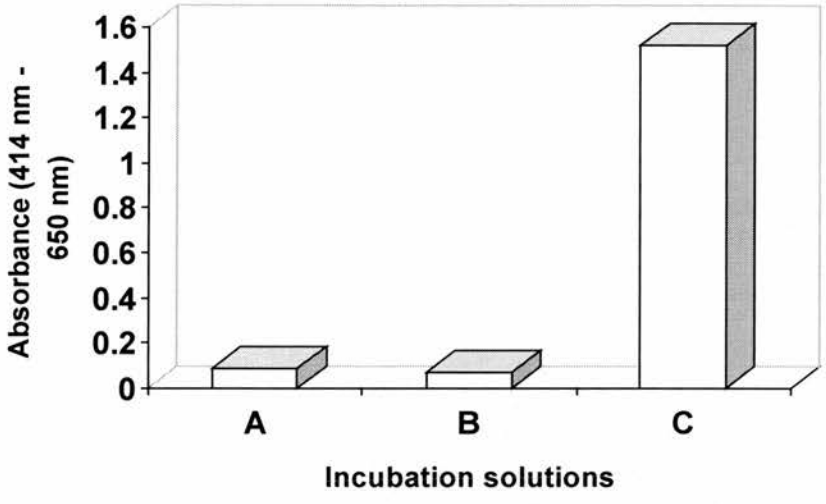


Figure 5.13 Haemolysis assay of purified TBAP from *P. tricornutum*. Solution A contains erythrocyte suspension plus stock TBAP; B: erythrocyte suspension plus ultra pure deionised water; C: erythrocyte lysate plus ultra pure deionised water. Each solution was incubated for 30 min in the dark at 25 °C, and then centrifuged. Cell lysis was measured as absorbance of the supernatants, read at 414 nm minus absorbance at 650 nm with diluent used as blank. The final absorbance of solution C corresponds to complete lysis of the erythrocyte suspension. The final absorbance reading of the test solution (A) was similar to that of the negative control, B.

5.4 Discussion

By means of these experiments, it was established that the antibacterial factor produced by *P. tricornutum* was an ammonium salt, tertrabutyl-ammonium phenolate, consisting of a tetrabutyl-ammonium cation ($C_{16}H_{36}N$) and a phenolate anion (C_6H_4O *). At present it is unknown which ion is responsible for the activity observed. The molecular weight of the ammonium ion was determined by electronspray mass spectrometry to be 242.4 Da, which correlates exactly to that calculated by IsisDraw software. Unfortunately, it was not possible to assign the functional group (*) at substitution position 4 on the phenolate. To the best of my knowledge, this is the first report of the isolation of TBAP from a natural source.

The first RP-HPLC fractionation stage of the methanol extract appeared to be successful in isolating an antibacterial protein of high molecular weight that was resistant to digestion by Proteinase K with the conditions used. However, upon the second HPLC, two protein bands were shown to be present in the antibacterial fraction. The low intensity of these protein bands stained by silver indicated that they were of low concentration. This was surprising as the very high peak of absorbance at 214 nm detected for the antibacterial fraction was indicative of a high protein concentration. The resistance to Proteinase K digestion of the antibacterial factor was confirmed by repeating the assay with the concentrated antibacterial fraction gained from the second chromatography. Surprisingly, given the failure of Proteinase K to digest the sample, amino acids were detected. However, as the amino acid analyses were at the limit of sensitivity for the techniques used, caution must be used for interpretation of the data obtained. The presence of amino acids in

the sample may have been due to contamination. Chromatographic spectra analyses of the antibacterial factor showed absorption maximum at 226 and 278 nm, indicative of an aromatic component, not necessarily a protein.

The harvesting of *P. tricornutum* cells from culture at early exponential phase was optimal for a high yield of TBAP compared with late exponential or stationary phases. By contrast, the isolation of secondary metabolites with antibacterial activity has often been reported from microalgae in late exponential or stationary phase (Pratt *et al.* 1945; Pinter and Altmeyer 1979; Harrison *et al.* 1990; Morton and Bomber 1994; reviews by Borowitzka 1995; Skulberg 2000). If the extinction coefficient is determined for TBAP, then it could be quantified by spectral analysis. However, care must be taken when relating such values to the concentration per cell as complete extraction cannot be determined until the cellular location of TBAP is investigated.

The TBAP, purified from *P. tricornutum*, was found to be active against both the test Gram-positive and Gram-negative bacteria. Importantly, it was active against MRSA. MRSA is considered a serious threat to public health as the most common cause for nosocomial wound infections worldwide (Lohner and Staudegger 2001). Furthermore, TBAP was also found to be active against *E. coli* (12210), an international reference strain used in studies of antibiotic susceptibility (Young and McFarlane 1994). However, TBAP was not haemolytic against one type of mammalian erythrocyte.

As the ion complex, TBAP is interesting as it is relatively stable due to its overall neutral charge, whereas most antimicrobial surface-active agents are either cationic or anionic (Gale *et al.* 1972). However, examining the distribution of charge for each ion reveals the negatively charged hydrophobic phenolate anion and the positively charged hydrophilic quaternary ammonium cation. Possibly, the mode of action of TBAP resembles that of a detergent, in as much as the ammonium cation is adsorbed onto and penetrates into the cell wall of a negatively charged bacterium. It is also possible that the hydrophobic phenolate may interact with lipid-protein complexes leading to the disorganisation of the membrane (Gale *et al.* 1972). In turn this could cause leakage of cytoplasmic constituents of low molecular weight and degradation of proteins or nucleic acids with the eventual lysis of the cell by the action of wall-degrading enzymes (Gale *et al.* 1972). Further investigations into the mode of action of TBAP are required before this can be confirmed.

Halogenation of phenols is known to increase antimicrobial activity, particularly as *para*-substitution (Gale *et al.* 1972). Coincidently, the phenolate of TBAP is *para*-substituted. At present, the functional group is unknown, although a halogen may be a likely candidate (Philp, D., University of St Andrews, personal communication). The presence of nitrogen in the ammonium cation may also contribute to the antibacterial activity of TBAP, perhaps due to the quaternary structure (Gale *et al.* 1972). It is interesting to note that more than 40% of secondary metabolites that have been characterised from marine microbes, including cyanobacteria, contain nitrogen (Kleinkauf and Von Dohren 1990). Furthermore, Nagle and Paul (1999) reported that most secondary metabolites from cyanobacteria contain nitrogen and may derive from amino acid or mixed biogenesis pathways.

Microalgae have been found to produce phenolic compounds with a variety of biological activity (reviews by Aubert *et al.* 1979; Metting and Pyne 1986; Borowitzka 1995). Many of the toxins produced by cyanobacteria have been identified as alkylphenols (reviewed by Metting and Pyne 1986; Skulberg 2000; Burja *et al.* 2001). The majority of the phenolic toxins produced by cyanobacteria have complex structures with numerous functional groups, with the exception of a simple brominated phenol with antibacterial and antialgal activity isolated from the cyanobacterium *Calothrix brevissima*un (Pedersen and DaSilva 1973). TBAP is unusual amongst phenolic compounds isolated from algae in respect of the simple structure of the phenolate ion, with the possibility of just a single substituted halogen, and the ion complex with the ammonium cation, if they are indeed present within an algal cell or in seawater as such a complex.

The antioxidant activity of halogenated phenols from algae has also been reported (Fujimoto 1990). A methanol extract of the red macroalga, *Polysiphonia ureolata* has antioxidant activity, and four different simple brominated phenols have been identified from the extract (Fujimoto *et al.* 1985, 1986). Antihypoglycemic activity has also been found for a phenolic product gained from the hydrolysis of Sargalin (Saito and Nakamura 1951); an alkaline phenolic compound isolated from the brown macroalga, *Sargassum confusum* (Takaoka and Saito 1949). Saito and Nakamura (1951) showed that the phenol product has suppressive action against diabetes mellitus.

Phenolic compounds are well known for their antimicrobial activity (Gale *et al.* 1972; Franklin and Snow 1981). Indeed, crude mixtures of phenolics with a detergent or alkalis have been used for many years as general disinfectants (Franklin and Snow 1981). Halogenated phenolic solutions are extensively used as antiseptics, such as chlorinated cresols or xylenols (Dettol). The bactericidal mode of action of phenolic compounds is thought to be the disorganisation of the cytoplasmic membrane, causing leakage of cytoplasmic constituents of low molecular weight and cell death (Franklin and Snow 1981). Hexachlorophene, at sub-lethal concentration, was reported to inhibit oxygen consumption of *Staphylococcus aureus*, indicating a direct effect on the respiratory system, associated with the cytoplasmic membrane (Franklin and Snow 1981).

From an ecological perspective, the antibacterial activity of TBAP against the marine strains *P. citreus* and *P. immobilis* is significant, as the salt concentration of the agar media used for the RDA of these bacteria, equivalent to the salt concentration of natural seawater, did not appear to affect the activity of TBAP. The implications of this are that if *P. tricornutum,* in the marine environment, produces and releases TBAP into it's surroundings the compound may retain biological activity. The release of TBAP by *P. tricornutum* either by active transport of passive diffusion into the environment would be very interesting as it may play a role in the milieu of chemical ecology within the marine environment and warrants further study.

Chapter 6

General Discussion

The overall objective of this study was to investigate the production of antibacterial activity by a model species of microalgae and to isolate and fully characterise the compound(s) responsible for the activity observed. Specifically, it was aimed at:

1. Determining antibacterial activity of *P. tricornutum* cells using a range of extraction solvents/buffers and using an empirical approach of protein purification techniques to fractionate extracts whilst retaining biological activity.
2. Isolating and characterising antibacterial agents in fractionated extracts of *P. tricornutum*. In particular, it was aimed at isolating at least one antimicrobial peptide from this alga.

The first aim was fulfilled by the screening of a wide range of buffer/solvent cellular extracts of *P. tricornutum* to ascertain the methods of extraction that resulted in the strongest antibacterial activity, described in Chapter 2. Antibacterial activity was detected in 18 of 23 extracts against the Gram-positive strains Y# or *Aerococcus viridens*. Interestingly, 60 % ethanol and 100 % methanol extracts were the only solutions active against both Gram-negative and Gram-positive bacteria, indicating that more than one type of antibacterial compound might be present in *P. tricornutum* cells. The strongest antibacterial activity against Y# was found for the 100 % methanol, 60 % ethanol and phosphate buffer extracts, so were chosen for further investigation.

Empirical approaches were used in Chapters 3, 4 and 5, in order to facilitate isolating the compound(s) responsible for antibacterial activity of the extracts selected in Chapter 2. Unfortunately, the factor(s) present in the phosphate buffer or 60 % ethanol extracts were not isolated as the biological activities were lost upon a second stage of chromatography. However, an antibacterial lipid was successfully fractionated from a methanol extract of *P. tricornutum*, although the biochemical characterisation of the lipid was not performed due to the lack of sufficient sample for further investigation.

Chapter 5 incorporated both aims, where an ammonium salt, tetrabutyl-ammonium phenolate (TBAP) was identified from a fractionated methanol extract of *P. tricornutum* by a series of biochemical and structural characterisation techniques. From a preliminary experiment, the optimal stage of growth for the production of TBAP by *P. tricornutum* was shown to be at early exponential phase. The purified TBAP was found to have a broad spectrum of antibacterial activity, being active against both Gram-negative and Gram-positive bacteria, including a pathogenic strain, methicillin-resistant *Staphylococcus aureus* (MRSA). TBAP was also found not to lyse mammalian erythrocytes under the conditions used.

Unfortunately, antimicrobial proteins or peptides were not found in the cellular extracts of *P. tricornutum* for any of the conditions tested. This was surprising as antimicrobial peptides (AMP) have been isolated from representatives of higher and lower organisms, including the microalga, *Stichochrysis immobilis* (Berland *et al.* 1972). There could be many reasons for the failure to isolate an AMP from *P. tricornutum* cells. One possibility is that the species or strain used does not produce

AMPs. Alternatively, the culture conditions used, particularly the phase of growth at harvest, cell density, enriched nutrient availability or lack of microbial challenge may have stimulated the alga to reduce or 'turn off' the level of expression of AMPs. The expression of some AMPs is known to be induced when the organism is challenged by microbes (Cammue *et al.* 1994; Cocianich *et al.* 1994; Broekaert *et al.* 1997; Fritig *et al.* 1998). In addition, the extraction methods used may not have facilitated the solubilisation of AMPs. Further, if AMPs were solubilised by the extraction method, they may have become degraded by the action of any cellular proteases present in the extracts. However, this is unlikely as the phosphate buffer included a cocktail of protease and phenolic degradation inhibitors. It is also possible that AMPs were present in the extracts but were either not active against the strains of bacteria tested or that the concentration of these AMPs was so low that they were not detectable by the assays. For example, the yield of *P. tricornutum* cells obtained from 18 l of culture at mid-exponential phase was typically 10 ml of a wet cell pellet and this was used for a single extraction and subsequent fractionation. By comparison, 1 kg of plant seeds was used as the starting material for the isolation of AMPs by Tailor *et al.* (1997) and Cammue *et al.* (1992). However, in the present study, the yield of cells was always limited by the size of the culture vessel.

This study has generated findings relevant to future studies of antimicrobial activity by microalgae. The screening of different methods of extraction and subsequent fractionation of selected extracts showed that the choice of extraction solvent/buffer (and concentration) is important for the yield of antibacterial activity. Moreover, a single algal species seems to capable of producing different compounds with

antibacterial activity, some which have a narrow range of activity and others have a broader range.

The current concern for the incidence of antibiotic-resistant pathogens has increased the call for novel antimicrobial agents (Cooksey 1991; Lancini *et al.* 1995; Lohner and Staudegger 2001). Many of the antimicrobial agents used today were first identified from natural sources. The early studies of natural product chemistry mainly focused on terrestrial plants and microorganisms and resulted in the discovery of a multitude of compounds, which have found applications in pharmacology (Davidson 1995). However, our attention has only recently turned to the marine environment. This targeting of marine organisms has revealed thousands of new compounds, and many of these are unlike those found in terrestrial organisms (Bernan *et al.* 1997; Faulkner 1998).

Although the main direction of the present study was applied phycology, the work has potential ecological significance. TBAP may be produced by microalgae or other organisms in the environment. The production of so called 'secondary metabolites' in chemical defence is widespread. Higher plants have been shown to accumulate a large number of 'secondary metabolites' (Harborne 1990; Metraux 1994; Bourgaud *et al.* 2001). These include cyanogenic glucosides or glucosinolates, flavonoids, isoflavonoids, alkaloids, coumatins, terpenoids, phenolic compounds and hydroxamic acids (reviews by Barz *et al.* 1990; Metraux 1994). Amongst the induced biochemical compounds are the phytoalexins. They have antimicrobial properties and are produced at or around sites of pathogen infection (reviews by Bailey and Mansfield 1982; Barz *et al.* 1990). So far, these comprise more than 300

natural products including peptides (reviewed by Broekaert *et al.* 1997), phenolics, terpenoids, polyacetylenes, fatty acids derivatives plus other classes (reviewed by Barz *et al.* 1990). Pathogenesis-related proteins are also induced at the site of infection; these are acid extractable low-molecular weight, proteinase resistant proteins, which accumulate intracellularly and are synthesised in response to both pathogens and abiotic stress and induction is transcriptionally regulated (reviews by Cammue *et al.* 1994; Metraux 1994). Certainly, higher plants liberate defence compounds during the plant-pathogen interaction (Harborne 1990; Metraux 1994). These are called elicitors and are recognised by other plants where they induce defence reactions (Metraux 1994).

It follows that some of these defences could be employed by microalgae. Indeed, the production and release of antimicrobial compounds by microalgae living in a biofilm may play a significant role in the regulation of the microbial consortia by inhibition (Bakus *et al.* 1986; Steinberg *et al.* 2001), Connell and Slatyer's third model of succession (Connell and Slatyer 1977).

Apart from continuing to resolve the question as to whether or not microalgae express AMPs, one of the first priorities for future work on TBAP in *P. tricornutum* must be to determine whether TBAP is produced when the alga is cultured in different media, specifically using a natural seawater base. This will enable the scaling-up of culture for high biomass more economical and less time-consuming. In addition, this will also show whether the production of TBAP by *P. tricornutum* was a consequence of the constituents of the media and artificial seawater used for this

study. This could be achieved by screening a series of batch cultures of *P. tricornutum* grown in different media for TBAP.

The high biomass yield gained by the scaling-up of culture should provide enough starting material for the purification of TBAP for further structural characterisation to elucidate the remaining unknown functional group that is *para*-substituted on the phenolate ion. This would permit synthesis of the compound for further investigations, such as the full characterisation of biological activity of TBAP. This would include assessing antialgal, antifungal, antiviral or anticancer activities, as well as assaying other potential biological activities of interest for pharmacology. Detailed toxicological, and mode of action studies of TBAP for the different sentinel organisms would also be interesting questions to address.

To determine the potential role of TBAP in microalgal chemical ecology, it is necessary to determine the presence of, and quantify TBAP in cell-free culture supernant of *P. tricornutum*. Additionally, it would be interesting if the concentration gradient of TBAP from a single cell of the alga could be measured, as this may indicate the level of influence that *P. tricornutum* has on it's surrounding biota in a scale of three-dimensional space and time, although technical difficulties may prevent this from being accomplished at this time. This region, termed the "phycosphere" was originally defined as the stimulation of bacterial growth (Bell and Mitchell 1972). However, it may equally apply to inhibitory activity. Finally, the effects of environmental conditions, e.g. irradiance, light/dark cycle and temperature needs to be studied, and to determine if other strains and which morphotype of *P. tricornutum*, and indeed other species of microalgae produce TBAP.

Appendices

Appendix 1 Formulae

Unless specified, chemicals / reagents were acquired from Sigma-Aldrich. All solutions are prepared with deionised or ultra pure deionised water (Elga), except where specified.

(a) Modified Erd-Schreiber medium (Foyn 1934)

Preparation of stock solutions:

Soil extract:

One kg of garden soil / potting compost (free from biocides or fertilisers) was mixed with 2 l of deionised water, autoclaved for 60 minutes at 107 °C (15 psi), and allowed to settle overnight. The supernatant was decanted and filtered (Whatman no.1), followed by glass-fibre (GF/C, Whatman) paper. The filtrate was stored at -20°C in 25ml sterile universal bottles until use.

Nitrate / phosphate solution:

20 % (w/v) $NaNO_3$, 2 % (w/v) (Na_2HPO_4) in sterile deionised water.

Silicate solution:

4 % (w/v) ($Na_2SiO_3.5H_2O$) in sterile deionised water.

For the final media, 50 ml of soil extract, 1 ml of nitrate / phosphate solution and 1 ml of silicate solution was combined and made up to 1 l with unfiltered aged seawater and adjust to pH. 8 with HCL or NaOH. This final solution was then sterilised by autoclaving for 35mins at 107°C (15 psi), and allowed to stand for at least 2 days before use.

(b) Enriched artificial seawater medium (ESAW)

(modified from Harrison *et al.* 1980)

The two AW salt solutions were prepared separately, autoclaved at 121°C for 15 min then left to stand in a cool dark place for 48 hrs for gaseous exchange to take place before combining 1:1 (v/v). The nutrient and trace metal solutions were mixed in the specified proportions, autoclaved as above and stored at 4 °C. 10 ml of this enrichment was added to 1 l of AW and mixed well prior to inoculation.

Compound	RMM	g/l soln.	g/ 10 l DW	Final conc. mmol. l^{-1}
AW Solution I (Anhydrous salts)				
NaCl	58.44	20.758	207.58	362.661
Na_2SO_4	142.04	3.477	34.77	24.993
KCl	74.56	0.587	5.87	8.038
$NaHCO_3$	84.00	0.170	1.70	2.066
KBr	119.01	0.0845	0.85	7.249×10^{-1}
H_3BO_3	61.83	0.0225	0.23	3.715×10^{-1}
NaF	41.99	0.0027	0.03	6.570×10^{-2}
AW Solution II (Hydrated salts)				
$MgCl_2 . 6H_2O$	203.33	9.395	93.95	47.176
$CaCl_2 . 2H_2O$	147.03	1.316	13.16	9.139
$SrCl_2 . 6H_2O$	266.64	0.0214	0.03	8.200×10^{-2}

Nutrient and trace metal mix

Compound	RMM	g/ l	Final conc. µmol. L^{-1}
$NaNO_3$	84.99	4.667	549.09
$Na_2SiO_3 . 9H_2O$	212.1	3.000	105.60
Na_2glyceroPO$_4$	216.0	0.667	21.79
$C_{10}H_{14}N_2O_8Na_2 . Na_2$EDTA	372.2	0.553	14.86
H_3BO_3	61.83	0.380	61.46
$Fe(NH_4)_2(SO_4)_2 . 6H_2O$	392.1	0.234	5.97
$FeCl_3 . 6H_2O$	270.3	0.016	5.92×10^{-1}
All the above were in solution before adding the following trace metals			

Trace metal solution (×50), add 2ml per l of nutrient soln:

Compound	RMM	g / 100 ml	g/ l of ES	Final conc. µmol. L^{-1}
$MnSO_4 . 4H_2O$	169.0	2.70	0.054	2.42
$ZnSO_4 . 7H_2O$	287.5	0.37	0.0073	2.54×10^{-1}
$CoSO_4 . 7H_2O$	281.1	0.08	0.0016	5.69×10^{-2}

(c) Radial Diffusion Assay (RDA)

(Modified from Lehrer *et al.* 1991)

Preparation of agars for the RDA:

The following constituents for each type of agar was added to 500 ml of deionised water and boiled for 2 min to dissolve the agar, mixing constantly. After cooling at room temperature for a short while, 15 ml aliquots were decant into universal bottles, sterilised by autoclaving at 121°C for 15 min, and stored at 4 °C until use.

For marine bacteria	Marine Broth 2216 (Difco)	Agarose	NaCl
Base Agar	3.74 g l^{-1}	6.0 g l^{-1}	3 % (w/v)
Top Agar	37.5 g l^{-1}	6.0 g l^{-1}	none

For non-marine bacteria	Nutient Broth CM1 (Oxoid)	Agarose
Base Agar	1.3 g l^{-1}	6.0 g l^{-1}
Top Agar	13.0 g l^{-1}	6.0 g l^{-1}

Appendix 2 Gel electrophoresis

(a) Tris-tricine SDS-PAGE

(Schägger & Von Jagow 1987)

Preparation of the following 'ready to use' stock solutions using deionised water (DW) or ultra pure deionised water (UPDW):

Gel Buffer (100ml):	36.33 g Tris base (3M)
(UPDW)	0.3 g SDS (0.3%)
	pH 8.45 (using HCl)
Anode Buffer (500 ml):	12.11 g Tris Base (0.2M)
(DW)	PH 8.9 (using HCl)
Cathode Buffer (500 ml):	6.05 g Tris Base (0.1M)
(DW)	8.96 g Tricine (0.1M)
	0.5 g SDS (0.1%)
	pH 8.25 (using HCl)
80% Glycerol:	80 ml Glycerol
(UPDW)	20 ml H_2O
10% APS:	0.1 g APS (prepared prior to use)
(UPDW)	0.9 ml H_20
Sample Buffer (100 ml):	0.606 g Tris Base
(UPDW)	4 g SDS
	12 g Glycerol
	pH 6.5 (using HCl)
	0.01 g Bromophenol blue

Just prior to use 20 µl of β-Mercaptoethanol was added to 980 µl of the sample buffer.

For two gels the following three solutions were prepared in sterile disposable 15 ml tubes:

Constituant	**Separating**	**Spacer**	**Stacking**
40% acrylamide (29:1)	3.1 ml	0.77 ml	0.62 ml
Gel Buffer	2.5 ml	1.0 ml	1.55 ml
UPDW	0.65 ml	1.23 ml	4.08 ml
80% Glycerol	1.25 ml	NONE	NONE
Final constituants			
10% APS	65 µl	30 µl	100 µl
TEMED	6.5 µl	3.0 µl	10.0 µl

Procedure:

The final constituents (above) were added to the separating solution and mixed quickly before pouring half (4-4.5 cm of height) into each side of the set up gel kit. The final constituents were immediately added to the spacer solution, mixed and poured on top of the separating solution to add 1 cm of height to each side. Each side was then topped up with water-saturated butan-1-ol to level-off spacer and allow polymerisation to take place. After the separating and spacer solutions solidified the water saturated butan-1-ol was poured out and the gels washed thoroughly with ultra pure deionised water. The remaining final constituents for the stacking solution were added, mixed as before and poured on top of the polymerised spacer to the top of the glass plates so that there was a height of 0.8-1.0 cm between the bottom of the wells and the spacer. The well forming comb was placed in between the glass plates before the stacking solution polymerised, taking care not to form air bubbles. When the stacking gel solidified the comb was removed and cathode buffer was poured into the centre compartment to above the top of the gels and the anode buffer poured into the outer bays. The samples to run were mixed with the sample buffer 1:1 (v/v) and boiled for 5 mins, mixed briefly on a vortex mixer and centrifuged at 10,000 × g for 30 seconds before pipetting each sample into an individual well on the gel. A low molecular weight marker (Promega) was treated the same as a sample. Sample

buffer was typically pipetted into the well on each end of a gel and where possible between samples. The gel was run at 30 mA set to constant current, until the dye-front migrated down into the separating gel, then the current was increased to 40 mA until the dye-front was nearly at the bottom of the gel. The gels were then removed from the gel kit and the stacking gel section cut off, leaving the remaining gel to be stained with silver.

(b) Nondiamine chemical development silver staining of SDS-PA gels

(modified from Rabilloud 1990)

	Step	**Constituents**	**Duration (min)**
1.	Fixative:	40 % (v/v) methanol 10 % (v/v) acetic acid	20
2.	Fixative:	40 % (v/v) methanol	10
3.	Wash:	ultra pure deionised water	2 × 5 min.
4.	Oxidiser:	0.02 % (w/v) $Na_2S_2O_4$	1
5.	Wash:	ultra pure deionised water	2 × 20 sec.
6.	Silver reagent:	0.1% (w/v) Chilled $AgNO_3$ (stored at 4°C)	20
7.	Wash:	ultra pure deionised water	30 sec.
8.	Developer:	0.05 % (v/v) 37%HCHO 3 % (w/v) Na_2CO_3	~30 sec
9.	Developer:	0.05 % (v/v) 37%HCHO 3 % (w/v) Na_2CO_3	~10 min
10.	Stop:	5 % (v/v) acetic acid	5

Appendix 3 List of Suppliers

BDH, Poole, Dorset, UK

Biochrom Ltd, Cambridge, Cambridgeshire, UK

BioRad, Hemel Hempstead, Hertfordshire, UK

BOC Edwards, Crawley, West Sussex, UK

Capital HPLC, Broxburn, West Lothian, UK

Corning, High Wycombe, Buckinghamshire, UK

Daikin, Woking, Surrey, UK

Difco, West Molesey, Surrey, UK

Dynatech, Chantilly, VA, USA

Elga, High Wycombe, Buckinghamshire, UK

Greiner, Nűrtingen, Germany

Kratos, Manchester, U.K

LI-COR Biosciences, Cambridge, Cambridgeshire, UK

Oxoid, Basingstoke, Hampshire, UK

Pharmacia (Amersham), Little Chalfont, Buckinghamshire, UK

Philip Harris Scientific, Macclesfield, Cheshire, UK

Pierce, Rockford, Illinois, USA

Promega, Madison, WI, USA

Sarstedt, Beaumont Leys, Leicester, UK

Varian, Walton on Thames, Surrey, UK

Waters, Watford, Hertfordshire, U.K

Whatman, Maidstone, Kent, UK

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