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Seaweed Biotechnology: Isolation of Useful Compounds from Seaweed and Strain Improvement

by

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해조 생물공학: 해조류로부터 유용물질의 분리 및 품종개량

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by
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Abstract

Seaweed biotechnology is a multidisciplinary subject to produce food, pharmaceuticals, chemicals, and environmental remediation materials from seaweed resources. For centuries, seaweed has been of botanical, industrial and pharmaceutical interest. Compounds extracted from the seaweed are important in pharmaceutical industries. In particular, our survey effort in this area have led to two main themes in studies: (1) screening, isolation and biological analysis of biologically active substances, such as microalgal growth enhancer, antifouling agent and anti-inflammatory (2) tissue culture and mutant selection to overcome supply issues of useful substances. These studies are highly interdisciplinary in nature and draw on diverse methodologies in marine biotechnology. Firstly, in an attempt to identify the microalgal growth enhancer, we isolated the compound levoglucosan from the green seaweed *Monostroma nitidum*, which enhances cell growth in several microalgae in various culture media. Yield of the compound from seaweed powder was 5×10^{-3}% (w/w). At 10 mM concentration, levoglucosan enhanced cell growth and the specific growth rate of all feed microalgal

species tested (*Chaetoceros gracilis*, *Chlorella ellipsoidea*, *Dunaliella salina, Isochrysis galbana, Nannochloris oculata, Navicula incerta, Pavlova lutheri, Tetraselmis suecica*) in most culture media by approximately 150%. Cellular fatty acid profiles and cell size differed marginally between cultures with and without levoglucosan. Secondly, during our studies on allelopathic compounds as environmentally friendly antifouling agents led to the isolation of a novel fatty acid, heptadeca-5,8,11-trienoic acid (HDTA: C17:3). HDTA, an odd-numbered carbon atom fatty acid, showed more than 50% lysis at a concentration of 5 μg/mL against the spores of three chlorophyte species, eight rhodophytes, two phaeophytes, and the cells of three phytoplanktons. Lysis activity increased as the number of double bonds and carbon atoms in the fatty acid increased. HDTA showed ten-fold stronger activity with LC_{50} of 3.1 μg/mL than α-linolenic acid (C18:3). Thirdly, we summarize our study on the anti-inflammatory effects of *C. fragile* involving isolation and structure determination of active substances. The main active compound was isolated by acetonitrile extraction, fractionating by polarity, silica gel column chromatography, and reverse-phase HPLC to give pure compounds CF-2, CF-6 and CF-7. The structure of compound CF-2 was identified by 1D and 2D of ^{1}H and ^{13}C NMR spectroscopy and GC-MS data and suggested as eicosa-5,8,11,14,17- pentaenoic acid (EPA) (C20:5 ω-3). The structure of compound CF-6 and CF-7 were also studied by the NMR and MS experiments and its structures will be constructed. Purified compounds of EPA, CF-6 and CF-7 were tested for anti-inflammatory activities against the PMA-induced mouse ear inflammation symptoms of edema and erythema. The inhibitory effects of different concentrations of EPA, CF-6 and CF-7 topically applied to mouse ears were dose-dependent. The EPA concentrations producing IC_{50} were 230 and 462 μg per ear for edema and erythema, respectively. Finally, a strain improvement program was initiated based on mutagenesis with the goal of increasing the content of tryptophan and lysine (essential amino acids) of

Porphyra. Three rounds of ultraviolet radiation and selected mutagenic agents (DL-5-methyltryptophan and aminoethyl-L-cysteine) were conducted using monospores of *Porphyra* as the parent strain. When analyzing the IC_{100} of selected monospores of the first stage, value of 8 mM and 116 mM, which is higher than original stage, were obtained for DL-5-methyltryptophan and aminoethyl-L-cysteine, respectively. When analyzing the IC_{100} of selected monospores of the final stage, value of 9.3 mM and 135 mM were obtained for DL-5-methyltryptophan and aminoethyl-L-cysteine, respectively. Our results appear promising that it is possible to increase the volumetric productivity of both essential amino acids using this method.

Keywords: algal growth enhancer, levoglucosan, microalgal growth, *Monostroma nitidum*, allelopathy, crustose coralline algae, *Lithophyllum* spp., monospore, *Codium fragile,* anti-inflammatory, strain improvement, tryptophan, lysine, essential amino acids, *Porphyra* sp..

Chapter 1

Seaweed Biotechnology: A Review

Drug Discovery from Natural Sources

For thousands of years, natural products have played an important role throughout the world in treating and preventing human diseases. Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates. 1 The importance of natural products in modern medicine has been discussed in recent reviews and reports (Newman et. al., 2000, 2003; Koehn & Carter, 2005; Paterson & Anderson, 2005; Balunas & Kinghorn, 2005; Jones et. al., 2006)

An analysis of the origin of the drugs developed between 1981 and 2002 showed that natural products or natural product derived drugs comprised 28% of all new chemical entities (NCEs) launched onto the market (Newman et. al., 2003). In addition, 24% of these NCEs were synthetic or natural mimic compounds, based on the study of pharmacophores related to natural products (Newman et. al., 2000). This combined percentage (52% of all NCEs) suggests that natural products are important sources for new drugs and are also good lead compounds suitable for further modification during drug development. The large proportion of natural products in drug discovery has stemmed from the diverse structures and the intricate carbon skeletons of natural products. Since secondary metabolites from natural sources haven been elaborated within living systems, they are often perceived as showing more “drug-likeness and biological friendliness than totally synthetic molecules” (Koehn & Carter, 2005) , making them good candidates for further drug development (Balunas & Kinghorn, 2005; Drahl et. al., 2005).

Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorized human diseases (48/55), including as antibacterial, anticancer, anticoagulant, antiparasitic, and immunosuppressant agents, among others (Newman et. al., 2003). There was no introduction of any natural products or related drugs for 7 drug categories (anesthetic, antianginal, anti histamine, anxiolytic, chelator and antidote, diuretic, and hypnotic) during 1981 to 2002 (Newman et. al., 2003). In the case of antibacterial agents, natural products have made significant contributions as either direct treatments or templates for synthetic modification. Of the 90 drugs of that type that became commercially available in the United States or were approved worldwide from 1982 to 2002, ~79% can be traced to a natural product origin (Newman et. al., 2003). Frequency of use of natural products in the treatment and/or prevention of disease can be measured by the number and/ or economic value of prescriptions, from which the extent of preference and/or effectiveness of drugs

can be estimated indirectly. According to a study by Grifo and colleagues (Grifo et. al., 1997), 84 of a representative 150 prescription drugs in the United States fell into the category of natural products and related drugs. They were prescribed predominantly as anti-allergy/ pulmonary/respiratory agents, analgesics, cardiovascular drugs, and for infectious diseases. Another study found that natural products or related substances accounted for 40%, 24%, and 26%, respectively, of the top 35 worldwide ethical drug sales from 2000, 2001, and 2002 (Butler, 2004). Of these natural product-based drugs, paclitaxel (ranked at 25 in 2000), a plant-derived anticancer drug, had sales of $1.6 billion in 2000 (Thayer, 2003; Oberlies & Kroll, 2004). The sales of 2 categories of plant-derived cancer chemotherapeutic agents were responsible for approximately one third of the total anticancer drug sales worldwide, or just under $3 billion dollars in 2002; namely, the taxanes, paclitaxel and docetaxel, and the camptothecin derivatives, irinotecan and topotecan (Thayer, 2003; Oberlies & Kroll, 2004).

Beneficial Role of Oceans

Humankind has explored and exploited the terrestrial environment for more than 3000 years, leading to the examination of almost every possible resource on land. As we have benefited from life on land, it is reasonable to predict that the next few decades will be filled with new discoveries from our greatest untapped resource, the world's oceans. The marine environment provides amazing source of both biological and chemical diversity, we have much to learn. Among 34 fundamental phyla of life, 17 occur on land whereas 32 occur in the sea (with some overlap). This diversity has been the source of unique chemical compounds with the potential for industrial development as new medicinal products, cosmetics, nutritional supplements, molecular probes, agrochemicals, industrial chemicals, and new, environment-friendly industrial processes (Fenical, 2006).

In recent years, a significant number of novel metabolites with potent pharmacological properties has been discovered from the marine organisms. Although there are only a few marine-derived products currently on the market, several robust new compounds derived from marine natural products are now in the clinical pipeline, with more clinical development. While the marine world offers an extremely rich resource for novel compounds, it also represents a great challenge that requires inputs from various scientific areas to bring the marine chemical diversity up to its therapeutic potential (Fenical, 2006).

Until 2004, researchers have isolated approximately 7000 marine natural products, 25 percent of which are from algae, 33 percent from sponges, 18 percent from coelenterates (sea whips, sea fans and soft corals), and 24 percent from representatives of other invertebrate phyla such as ascidians (also called tunicates), opisthobranch molluscs (nudibranchs, sea hares etc), echinoderms (starfish, sea cucumbers *etc*) and bryozoans

(moss animals) (Kijjoa & Sawangwong, 2004). A simplistic analysis of these data reveals that as the search for "Drugs from the Sea" progresses at the rate of a 10 percent increase in new compounds per year, researchers are concentrating their efforts on slow-moving or sessile invertebrate phyla that have soft bodies, and lack of spines or a shell, *i.e.* animals that require a chemical defence mechanism (Faulkner, 1995)

Natural Products and the Treatment of Human Disease

More than 3000 years ago, early societies recognized that the diversity of plant life around them could be used for the treatment of human illness. Natural "preparations," in the form of teas or salves derived from plants, were commonly used to treat pain, infections, gastrointestinal maladies, inflammation, cancer, and many other common illnesses. Traditional healers evolved who were consulted to treat illness, and the knowledge of these individuals was passed down to understudies or apprentices who continue to practice today. Today, for economic as well as traditional reasons, much of the developing world still relies on natural medicines (e.g., ethnomedicines or traditional medicines) for the treatment of human disease (Fenical, 2006).

Over time, the "active ingredients" from traditional medicines were chemically purified, and during the 19th and 20th centuries some of these drugs (e.g., morphine, quinine, the salicylates [aspirin]) were utilized in single-ingredient formulations (i.e., "drugs"). As time passed, these molecules became the foundation of the new discipline of organic chemistry. The developing pharmaceutical industries evolved to focus their efforts on purifying new drugs from these traditional ethnomedicines (Therapeutic Research Faculty, 2006).

The discovery of penicillin in the late 1920s by Alexander Fleming was perhaps the single most important medical discovery in modern times. This, and subsequent discoveries by Selman Waksman (i.e., actinomycin and other antibiotics) and other researchers, changed how drugs were discovered and how Nature was explored (Bérdy, 2005). The pharmaceutical industry, worldwide, quickly evolved by embracing these findings, and subsequently discovered hundreds of "wonder drugs" that had the capability to cure pneumonia and almost all bacterial infectious diseases. These natural "wonder drugs"
saved millions of lives during and after World War II, and gave us the false sense that the great plagues of the past (e.g., cholera) would never again be seen (see Laws case study, this issue). More than 120 antibiotics, anticancer agents, and other therapeutics originally derived from microorganisms that are found in soil are still prescribed today.

Discoveries and Successes - Current

Many natural products were developed into medicines in the mid 20th century, but

the challenges and difficulties to do so now have dramatically changed. In the 21st century, we live in a more complex world in which diseases are complex and resistant to cures. With the past in mind, society now places exceptionally strong demands on drug safety and efficacy. As a consequence, many of the drugs developed in past years would not survive today's high-level expectations. Drug resistance in the treatment of cancer and infectious diseases is emerging at a frightening rate, just when the pharmaceutical industries are turning away from some of these areas, only to focus their attention on the development of "block-buster drugs" (sales in excess of $2 billion) in therapeutic areas requiring chronic treatment over a lifetime. Explorations for new anti-infective agents, especially antibacterial drugs, have all but ceased. Given these new realities, how can the biomedical potential of the oceans best serve human medicine ?

Over the past ten years, the oceans have provided exciting medical discoveries that are now yielding drugs. The first marine drug was Ziconotide (Pralt™), a potent pain medication that was developed based upon knowledge of the highly toxic small peptide ω-conotoxin MVIIA extracted from the venomous gastropod mollusk *Conus magus* (Olivera et al., 1987). Ziconotide, a potent calcium channel blocker, is the only drug in this class of agents that provides relief from severe neurogenic pain. Although this is the first marine drug generally recognized, in the 1950s, Werner Bergman, a pioneer in marine sterol chemistry, isolated two modified nucleosides, spongothymidine and spongouridine, from the sponge *Cryptotethia crypta* (Bergmann and Feeney, 1951). These compounds possessed unique antiviral activities, and over the decades were the inspiration for the development to two related antiviral drugs, Ara-A (Vidabarine) and Ara-C. As might be expected, the large pharmaceutical industries have had only modest interest in embracing marine drug discovery. This area continues today to be one of uncertainty and high predicted risk, and one that industry has not learned to reliably control. As a consequence, more and more discoveries are being made by academic researchers and by the flourishing small biotechnology industries. Increasingly, the large pharmaceutical industries rely on the "inlicensing" of drugs discovered elsewhere (Table 1). Thus, in the decades to come, it can be predicted that marine biotechnology companies will evolve as opportunities arise to exploit marine biodiversity.

Cancer continues to be a major disease worldwide; in the United States, Europe, and Japan it is a major cause of human mortality. It is thus no surprise that the NCI has invested heavily over the past three decades in the discovery and development of anticancer drugs from marine sources (Cragg et al., 2005). What is not widely known is the degree of their success. In 2006, more than 30 marine derived molecules are in preclinical development or clinical trials against a wide diversity of cancers. A significant number of these new drug candidates was developed with direct or indirect NCI assistance, and once brought to the point of perceived utility, they were then licensed to

pharmaceutical partners for clinical development, manufacture, and sales. Of particular importance over the past 15 years is the NCI's "National Cooperative Drug Discovery Groups" or NCDDGs, and productive researchers such as G.R. Pettit at Arizona State University's Cancer Research Institute, who have dedicated their work to cancer-drug discovery. The NCDDG collaborative grants were cleverly crafted to require the close collaboration of academic researchers and industrial scientists, whose respective abilities to focus on new sources for possible drugs were coupled with the pharmacological strengths and developmental expertise of industry. The result has been productive collaborative programs that link these diverse scientific endeavors. Table 1 lists the cancer drugs discovered in these and related programs, their sources, and discoverers. Seventeen novel molecules, produced by marine bacteria, sponges, ascidians, mollusks, bryozoans, and sharks, are currently in clinical trials. This impressive list can leave no doubt that the oceans have the ability to offer new pharmaceuticals, particularly for the treatment of cancer. It is often asked why marine sources should yield new anticancer agents? Is it simply that the oceans contain many potent toxins, and these are useful in killing cancer cells but have no other utility? This is an inaccurate view of the medical potential of the world's oceans created by the significant funding available worldwide to discover new anticancer drugs. Marine life produces a massive diversity of complex, bioactive molecules only a small percentage of which is "toxic" to humans and other species in the traditional sense. These molecules are now being shown by academic researchers to possess diverse and highly complex pharmacological properties with applications to many diseases. Many unique molecular probes with activities relevant to fundamental processes have been isolated and defined. The problem of developing a greater diversity of marine drugs lies in the lack of funding for the discovery of drug leads in other therapeutic areas. As of 2006, the NCI is the only NIH institute that had a dedicated drug-discovery program (Cragg et al., 2005). Furthermore, there is an undeveloped relationship between those who discover new marine molecules and those who have the biological expertise and screening capacity to develop new drugs in diverse therapeutic areas. Impressively, three new marine drugs are in clinical trials for acute pain, three more are in clinical evaluation for the treatment of asthma, and one drug is in clinical assessment for Alzheimer's Disease (Table 1). Other molecules are being shown to be effective against malaria and other infectious diseases. Indeed, today, the study of bioactive marine molecules continues at a spectacular pace (Blunt et al., 2003, 2004, 2005).

While the ocean is clearly a new frontier in drug discovery, it remains isolated from the mainstream discovery and developmental processes, which require hundreds of millions of dollars of investment. How can we change this? One can predict that the next decade will see major changes in the pharmaceutical industry and in how NIH will respond to medical discoveries and human medical needs. More academia-industry

linkages will be observed, and the responsibility for drug discovery, particularly in the less-profitable areas such as antibiotics discovery, will be more greatly embraced by the NIH. These changes are beginning now with industry reconsidering natural product-based drug discovery and the NIH planning their own drug-discovery efforts as part of the "NIH Roadmap for Medical Research" (for more information go to http://nihroadmap.nih.gov).

Benefical Role of Seaweeds

Marine macroalgae, better known as seaweeds, belong to a rather ill-defined assemblage of plants known as the algae. The term "seaweed" itself does not have any taxonomic value, but is rather a popular term used to describe the common large attached (benthic) marine algae found in the groups Chlorophyceae, Rhodophyceae, Phaeophyceae or green, red and brown algae according to their pigmentation, respectively.

The algae differ from the higher plants in that they do not possess true roots, stems or leaves. However, some of the larger species, upon which the industries are primarily based, possess attachment organs, or holp-fasts, that have the appearance of roots, and there may also be a stem-like portion called a stipe, which flattens out into a broad leaf-like portion of lamina (eg. *Laminaria*). Some species consist simply of a flat plate of tissue (eg. *Ulva*), whilst in others the plant body, or thalus, is composed of a narrow, compressed or tubular axis with similar branches arising from it (eg. *Gelidium*). The smaller species differ from those described above in that they are mainly filamentous (Chapman V. J., 1980).

Seaweeds are widely used as food, as ingredients in cosmetics and fertilizers, and in hydrocolloid production (e.g. agar and alginate). Seaweeds are of ecological importance because they assist in supplying oxygen to the sea and act as one of the primary producers in the marine food chain. Some seaweed have the capacity to remove heavy metals from the water and can potentially be used in biomonitoring and in the bioremediation of such pollutants. Seaweeds also possess excellent survival strategies to withstand the many environmental stresses that they are exposed to. For all these reasons, together with their unique life-cycle and physiology, seaweeds are interesting study subjects (Chan et al. 2006).

Global utilization of macroalgae is a multi-billion dollar industry. Much of this is based on farming of edible species or on the production of agar, carrageenan and alginate. Of all seaweed products, hydrocolloids have had the biggest influence on modern western societies. They have attained commercial significance through their use in various industries which exploit their physical properties such as gelling, water-retention and their ability to emulsify (Renn, 1997). Little commercial exploitation of products extracted from seaweeds occurs outside the hydrocolloid industry. However, in recent years pharmaceutical firms have started looking towards marine organisms, including seaweeds,

in their search for new drugs from natural products. These products are also increasingly being used in medical and biochemical research.

Since the 1940s, when the potential of agar production from seaweeds was recognized, taxonomy, physiology and biochemistry have been the main research focus. Physiological aspects related to the production of hydrocolloids and pigments, and to mass cultivation of seaweeds, have been of particular interest. These areas of research laid the foundation of our understanding of seaweed biology. Prior to the 1950s, the medicinal properties of seaweeds were restricted to traditional and folk medicines (Lincoln et al., 1991). During the 1980s and 90s, compounds with biological activities or pharmacological properties (bioactivities) were discovered in marine bacteria, invertebrates and algae (Mayer & Lehmann, 2000). In the last three decades the discovery of metabolites with biological activities from macroalgae has increased significantly. However, despite the intense research effort by academic and corporate institutions, very few products with real potential have been identified or developed. Based on Silver platter MEDLINE and Aquatic Biology, Aquaculture & Fisheries Resources databases, the literature was searched for natural products from marine macroalgae in the Rhodophyta, Phaeophyta and Chlorophyta with biological and pharmacological activity. Substances that currently receive most attention from pharmaceutical companies for use in drug development, or from researchers in the field of medicine-related research include: sulphated polysaccharides as antiviral substances, halogenated furanones from *Delisea pulchra* as antifouling compounds, and kahalalide F from a species of *Bryopsis* as a possible treatment of lung cancer, tumors and AIDS. Other substances such as macroalgal lectins, fucoidans, kainoids and aplysiatoxins are routinely used in biomedical research and a multitude of other substances have known biological activities. The potential pharmaceutical, medicinal and research applications of these compounds are discussed.

Antiviral Activity

Some sulphated polysaccharides from red algae show antiviral activities towards viruses responsible for human infectious diseases. Most notable are *Aghardhiella tenera* and *Nothogenia fastigiata*. Witvrouw et al. (1994) tested a galactan sulphate from *Aghardhiella tenera*, and Damonte et al. (1994) and Kolender et al., (1995) a xylomannan sulphate from *Nothogenia fastigiata* against human immunodeficiency virus (HIV), Herpes simplex virus (HSV) types 1 and 2 and respiratory syncytial virus (RSV). These polysaccharides are active during the first stage of the RNA virus replication when the virus adsorbs onto the surface of the cell (De Clercq, 1996, 2000). An important requirement of an antiviral polysaccharide is that it must have very low cytotoxic activities towards mammalian cells, and most of the algal polysaccharides, particularly those of *Aghardhiella tenera* and *Nothogenia fastigiata*, have this characteristic (De Clercq, 1996).

Carrageenans (Figure 1) demonstrate potential *in vitro* antiviral activity. Carlucci et al. (1997, 1999a, 1999b) noted that λ-carrageenan and partially cyclized μ/ί-carrageenan from *Gigartina skottsbergii* have potent antiviral effects against different strains of HSV types 1 and 2 during the virus adsorption stage. Carrageenans from cystocarpic and tetrasporophytic stages of *Stenogramme interrupta* show similar antiherpetic activity (Caceres et al., 2000). Zeitlin et al. (1997) tested a range of antiviral substances for their possible effectiveness as vaginal microbicide against genital herpes in mice, and found that carrageenan and fucoidan, or fucoidin, are good candidates for further development. None of these studies have shown that carrageenans exhibit significant levels of cytotoxicity or anticoagulant activity. A carrageenan-based vaginal microbicide called Carraguard has been shown to block HIV and other sexually transmitted diseases *in vitro*. Carraguard entered phase III clinical trials involving 6000 non-pregnant, HIV-negative women in South Africa and Botswana in 2003 (Spieler, 2002).

A sulphated polysaccharide from *Schizymenia pacifica* inhibits HIV reverse transcriptase *in vitro* (Nakashima et al., 1987a, 1987b), a later stage in HIV replication. It has minimal effect on human DNA and RNA polymerase activity. Some agaroids such as high molecular weight galactan sulphate from *Gracilaria corticata* have antiviral properties against HSV types 1 and 2, and this action is likely due to an inhibition of the initial virus attachment to the host cell (Mazumder et al., 2002).

Fucoidan has potent antiviral properties towards viruses such as RSV (Malhotra et al., 2003), HIV, (Sugawara et al., 1989), HSV types 1 and 2 and human cytomegalovirus (Feldman et al., 1999; Majczak et al., 2003; Ponce et al., 2003). The antiviral properties of fucoidan seem to stem from inhibiting binding of the viral particle to the host cell (Baba et al., 1988). It has the additional benefit of inhibiting binding of sperm to the zona pellucida in humans (Oehninger et al., 1991), thus allowing for the compound to be developed into a possible vaginal microbicide with contraceptive properties. Uncharacterised polysaccharide fractions obtained from *Caulerpa* sp., *Corallina* sp., *Hypnea charoides*, *Padina arborescens* and *Sargassum patens* also have high antiviral activity against HSV types 1 and 2 while maintaining low levels of cytotoxicity (Zhu et al., 2003). The antiviral activities discussed thus far are for algal polysaccharides, but other compounds exhibit similar properties. Chondriamide A (Figure 1) from *Chondria atropurpurea* shows antiviral activity against HSV type II (Palermo et al., 1992). Kahalalide F (Figure 1) produced by a species of *Bryopsis* has also been noted for its effectiveness in some AIDS study cases, and its antiHIV qualities are being further studied in clinical trials (Hamann et al., 1996; Haefner, 2003).

Antibiotic Activity

Chemicals responsible for antibiotic activities are widespread in macroalgae.

Interesting substances in particular are the halogenated compounds such as haloforms, halogenated alkanes and alkenes, alcohols, aldehydes, hydroquinones and ketones (Lincoln et al., 1991). The list of terpenoids with antibiotic qualities is especially long, andmany of these are also halogenated. Compounds such as sterols and heterocyclic and phenolic compounds sometimes have antibiotic properties. Many of these could be developed into antiseptics and cleansing agents, but their antibiotic activity *in vivo* is often only achieved at toxic concentrations (Lincoln et al., 1991).

The depsipeptides kahalalide A (Figure 1) and F from *Bryopsis* sp. were noted for their *in vitro* activity against *Mycobacterium tuberculosis* (el Sayed et al., 2000), but the future of these peptides seems to lie with the development of kahalalide F for treatment of lung cancer, tumors and AIDS. A promising antibacterial agent is a halogenated furanone, or fimbrolide, that belong to a class of lactones (Figure 1) from *Delisea pulchra*. It has been examined for its effectiveness as an active ingredient in bacterial antifouling agents (Kjelleberg & Steinberg, 2001), and as a possible treatment for chronic *Pseudomonas aeruginosa* infection. *Pseudomonas aeruginosa* infection is characterised by the production of mucoid alginate and formation of a 'biofilm' in the lungs of cystic fibrosis sufferers (Høiby, 2002). Inhibition of bacterial colonisation is achieved by the inhibiting effect of furanone on the quorum sensing mechanism of cells by functioning as an intercellular signal antagonist. The result is a disruption of intra and inter-species cell-cell communication (Rasmussen et al., 2000). The effect has been observed in a wide range of Gram-negative bacteria. Effects are seen on the swarming of *Serratia liquefaciens* (Rasmussen et al., 2000) and the bioluminescence and virulence in several pathogenic *Vibrio* species (Manefield et al., 2000; Kjelleberg & Steinberg, 2001). It also inhibits carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora* (Manefield et al., 2001).

Agglutination, Coagulation and the Stimulation of Cell Migration

Macromolecule recognition processes are common in cells and their specificity is their most important characteristic. Many research programs exploit recognition events and these have become focus areas of research in biology, chemistry, medicine and pharmacology. Biological reactions that involve recognition events include processes such as cell agglutination and coagulation, the stimulation of cell migration and fertilisation. Lectins, sometimes referred to as haemagglutinins or agglutinins, are glycoproteins with an ability to agglutinate red blood cells (Boyd&Reguera, 1949).Various polysaccharides are present on cell surfaces, and as a result many cells including microbes and yeasts (e.g. Patchett et al., 1991; Bird et al., 1992; Cisar et al., 1995), tumor cells (Hori et al., 1986) and erythrocytes are selectively agglutinated by lectins (Chen et al., 1995).

Lectins are inhibited by sugars of the same type as those on the surface of the cells

being agglutinated (Sharma& Sahni, 1993). They are useful in exploring properties of biological structures and processes, and have found applications in biology, cytology, biochemistry, medicine and food science and technology. Lectins from *Codium* spp. have been developed into commercially available reagents and are routinely used in biochemical studies. Lectins with haemagglutinating properties occur in a variety of red, green and brown algae (e.g. Rogers & Hori, 1993; Benevides et al., 1998; Shanmugam et al., 2002). They react with a wide array of erythrocytes, including human blood group types. Agglutination reactions with human blood groups have led to their use in assays for blood typing. Lectins are also used to characterize cell-surface polysaccharides or to examine cell binding patterns in lectinosorbent assays (Llovo et al., 1993; Wu et al., 1996; Wu et al., 1998). Lectins from *Codium fragile* subsp. *tomentosoides* have been developed into a histochemical reagent by coupling them to colloidal gold, forming a lectin-gold conjugate. This conjugate is useful for studies of the surface topography of cells of animal tissues (Griffin et al., 1995). Other common examples of lectins from macroalgae are hypnins A-D in *Hypnea japonica* (Hori et al., 1986), a sulphated polysaccharide in *Gracilaria verrucosa* (Kakita et al., 1997) and a haemagglutinin in an ammonium sulphate fraction of a buffer extract of *Gracilaria chorda* (Kakita & Kitamura, 2003). Anticoagulant effects are often related to the sulphate and sugar content of the components (Jurd et al., 1995; Shanmugam et al., 2002). Cell migration is also stimulated by lectins. For example, the lectin amansin from *Amansia multifida,* induces neutrophil migration *in vitro* and *in vivo* in the peritoneal cavity or dorsal air pouch of mice (Neves et al., 2001). Lastly, lectins from *Gracilaria verrucosa* induce morphological changes and growth suppression in the dinoflagellate *Chatonella antiqua* (Tanabe et al., 1993).

Activities Related to Cellular Growth

Mitogenic activity

Mitogenic activities, the stimulation of mitosis in previously non-dividing cells, have been demonstrated in mouse lymphocytes using lectins from *Eucheuma serra* (Kawakubo et al., 1997). Amansin isolated from *Amansia multifida* has been found to stimulate peripheral blood mononuclear cells and causes a gradual reduction in mitogenic capacity with progressive increase in the lectin concentration (Lima et al., 1998). Fucoidan enhances new blood vessel formation by modulating the expression of surface proteins (Matou et al., 2002), and lipogenic activity has been demonstrated for lectins from *Codium fragile* in isolated rat and hamster adipocytes (Ng et al., 1989). *Effects on fertilisation and larval development* Various seaweed-derived compounds affect fertilization and larval or embryonic development in both invertebrates and vertebrates. Fucoidan inhibits the initial binding of sperm and subsequent recognition events necessary for penetration of the human zona pellucida (Oehninger et al., 1991; Patankar et al., 1993). This property of

fucoidan, together with its antiviral activity, makes it a potential candidate for development into vaginal microbicide with contraceptive properties (Baba et al., 1988; Zeitlin et al., 1997). Premakumara et al. (1996) identified a sphingosine derivative from *Gelidiella acerosa* as a post-coital contragestative agent in studies on pregnant rats. The action of the orally administered substance is via an antiprogesterone mechanism with no maternal toxicity (Premakumara et al., 1995). The lectin diabolin isolated from *Laminaria diabolica* causes the development of a fertilisation envelope around unfertilised eggs of the sea urchin *Hemicentrotus pulcherrimus*, thus preventing cleavage (Nakamura & Moriya, 1999; Nomura et al., 2000). Terpenoids are also known for their effects on fertilisation and subsequent development of embryos. For example, caulerpenyne, a sesquiterpene from *Caulerpa taxifolia*, affects embryogenesis, larval development and metamorphosis of the sea urchin *Paracentrotus lividus* (Pesando et al., 1996, 1998). It also interferes with microtubule-dependent events during the first mitotic cycle of sea urchin eggs (Pedrotti & Lemee 1996), and affects regulation of intracellular pH in sea urchin eggs and sea bream hepatocytes (Galgani et al., 1996). *Cytotoxicity, antimitogenic, anticancer and antitumour properties* Kahalalide F which is produced by *Bryopsis* sp. And subsequently assimilated by the grazer *Elysia rufescens* has anticancer and antitumour properties (Hamann & Scheuer, 1993; Hamann et al., 1996). It is effective in controlling tumours that cause lung, colon and prostate cancer (Horgen et al., 2000; Nuijen et al., 2000; Sparidans et al., 2001), and has been patented for use as a possible active substance in therapeutics for the treatment of human lung carcinoma (Scheuer et al., 2000). It has entered phase II clinical studies for liver carcinoma treatment. Kahalalide F functions by acting on the lysosomal membrane (Stokvis et al., 2002), a mechanism that distinguishes it from all other known antitumour agents. It also induces cell necrosis *in vivo* and selectively targets tumour cells *in vitro*. The cytotoxic activity of Kahalalide F is not mediated by mRNA and protein synthesis de novo, nor caspase activation. Kahalalides O and G, also from *Bryopsis* sp. (and *Elysia ornata*) do not show significant cytotoxicity towards the cancer lines tested (Hamann et al., 1996; Goetz et al., 1997; Horgen et al., 2000; el Sayed et al., 2000). Several sulphated macroalgal polysaccharides have cytotoxic properties. Fucoidans are known to have antitumour, anticancer, antimetastatic and fibrinolytic properties in mice (Coombe et al., 1987; Maruyama et al., 1987), and they also reduce cell proliferation (Religa et al., 2000). Translam, the 1→3:1→6-β-Dglucans produced by enzymatic action on laminaran (laminarin), has antitumour properties (Saito et al., 1992). Kaeffer et al. (1999) noted that ulvan has cytotoxicity or cytostaticity targeted to normal or cancerous colonic epithelial cells. Chondriamide A (Figure 1) isolated from *Chondria atropurpurea* shows cytotoxicity against human nasopharyngeal and colorectal cancer cells (Palermo et al., 1992). Terpenes are exceptionally wide in their range of cytotoxic and antitumour activities. Examples include (*S*)-12-

hydroxygeranylgeraniol and (*S*)-13-hydroxygeranylgeraniol derivatives from *Bifurcaria bifurcata* which are toxic towards fertilised sea urchin eggs (Valls et al., 1995; Culioli et al., 2001); caulerpenyne from *Caulerpa taxifolia* which is cytotoxic towards several human cell lines and as such has anticancer, antitumour and antiproliferative properties (Fischel et al., 1995; Parent-Massin et al., 1996; Barbier et al., 2001); the hydroquinone diterpene, mediterraneol, from *Cystoseira mediterranea* which is an inhibitor of mitotic cell division (Francisco et al., 1985); and the meroterpenes, usneoidone E and Z, from *Cystophora usneoides* which have antitumour properties (Urones et al., 1992).

Antithrombic and Anticoagulant Activities

Fucoidans have *in vivo* and *in vitro* heparin-like antithrombic and anticoagulant activities that are mediated by blood coagulation inhibitors such as heparin cofactor II or antithrombin III (Church et al., 1989; Colliec et al., 1991; Matou et al., 2002). The anticoagulation activity is the result of direct fucan-thrombin interaction (Graufel et al., 1989), and it usually increases with the amount of sulphation (Nishino & Nagumo, 1991, 1992). Sulphated fucans from *Fucus vesiculosus* and *Ascophyllum nodosum* have been patented as anticoagulant substances. The work was motivated by the need to find a potential replacement for cattlederived heparin and the fear of the transmission of bovine spongiform encephalitis (BSE) through the use of bovine-derived products (Trento et al., 2001). Sulphated fucoidan has several advantages over heparin. It shows concentration-dependent inhibition of thrombin generation from platelets; it exhibits concentration dependent inhibition of thrombin-induced platelet aggregation; it lacks the hypotensive effect found in thrombin; it reduces the sticking of polymorphonucleated leukocytes to rabbit aorta; and it shows a dose dependent inhibition of thrombin-induced thrombosis (Trento et al., 2001). Some older literature reports laminaran as having anticoagulant properties (Hoppe & Schmid, 1962, cited by Chapman, 1970), but it is possible that this activity comes from fucoidan which is often present in the same extracted fraction as laminaran.

Anti-inflammatory Activity and Effects on the Immune Response

Macroalgae, especially red algae, are rich in 20-carbon atom polyunsaturated fatty acids (PUFAs), chiefly eicosapentaenoic and docosahexanoic acids (Stefanov et al., 1988; Gerwick & Bernart, 1993). Seaweeds are capable of metabolising various C20-PUFAs via oxidative pathways (Gerwick et al., 1993) and in the Gracilariales, prostaglandins are one of the products. Two alternative pathways for the production of prostaglandin have been proposed. The first involves fatty acid cyclooxygenase acting on arachidonic acid, as in mammalian systems (Noguchi et al., 1994). The other mechanism uses lipoxygenase, also acting on arachidonic acid (Gregson et al., 1979). In many red algae, the metabolised

products of PUFAs, called oxylipins, resemble eicosanoid hormones in higher plants and humans which fulfil a range of physiologically important functions (Gerwick et al., 1993; Imbs et al., 2001). The anomalous production of these compounds underlies a number of diseases related to inflammation (Gerwick & Bernart, 1993), and so eicosanoids and their derivatives have received much research attention in the search for development of new classes of antiinflammatory drugs (Jacobs et al., 1993). Gerwick and Bernart (1993) list studies on macroalgal eicosanoids with antiviral, antimicrobial and antihypertensive properties and showing various enzyme-inhibiting activities. The eicosanoid prostaglandin E_2 (PGE_2) (Figure 2) is the likely agent responsible for 'ogonori' poisoning resulting from the human consumption of a species of *Gracilaria* (Fusetani & Hashimoto, 1984; Noguchi et al., 1994). The cases of ogonori poisoning appear to have been brought about by soaking the seaweed in freshwater, thus causing the production of PGE_2. PGE_2 productionwas further enhanced interacting with a diet rich in seafood, leading to the high availability of arachidonic acid, the precursors of PGE_2. More PGE_2 was produced via the action of fatty acid cyclooxygenase, causing haemorrhaging of the victim's stomach. Symptoms included nausea, vomiting and hypotension and death resulted from hypotensive shock (Noguchi et al., 1994). Symptoms are similar to those of misoprostol overdose. Misoprostol is a PGE_1 analogue used for the prevention of nonsteroidal anti-inflammatory drug-induced gastropathy (Graber & Meier, 1991). Poisonings involving prostaglandins are rare but have been mentioned in medical literature (Al Hassan et al., 1987; Graber & Meier, 1991; Bond & van Zee, 1994).

PGE_2 and PGF_2 were detected in *Gracilaria lichenoides* (Gregson et al., 1979). PGE_2 and 15-keto- PGE_2 were found in *G. asiatica* (Sajiki, 1997; Sajiki & Kakimi, 1998) and *G. verrucosa* was found to contain PGA_2, PGE_2, PGF_2, and 15-keto-PGE_2 (Fusetani& Hashimoto, 1984; Imbs et al., 2001). Eicosanoids such as leukotrienes and hydroxyeicotetraenoic acid have physiologically active characteristics such as chemoattraction of neutrophils or smooth muscle cells, the contraction of muscles, and have connections with various kinds of diseases in mammals (Gurr & Harwood, 1991; Sajiki & Kakimi, 1998). The combined effect of prostaglandins and expansion of *Laminaria* stipes is also well-known in obstetrics and gynaecology where it is used as a cervical dilator (Blumenthal, 1988; el Refaey & Templeton, 1995; Lee et al., 1998). Translam, 1→3:1→6-β-D-glucans produced from laminaran, has immunostimulating activities in animals and plants and it has been suggested that they might serve as radioprotective substances in patients with radiation illness (Kuznetsova et al., 1994; Zaporozhets et al., 1995; Chertkov et al., 1999). Preparations containing 1→3:1→6-β-D-glucans, laminaran, and fucoidan are manufactured by the health industry and marketed for their beneficial properties on the immune system. The producers of these tablets cite numerous papers discussing the biological activities of the 1→3:1→6-β-D-glucans.

Laminarans themselves generally have very low levels of bioactivity, but their immunomodulatory effect on anterior kidney leukocytes of the salmon *Salmo salar* has been noted (Dalmo & Seljelid, 1995; Dalmo et al., 1996). Porphyran likely contributes to macrophage stimulating activity in mice (Yoshizawa et al., 1995). The compound 6-n-tridecylsalicylic acid was isolated from *Caulocystis cephalornithos* and shown to be active after oral administration in both acute and chronic animal models of inflammation such that it has similar antiinflammatory activity but less ulcerogenic activity on a molar basis than salicylic acid (Buckle et al., 1980; Kazlauskas et al., 1980). Lastly, carnosadine identified in *Grateloupia carnosa* has been patented as an antiinflamatory agent with positive carcinostatic and immunological effects (Wakamiya et al., 1984).

Enzyme Inhibitors and Stimulants

In humans, secreted phospholipase A_2 (PLA_2) is involved in the development of a variety of inflammatory diseases via the production of arachidonic acid, the precursor of prostaglandins and leukotrienes (Flower & Blackwell, 1976). Secreted phospholipase A_2 could therefore act as target for a class of anti-inflammatory drugs and a substantial research effort has been focused on this group of enzymes. Several macroalgae show potent bee venom-derived PLA_2 activity. Compounds active against PLA_2 include rhiphocephalin, a linear sesquiterpene from *Rhipocephalus phoenix*; caulerpenyne, a sesquiterpene from *Caulerpa prolifera*; cymopol and cyclocymopol, prenylated bromohydroquinones from *Cymopolia barbata*; an acetylene containing fatty acid derivative from *Liagora farinosa*; a macrocyclic enol-ether from *Phacelocarpus labillardieri*; and stypoldione, an orthoquinone from *Stypopodium zonale* (Mayer et al., 1993). Fucoidan also inhibits cytotoxic and myotoxic activities of several PLA2 myotoxins from crotaline snake venoms that result in muscle necrosis caused by snake bites (Angulo & Lomonte, 2003). An inhibitor of pancreatic lipase has been purified from an extract of *Caulerpa taxifolia* (Bitou et al., 1999). The substance, caulerpenyne, competitively inhibits lipase activities, and *in vivo* oral administration to rats demonstrated a reduced and delayed peak plasma triacylglycerol concentration. Phlorofucofuroeckol A is an antiplasmin inhibitor found in *Ecklonia kurome* (Fukuyama et al., 1989, 1990). The compound 5_-deoxy-5-iodotubercidin isolated from *Hypnea valentiae* strongly inhibits the action of adenosine uptake into rat and guinea-pig brain slices, and inhibits adenosine kinase obtained from guinea-pig brain and rat brain and liver (Davies et al., 1984). Its also causes muscle relaxation and hypothermia when injected into mice (Davies et al., 1984). Inosine-5_-monophosphate dehydrogenase is inhibited by the brominated diphenylmethane derivative, isorawsonol, which has been isolated from *Avrainvillea rawsonii* (Chen et al., 1994). The linear diterpenes eleganolone and elegandiol, isolated from *Cystoseira brachycarpa* var. *balearica*, inhibit contractile activities of acetylcholine

and histamine on ileum musculature of guinea pigs (Della Pieta et al., 1993, 1995). Patier et al. (1993) noted the effect of laminaran in enhancing D-glycanase activities in suspended cell cultures of *Rubus fruticosus*. A fucoidan isolated from *Cladosiphon okamuranus* has shown antipeptic activity and this characteristic has been suggested to protect the gastric mucosa from ulceration (Shibata et al., 2000).

Purposes of this Study

Seaweed biotechnology is a multidisciplinary subject to produce food, pharmaceuticals, chemicals, and environmental remediation materials from seaweed resources. In particular, our survey efforts in this area have led to two main themes in studies:

1. Screening, isolation and biological analysis of biologically active substances, such as microalgal growth enhancer, antifouling agent and anti-inflammatory.
2. A strain improvement program was initiated based on mutagenesis with the goal of increasing the content of tryptophan and lysine (essential amino acids) of *Porphyra*.

These studies are highly interdisciplinary in nature and draw on diverse methodologies in marine biotechnology. The detailed experimental procedures and the findings of the above mentioned tasks are discussed in the subsequent chapter.

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Table I-1. Status of marine-derived natural products in clinical and predinical trials

Compound Name	Source	Status (Disease)	Comment
Bryostatin 1	Bryozoan *Bugula neritina*	Phase II (Cancer)	Now in combination therapy trials; licensed to GPC Biotech by Arizona State University
TZT – 1027	Synthetic Dolastatin	Phase II (Cancer)	Also known as Auristatin PE and Soblidotin
Cematodin	Synthetic derivative of Dolastatin 15	Phase I /II (Cancer)	Some positive effects in melanoma
ILX 651, Synthatodin	Synthetic derivative of Dolastatin 15	Phase I/II (Cancer)	For melanoma, breast, and non-small cell lung cancer (NSCLC)
Ecteinascidin 743	Ascidian *Ecteinascidia turbinata*	Phase II/III (Cancer) in 2003-2005	Licensed to Ortho Biotech (J&J/Janssen Pharmaceuticals)
Aplidine	Ascidian *Aplidium albicans*	Phase II (Cancer)	Dehydrodidemnin B; made by total synthesis
E7389	Sponge *Lissodendoryx sp.*	Phase II (Cancer)	Eisai's synthetic halichondrin B derivative; breast and lung
Discodermolide	Sponge *Discodermia dissoluta*	Phase I (Cancer)	Licensed to Novartis by Harbor Branch Oceanographic Institution
Kahalalide F	Mollusk *Eylsia rufescens* and Alga *Bryopsis sp.*	Phase II (Cancer)	Licensed to PharmaMar by University of Hawaii
Zalypsis	Synthetic Safracin B derivative	Phase I (Cancer)	PharmaMar (based on saframycin molecule)
ES-285	*Spisula polynyma*	Phase I (Cancer)	Rho-GTP inhibitor
KRN-7000	Sponge *Agelas mauritianus*	Phase I (Cancer)	An agelasphin derivative
Squalamine	Shark *Squalus acanthias*	Phase II (Cancer)	Anti-angiogenic activity as well
Æ-941 (Neovastat)	Shark	Phase II/III (Cancer)	Defined mixture of < 500 kDa from cartilage; anti-angiogenic
NVP-LAQ824	Synthetic	Phase I (Cancer)	Derived from Psammaplin, Trichostatin and Trapoxin structures
E-7974 (Eisai)	Synthetic	Phase I (Cancer)	Carboxylate-end modified hemisasterlin
Salinosporamide A (NPI-0052)	Bacterium *Salinispora tropica*	Phase I (Cancer)	Proteasome inhibitor Nereus Pharma
GTS-21 (aka DMBX)	Marine worm	Phase I (Alzheimer's)	Licensed to Taiho by the University of Florida
IPL-576,092 (aka HMR-4011A)	Sponge *Petrosia contignata*	Phase II (anti-asthmatic)	Derivative of contignasterol; Inflazyme Pharma
IPL-512,602	Derivative of 576092	Phase II (anti-asthmatic)	With Aventis. No further data as of 08/2005
IPL-550,260	Derivative of 576092	Phase I (anti-asthmatic)	With Aventis. No further data as 08/2005
Ziconotide (aka Prialt)	Mollusk *Conus magus*	Approved FDA 28DEC04 (Neuropathic pain)	Licensed by Elan to Warner Lambert; launched in U.S. and Europe in 2005
CGX-1160	*Conus geographus*	Phase I (Pain)	Cognetix and Elan Corporation (Ireland); Phase II late 2005
ACV1	*Conus victoriae*	Phase I (Pain)	Metabolic Pharma (Australia)(06/2006), conotoxin Vc1.1

This table was adapted from information kindly provided by David J. Newman, National Cancer Institute, Bethesda, MD, USA.

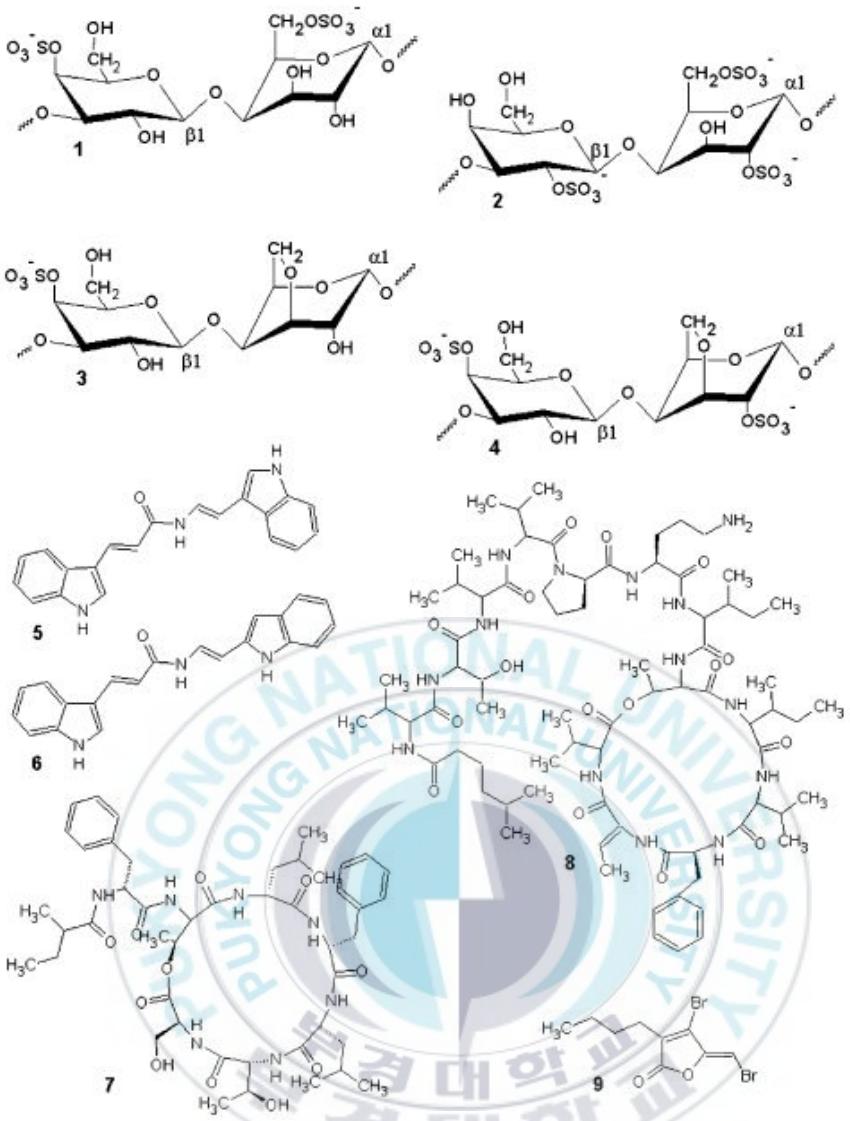

Figure 1. Some natural products

Idealised structures of *μ*-carrageenan (**1**), *λ*-carrageenan (**2**), *κ*-carrageenan (**3**) and *ι*-carrageenan (**4**). Some carrageenans have potent antiviral activities against several strains of HSV types 1 and 2. A carrageenan-based microbicide, Carraguard, is currently undergoing phase III clinical trials; it is used to block HIV and other sexually transmitted diseases *in vitro*. Chondriamide A (**5**) from *Chondria atropurpurea* shows antiviral activity against HSV type II and cytotoxicity against human nasopharyngeal and colorectal cancer cells. Chondriamide C (**6**), also from *Chondria atropurpurea*, displays cytotoxic and *in vitro* anthelmintic properties. The cyclic depsipeptides kahalalide A (**7**) and F (**8**) are produced by a species of *Bryopsis*. Both show *in vitro* activity against *Mycobacterium tuberculosis*. Kahalalide F has anti-HIV qualities which are being further studied in clinical trials and its effectiveness as treatment of lung cancers and tumours are also being studied. (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl- 2(5*H*)-furanone (**9**) is a halogenated compound from *Delisea pulchra* which displays strong antifouling properties.

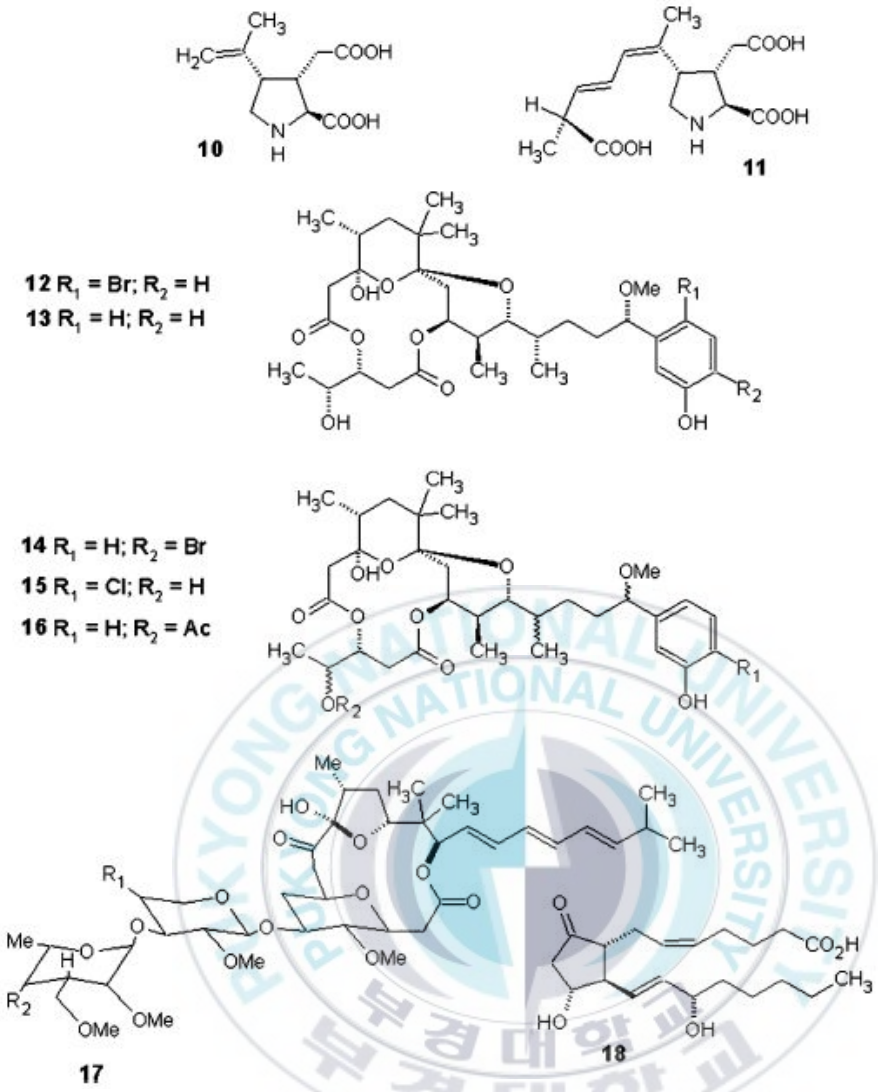

Figure 2. Some natural products (continuous)

α-Kainic (**10**) and domoic acids (**11**) are pyrrolidine dicarboxylates with excitatory and excitotoxic activities. Kainoids occur in some pennate diatoms where they cause amnesic shellfish poisoning, but they are also produced by some members of the Ceramiales. They are used as tools in research into neurophysiological disorders such as Alzheimer's and Parkinson's disease, and epilepsy. Domoic acid-containing extracts of *Digenea simplex* and *Chondria armata* have been used by the Japanese as anthelmintic agent, and it also has insecticidal properties. Aplysiatoxin (**12**) and debromoaplysiatoxin (**13**) are potent tumour promoters used in medical research, and are responsible for non-fatal poisonings associated with eating *Gracilaria coronopifolia*. The related manauealide A (**14**), manauealide B (**15**) and manauealide C (**16**) which also occur in *G. coronopifolia* were shown to induce diarrhoea in mice. Polycavernoside A (**17**) has been isolated from the red alga *Polycavernosa tsudae*. Polycavernosides are complex glycosidic toxins belonging to a class of macrocyclic lactones and are the causative agents for the fatal human poisonings following consumption of *Polycavernosa tsudae*.

Chapter 2

Microalgal growth enhancement by levoglucosan isolated from the green seaweed *Monostroma nitidum*

Abstract

Microalgal growth was enhanced by the addition of levoglucosan to the culture medium. The growth-enhancing compound levoglucosan was isolated from the green seaweed *Monostroma nitidum* using water extraction, molecular fractionation, DEAE-cellulose column chromatography, and high-performance liquid chromatography. Yield of the compound from seaweed powder was 5×10^{-3}% (w/w). At 10 mM concentration, levoglucosan enhanced cell growth and the specific growth rate of all feed microalgal species tested (*Chaetoceros gracilis*, *Chlorella ellipsoidea*, *Dunaliella salina, Isochrysis galbana, Nannochloris oculata, Navicula incerta, Pavlova lutheri, Tetraselmis suecica*) in most culture media by approximately 150%. Cellular fatty acid profiles and cell size differed marginally between cultures with and without levoglucosan.

Introduction

The mass culture of microalgae as feed for molluscs, crustaceans, and fish is an important component of the mariculture industry (Pulz & Gross, 2004). Microalgal feeds are especially valuable for early stages of marine species with fastidious dietary requirements that cannot be met by formulations of traditional agricultural products. Microalgal diets commonly have specific nutritional qualities and are in great demand because of their composition of protein, vitamins, pigments, and large amounts of docosahexaenoic acid [DHA; 22:6(n-3)] and eicosapentaenoic acid [EPA; 20:5(n-3)] (Mansour et al., 2005). Insufficient amounts of highly unsaturated fatty acids, especially DHA and EPA, cause high mortality and low growth rates, particularly during larval and early juvenile periods (Om et al., 2003; Park et al., 2006). Some microalgae have not been used extensively as nutritional diets because of difficulties in achieving high-density cultures, resulting in unreliable production (Wikfors & Ohno, 2001).

The development of conventional photo-bioreactors would be one way to solve this problem (Pulz, 2001).

Another way is to develop cell-growth-enhancing substances to increase cell biomass and thus the economic feasibility of small-scale production. Previous screening of seaweed extracts in our laboratory has shown that the aqueous extract from the green seaweed *Monostroma nitidum* Wittrock activates cell growth in several microalgae (Cho et al., 1999). In an attempt to identify the microalgal growth enhancer, we isolated the compound levoglucosan, which enhances cell growth in several microalgae in various culture media.

Materials and methods

Monostroma nitidum

Thalli of the green seaweed *M. nitidum* Wittrock (Monostromaceae, Ulvales) were collected in March 2004 and March 2005 from the upper intertidal zone of calm inlets at Imwhe (34°22′23″ N, 126°15′16″ E), Jindo Island, Korea. Seaweed thalli were dried completely for 1 week at room temperature and then ground to a powder for 5 min using a coffee grinder. The powder was stored at −20°C until use.

Isolation of the active compound

M. nitidum powder (100 g) was extracted three times with 5 L 100% methanol. The residual methanol was evaporated from the powder sample, after which 5 L distilled water was added to extract the water-soluble fraction for 1 day (once). The aqueous extract was evaporated using a rotary evaporator at 40°C (800 mg). The extract was then dissolved in 0.1 M Tris buffer (pH 8.0) and separated by molecular size fractionation using an Amicon diafilter membrane (Danvers, MA). An active fraction smaller than MW 500 was loaded onto a DEAE-cellulose column (50 g; 30 × 6 cm; Sigma, St. Louis, MO) equilibrated with the same Tris buffer. Elution was carried out with 500 mL of the equilibration buffer, followed by 500 mL 0.1 M NaCl in the same buffer. The 0.1 M NaCl fraction (121 mg) was separated by reverse-phase high-performance liquid chromatography (HPLC) using a μBondapak C-18 column (3.9 mm ID × 30 cm; Waters, Milford, MA). Analysis was performed on a Waters 600 gradient liquid chromatograph monitored at 220 nm. The mobile phase consisted of two solvent systems: acetonitrile

within Pfeffer et al. (1979) and Lafont et al. (1989).

Microalgal culture

To isolate the active compound, compare media, and analyze fatty acids, the axenic prasinophyte flagellate *Tetraselmis suecica* (Kylin) Butcher (CCAP-66; P-4) was cultured in f/2 medium (Guillard & Ryther, 1962) with an initial cell density of 1.2×10^5 cells mL^{-1}. Purified compound (10 mM) was added to the medium and cultured under 70 μmol $m^{-2}s^{-1}$ light intensity at 18°C for 4 days. Cells were counted under a microscope using a hemocytometer. Specific growth rate (λ) was calculated as a cell growth against the cultured day: $\log N - \log N_0 = \lambda(T - T_0)/2.303$, where N = cell numbers at day T, and N_0 = cell numbers at day T_0. Relative growth enhancement (%) was expressed as a relative rate: $(L/N) \times 100$, where L = cell numbers cultured with levoglucosan, and N = cell numbers cultured without levoglucosan. To examine the effect of levoglucosan, *T. suecica* cell growth was compared in six different media (100 mL): f/2, ESM, MF, MKM, M-ASP7, and W-ESM (Kasai et al., 2004). All microalgal strains were obtained from the Korean Marine Microalgae Culture Center.

Fatty acid analysis

For fatty acid analysis, extraction of lipids and methylation were performed using methanolic boron trifluoride (Xu et al., 1998). Cells ($\sim 5 \times 10^6$) were collected from 500 mL culture by centrifugation at 1,000 g for 10 min. The algal pellet was immediately sonicated with a mixture of 8 mL water, 10 mL chloroform, and 20 mL methanol for 10 min. Then, 10 mL chloroform and 10 mL water were added and the mixture was sonicated

for 10 min, respectively. After centrifugation, the lower chloroform layer was removed and evaporated under a vacuum. Lipids were methylated in 5 mL of a mixture of BF_3 (14% BF_3 in methanol), benzene and methanol (5:4:11). The tube was flushed with nitrogen gas and sealed tightly before heating at 90°C for 30 min. Fatty acid methyl esters were extracted with GC-grade hexane and analyzed on a Shimadzu GC-MS QP5050A chromatograph equipped with a flame ionization detector.

Statistics

Each independent assay was repeated at least three times with separate cultures. Treatment means were compared to controls using Student's t-test.

Results

Purification and identification of the active compound

The active compound was isolated as a white powdery compound (5 mg) from *M. nitidum* powder (100 g). The yield from the seaweed powder was 5×10^{-3}%. The mass spectrum of the purified compound showed a molecular ion peak [M^+] at m/z 162, which corresponded with the molecular formula $C_6H_{10}O_5$. The fragmentation pattern was compared to a library and found to match well with levoglucosan (Figure II-1). The ^{13}C NMR spectrum showed the presence of six oxygenated carbons (δ_C 68.5, 67.8, 71.6, 72.2, 82.1, and 107.8). These oxygenated carbons were determined based on chemical shift. The ^1H NMR spectrum revealed the presence of five oxygenated methine protons and an additional methylene proton (δ_H 3.40, 3.49, 3.73, 3.97, 4.98 and 3.86). Based on these spectral data, the compound was identified as levoglucosan and confirmed to be identical to data for authentic levoglucosan.

Effect of levoglucosan on microalgal growth

Tetraselmis suecica attained the stationary phase of growth within 8 days in f/2 medium under 70 μmol m^{-2} s^{-1} light intensity at 18°C. The half-life of the exponential phase was approximately 4 days; thus, cell numbers were counted at 4 days to determine growth enhancement. The purified levoglucosan compound was tested at different concentrations in f/2 medium using *T. suecica* (Figure II-2). At 10 mM, levoglucosan demonstrated strong growth enhancement of up to 169% relative to the reference culture without the extract (i.e., from 1.3×10^6 to 2.2×10^6 cells mL^{-1}), and this concentration was

used in all subsequent experiments. To confirm the effect in various culture media, 10 mM levoglucosan (Acros organics, New Jersey, USA) was added to *T. suecica* in six different media (100 mL): f/2, ESM, MF, MKM, M-ASP7 and W-ESM, and cultured for 4 days. *Tetraselmis suecica* overall cell numbers were increased by the addition of levoglucosan to the culture media ($P < 0.1$; Table II-1). Levoglucosan was most effective in media such as ESM, W-ESM and f/2, which are commonly used for marine microalgae. Both cell counts and specific growth rates of the cultures increased ($P < 0.1$). The addition of levoglucosan in f/2 and W-ASP7 increased the specific growth rate of the microalga almost two-fold in the early growth phase between 3 and 4 days. Cell size (approximately 20 μm in diameter) was similar in cultures with and without levoglucosan. Other useful feed microalgae were also tested to determine the growth enhancement effectiveness of levoglucosan (Table II-2). Levoglucosan enhanced the cell growth of all tested feed microalgae, especially *T. suecica*, *Isochrysis galbana* Parke, and *Chlorella ellipsoidea* Gerneck [K&H], with approximately 1.5-fold increases in cell density.

Fatty acid composition

Cellular fatty acid profiles were determined to ascertain whether the addition of levoglucosan caused changes in the lipid quality of the microalga as a feed organism. Polyunsaturated fatty acids (PUFA) are especially important in determining the nutritional value of diets for the survival and development of fish larvae. The proportions of PUFA, such as linoleic acid, linolenic acid, and EPA, differed marginally between cultures of *T. suecica* grown with and without levoglucosan (Table II-3).

Discussion

Live algae are produced mainly in outdoor tanks or fermenters for limited local use, and the risk of contamination and maintenance costs are high. Until now, much attention has been focused on the development of new biofermenter designs (Pulz, 2001), storage methods after harvest (Lim & Park, 1998), and the improvement of nutritional value (Lopez Alonso et al., 1992). Little has been done to develop growth enhancers to increase the productivity of live feed microalgae. We found that the cell count of cultured microalgae increased by approximately 50% when levoglucosan was added to the culture medium. However, little change occurred in the cellular fatty acid profiles and cellular size with the addition of levoglucosan. Previous research has determined that the gross biochemical composition and digestion efficiency were similar between cultures of *Isochrysis galbana* grown with and without *M. nitidum* crude extract (Cho et al., 1999). Among the 27 species of seaweed tested, only two - *M. nitidum* and *Grateloupia turuturu* - enhanced microalgal growth.

The seaweed *M. nitidum* is distributed in rocky areas in the upper intertidal zone of calm inlets. It grows during winter and early spring. It begins to mature, showing yellowish-green tissues along the thallus margin in February. Thalli are largest in March, at approximately 8 cm in length, 9 cm in width, and 2 g in wet weight. Frond cells form a monolayer. The fronds are edible and can be aquacultured using the same nets used for *Porphyra* (Kida, 1990). The aqueous extract inhibits the growth of the green alga *Enteromorpha prolifera* (Cho et al., 2001), decreases the total cholesterol and low-density lipoprotein in mouse (Jung et al., 1997), and enhances the cell growth of microalgae (Cho et al., 1999). We isolated levoglucosan as the main active growth-enhancing compound from *M. nitidum*; the compound enhances growth for diverse microalgal species and in

various culture media. Levoglucosan (CAS RN 498-07-7) contains an intramolecular glucosidic bond and has synonyms of 1,6-anhydro- β-d-glucopyranose, 1,6-anhydro-β-d-glucose, and anhydro-d-mannosan. Although levoglucosan can be easily hydrolyzed to glucose using an acid, none of the yeast and fungal strains tested showed levoglucosan hydrolyzing activity (Kitamura et al., 1991). In contrast, the formation of glucose 6-phosphate by an induced levoglucosan kinase was observed in reactions with cell extracts of levoglucosan-assimilating yeasts and fungi. Many eukaryotic microorganisms can utilize levoglucosan as a carbon and energy source for growth (Prosen et al., 1993; Zhuang et al., 2001). Very few prokaryotic organisms were found to use levoglucosan (Nakahara et al., 1994; Khiyami et al., 2005). Even though levoglucosan metabolism in microalgae has not been verified, its possible uses in microalgal culture are promising because most prokaryotic microorganisms are unable to metabolize levoglucosan, whereas all microalgae tested thus far have some ability to use it as a metabolite.

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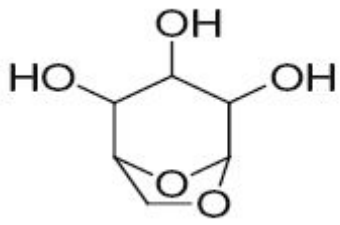

Figure II-1. Structure of levoglucosan isolated from the green seaweed *Monostroma Nitidum*.

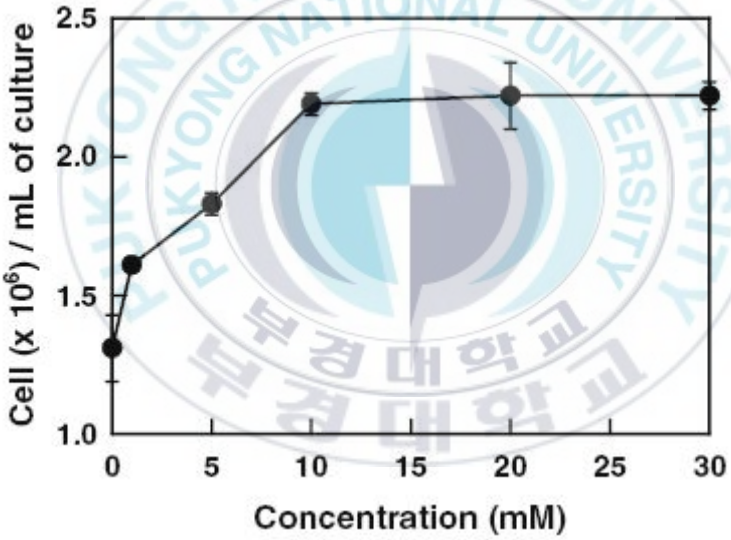

Figure II-2. Effect of various concentrations of levoglucosan on cell growth (mean number of cells ± SD; n ≥ 3) of the microalga *Tetraselmis suecica*. Levoglucosan was added to f/2 medium inoculated with 1.2×10^5cells mL^{-1} *T. suecica*, and cultured at 18℃ for 4 days

Table II-1. Cell growth of *Tetraselmis suecica* cultured for 4 days in various media with and without 10 mM levoglucosan. Values of cell numbers are mean × 10^6± SD ($n \geq 3$); $P < 0.1$

Medium	Number of cells		Specific growth rate per day	
	No levoglucosan	Levoglucosan	No levoglucosan	Levoglucosan
ESM	2.1 ± 0.1	3.1 ± 0.2	0.54	0.96
F/2	1.6 ± 0.2	2.3 ± 0.2	0.44	1.02
MF	1.9 ± 0.1	2.4 ± 0.1	0.98	1.66
MKM	3.0 ± 0.8	3.9 ± 0.2	0.18	0.30
W-ASP7	1.5 ± 0.1	2.0 ± 0.1	0.28	0.68
W-ESM	2.7 ± 0.1	4.0 ± 0.3	1.70	2.06

Table II-2. Effect of levoglucosan on cell growth enhancement for various microalgae. Values of cell numbers are mean × 10^6± SD ($n \geq 3$); $P < 0.1$. Each microalga was cultured for 4 days in f/2 medium with and without 10 mM levoglucosan

	Number of cells		Relative growth enhancement (%)
	No levoglucosan	Levoglucosan	
Chaetoceros gracilis B-015	1.2 ± 0.1	1.6 ± 0.1	133
Chlorella ellipsoidea C-021	1.6 ± 0.3	2.3 ± 0.4	144
Dunaliella salina C-248	1.0 ± 0.3	1.3 ± 0.1	130
Isochrysis galbana H-002	1.3 ± 0.2	1.9 ± 0.1	146
Nannochloris oculata C-031	1.6 ± 0.1	2.2 ± 0.1	138
Navicula incerta B-001	1.4 ± 0.2	1.8 ± 0.2	129
Pavlova lutheri H-003	1.9 ± 0.4	2.6 ± 0.3	137
Tetraselmis suecica CCAP-66	1.2 ± 0.2	1.9 ± 0.2	158

Table II-3. Profile of major fatty acids (% of total fatty acids) in *Tetraselmis suecica* cultured for 4 days in f/2 medium with and without 10 mM levoglucosan

Fatty acids[a]	No levoglucosan	Levoglucosan
14:0	2.8	3.2
16:0	21.9	22.7
17:0	2.7	2.9
18:0	47.1	49.4
18:1 (n-9)	1.9	1.7
18:2 (n-6)	2.0	1.7
18:3 (n-3)	14.5	13.2
20:5 (n-3)	2.7	2.5

[a] Peaks of <1% of the total area are omitted.

Chapter 3

Isolation of algal spore lytic C17 fatty acid from the crustose coralline seaweed *Lithophyllum yessoense*

Abstract

The algal spore lytic fatty acid of heptadeca-5,8,11-trienoic acid (HDTA: C17:3) was isolated from the crustose coralline seaweed *Lithophyllum yessoense*. HDTA, an odd-numbered carbon atom fatty acid, showed more than 50% lysis at a concentration of 5 μg/mL against the spores of three chlorophyte species, eight rhodophytes, two phaeophytes, and the cells of three phytoplanktons. Lysis activity increased as the number of double bonds and carbon atoms in the fatty acid increased. HDTA showed ten-fold stronger activity with LC_{50} of 3.1 μg/mL than α-linolenic acid (C18:3).

Key words: algal spore, C17 fatty acid, crustose coralline, spore lysis, *Lithophyllum yessoense*

Introduction

Many areas of the rocky shorelines of Korea and Japan are currently dominated by crustose coralline algae such as *Lithophyllum yessoense* Foslie (Suzuki *et al.,* 1998; Kim 2000). These non-articulated (non-geniculate) calcareous algae cover the surfaces of rocks in a pink or white-colored crust. The decrease in the seaweed flora of some rocky areas, known as alga whitening or barren ground, is associated with some species of crustose algae (Tokuda *et al.,* 1994).

Even though competitive allelopathy between crustose coralline algae and fleshy seaweeds has received little attention, coralline algae produce some allelopathic substances. Suzuki et al. (1998) isolated an allelopathic substance active against zoospores of the brown seaweed *Laminaria religiosa* from the crustose coralline alga *Lithophyllum* sp., but did not determine its structure. Kim et al. (2004) investigated the possible formation of multiple allelopathy-related substances by *L. yessoense* against the settlement and germination of spores from 17 species in 15 genera of seaweed. Kakisawa et al. (1988) isolated an allelopathic substance from the brown alga *Cladosiphon okamuranus* and identified it as octadeca-6,9,12,15-tetraenoic acid (C18:4) against 37 species of microalgae. Suzuki et al. (1996) isolated an allelopathic substance from the red alga *Neodilsea yendoana* and identified it as eicosapentaenoic acid (C20:5). Chiang et al. (2004) reported that the fatty acids α-linolenic acid (C18:3), oleic acid (C18:1), linoleic acid (C18:2), and palmitic acid (C16:0) of the green alga *Botryococcus braunii* showed allelopathic activity against a variety of phytoplankton and zooplankton. Alamsjah et al. (2005; 2007) reported the algicidal substances hexadeca-4,7,10,13-tetraenoic acid (C16:4), octadeca-6,9,12,15-tetraenoic acid, α-linolenic acid, and linoleic acid derived from *Ulva fasciata*.

Here, we describe the isolation and identification of the odd-numbered carbon atom C17 fatty acid from *L. yessoense* and its lytic activity against several types of seaweed spores, and compare its activity to that of other fatty acids (C16–C22).

Materials and methods

Lithophyllum yessoense samples

Pink crustose coralline tissues of *Lithophyllum yessoense* Foslie were collected from rocks in the lower intertidal zone of the shore at Chungsapo (35°09’28’’ N, 129°11’47’’ E), on the east coast of Busan, Korea. Stones covered with healthy algal tissue were transported in a seawater tank to the laboratory on the same day. White or grayish pink patches were removed from the crustose flats using a grinding drill and steel saw. After rinsing well with autoclaved seawater to remove potential contaminants, the tissues were brushed and cleaned three times with 30-s pulses of an ultrasonic water bath (low-intensity frequency of 90 kc s^{-1}) to eliminate micro-epiphytes. After sonication, the samples were dried completely for 2–3 days at room temperature using a fan.

Isolation of lytic compound

Approximately 12 kg of dried *L. yessoense* tissues were extracted with 50 L solution of methanol-water (4:1) at room temperature for 1 day, and then filtered through a Whatman GF/C filter under reduced pressure. This extraction procedure was repeated three times, and the extracts were combined. After filtration, the crude extract was concentrated to give a dark green residue (206 g) under reduced pressure in an evaporator.

The extract was successively fractionated into different classes according to polarity following Harborne (1998). The fraction that was acidified to pH 2 using sulfuric acid and extracted three times with chloroform, resulted in a moderately polar extract that contained the main lytic activity. This fraction was loaded on a silica gel column (5 x 80 cm; 70–230 mesh) and eluted sequentially with 500 mL of hexane:ether (8:2), ether, acetone, and EtOAc:MeOH (8:2). The acetone eluent (4.14 g) with activity was dried and dissolved in MeOH, then further fractionated on a Sephadex LH-20 (Pharmacia) column (2.5 cm x 100 cm) using 100% MeOH as the elutant. Each 2-mL fraction was collected at a flow rate of 0.5 mL min^{-1}. The active substance was obtained from fractions 80–100 (1.01 g), which were collected, dried, and dissolved in 3 mL MeOH for reverse-phase high performance liquid chromatography (RP-HPLC). Separation of each 150 μL was achieved using an Ultraspere C18 column (10 mm ID x 25 cm). The analysis was performed on a Waters 600 gradient liquid chromatograph monitored at 202 nm. The mobile phase consisted of two solvent systems: acetonitrile and water. Elution was performed with a linear gradient of 0–100% acetonitrile for 60 min at a flow rate of 2 mL min^{-1} to yield the pure compound (2.7 mg).

Analytical methods

The purified compound was analyzed on a JEOL JNM-ECP 400 NMR spectrometer (Tokyo, Japan), operating at 500 and 100 MHz for ^{1}H and ^{13}C, respectively, using methanol-d (CD_3OD). Electron Impact Mass Spectrometric (EIMS) data were measured on a JEOL JMX-700 spectrometer (Tokyo, Japan). Infrared spectrum was recorded on a Fourier Transform IR spectrophotometer (IFS-88; Brucker, Karlsruhe, Germany). The identity of the purified compound was confirmed by comparing it to

spectral data of a C17 carbon fatty acid (Saito and Ochiai, 1996).

Bioassay of algal spore lysis

The isolation procedure of the active compound was monitored using a lysis assay with monospores of *Porphyra suborbiculata* Kjellman. Juvenile blades were collected from the intertidal zone of the rocky shore at Chungsapo, Busan, Korea. Axenic isolation and culturing of the monospores followed Choi et al. (2002; 2005). For spore collection from various fleshy seaweeds, fertile thalli of 19 different seaweed species were collected from the coast of Korea. The thalli were cleaned, dried, and induced to release spores using sterilized seawater (Kim *et al.,* 2004). Microalgal cells were obtained from the Korea Marine Microalgae Culture Center.

Various amounts of the purified compound in MeOH (2 μL) were added to 198 μL Provasoli's enriched seawater (PES) medium (Provasoli, 1968) containing approximately 200 spores or cells, and then incubated under 40 μmol m^{-2} s^{-1} light at 20 ^{o}C for 4 h. The number of spores remaining was counted under a microscope (×100). Spore lysis (%) was expressed as a relative rate: [(C–S)/C] x 100, where C is spore number in controls (without the compound) and S is spore number of samples (with the compound). Assays for anti-attachment and anti-germination followed Kim et al. (2004). The minimum detectable lysis, anti-attachment, and anti-germination activities of spores by MeOH occurred at 1% (data not shown). Therefore, the final concentration of MeOH was kept below 1% in all tests.

Statistics

Each independent assay was repeated at least three times with separate cultures. Treatment means were compared to controls using Student's *t*-test.

Results

Isolation of the active compound

The active compound was eluted at 100% (in 70.9 min) acetonitrile by RP-HPLC. It was a colorless oily compound, weighing 2.7 mg, and yielding 2.3 x 10^{-5}% or 1.3 x 10^{-3}% from the dried seaweed tissue or the crude extract, respectively. Infrared (dry film) analysis of the purified compound showed absorptions for OH (3400–3000 cm^{-1}) and carbonyl function (1708 cm^{-1}). The ^{1}H NMR spectrum revealed a methyl proton at δ 0.88 (3H, t, H-17), nine methylene protons at [δ 1.33–1.29 (6H, m, H-14, H-15, H-16), δ 1.64 (2H, m, H-3), δ 2.05 (2H, m, H-13), δ 2.11 (2H, m, H-4), δ 2.26 (2H, t, H-2), δ 2.84–2.81 (4H, m, H-7, H-10)], and six methine proton signals at δ 5.38–5.33 (6H, m, H-5, H-6, H-8, H-9, H-11, H-12); the proton of the carboxyl group (H-1) was at δ 3.33. The ^{13}C NMR spectrum revealed one carbonyl carbon at δ_C 177.8 (C-1), one methyl carbon at δ_C 14.43 (C-17), nine methylene carbons [δ_C 23.64 (C-16), δ_C 26.11 (C-3), δ_C 26.55 (C-7, C10), δ_C 27.63 (C-4), δ_C 28.19 (C-13), δ_C 30.47 (C-14), δ_C 32.67 (C-15), δ_C 34.62 (C-2)], and six methine carbons [δ_C 128.78 (C-11), δ_C 129.11 (C-6), δ_C 129.43 (C-9), δ_C 129.81 (C-8), δ_C 130.13 (C-5), δ_C 131.18 (C-12)]. Assignments were made by analyzing the HMQC, HMBC, and COSY spectra. From the COSY spectrum, we determined the first double-bond position from the terminal methyl carbon (C17). The terminal methyl proton H-17, the methylene protons H-16, H-15, H-14, and H-13, and the methine proton H-12 showed a series of COSY correlations with each other, demonstrating that the first double bond

was at the sixth position (n-6) (Fig. 1-6). From these spectral data, we identified the compound as the polyunsaturated fatty acid heptadeca-5,8,11-trienoic acid (C17:3 n-6; Fig. 7).

Spore lytic activity of the isolated HDTA

When the isolated HDTA was added to PES medium containing monospores of *P. suborbiculata*, a lysis reaction occurred quickly and neared completion within 4 h at each concentration tested (Fig. 8).

Lysis activity on the spores of 15 seaweed species and the vegetative cells of four microalgae was measured (Table 1). In general, HDTA showed significant lytic activity against most spores and cells. Adding 25 μg/mL HDTA to spores, especially those of *Lomentaria catenata* and *Dictyota dichotoma*, did not cause the spores to burst. *Alexandrium catenella* was also little affected at this concentration. These data imply that *L. yessoense* produces a lytic compound against spore settlement of most, if not all, seaweeds that occur on crustose coralline algae flats, and thereby decreases the seaweed flora in such areas. Because the spores of *L. catenata* and *D. dichotoma* showed low levels of lysis, restoration of algal whitening areas might be possible by first transplanting these species, and later adding other seaweeds.

HDTA at a concentration of 0.5 μg mL^{-1}, which showed no lysis activity, was tested for inhibitory activity against settlement and germination of seaweed spores. The compound showed almost no effects on spore settlement of most seaweed species, although it reduced spore settlement to less than 80% of controls in *D. dichotoma*, *Gymnogongrus flabelliformis, L. catenata*, and *Ulva pertusa*. There was no substantial inhibition of spore germination by HDTA (data not shown).

Spore lytic activity of eight different C16–C22 fatty acids was measured on monospores of *P. yezoensis*. Using a dose-response curve, we determined the concentration resulting in 50% lysis (LC_{50}). HDTA showed an LC_{50} value of 3.1 μg/mL (Table 2). Fatty acids with a higher degree of unsaturation in the carbon chain exhibited higher activity. Although α-linolenic acid (n-3) and HDTA (n-6) had the same level of unsaturation, the LC_{50} value of HDTA was ten times higher. As the number of carbon atoms with unsaturation increased, lysis activity increased. Docosahexaenoic acid, which had the highest number of unsaturated carbons, showed the strongest LC_{50} (0.8 μg/mL).

Discussion

In areas with algal whitening, there are commonly no fleshy seaweed epiphytes on the pink crustose coralline flats. Although causes such as herbivore induction (Kitamura *et al.,* 1993), grazing (Agateuma *et al.,* 1997), physical sloughing (Masaki *et al.,* 1984; Johnson and Mann, 1986), and iron deficiency (Suzuki *et al.,* 1995) may be sufficient to prevent recruitment of fleshy seaweeds, allelopathic substances may also destroy (Suzuki *et al.,* 1998) and/or help prevent the settlement or germination of seaweed spores (Kim *et al.,* 2004).

We isolated a novel polyunsaturated fatty acid (PUFA) of HDTA (C17:3) that shows potent lysis activity against seaweed spores. Odd-numbered carbon atom fatty acids are typically toxic (Mackay *et al.,* 1940), and we found that HDTA showed more potent lysis against seaweed spores than α–linolenic acid (C18:3). In general, PUFAs, whether free acids, phospholipids, or glycolipids, are usually found in algal extracts and are considered components of the membrane. The composition of PUFAs can be used as an indicator of food quality (Wood *et al.,* 1999). However, high concentrations of PUFAs may be toxic (Yasumoto *et al.,* 1990). For example, EPA (C20:5 n-3) exhibits lethal toxicity to various microalgae and macroalgae. We isolated EPA also from *L. yessoense* that lyses seaweed spores, although the extractable amount was lower than that of HDTA. Jüttner (2001) proposed that the PUFAs observed in algal extracts are in fact the first products of the lipoxygenase cascade that starts upon cell disruption. Fu et al. (2004) assumed that PUFAs could be part of a defense strategy that rapidly converts an essential cell constituent into highly toxic compounds against grazers. Unsaturated fatty acids produce free radicals when oxidized in seawater, and they may attach to algae as toxins (Murata *et al.,* 1989). Thus, marine plants might commonly use PUFAs as allelochemicals

to inhibit the growth of competing macro- or microalgae. The high toxicity of HDTA may also be due to the amphiphatic properties of PUFAs, which probably disrupt the membrane integrity of seaweed spores and microalgal cells. Chemical structural features such as the number of unsaturated double bonds may also be involved in biological activity (Chiang *et al.,* 2004).

Even though *L. yessoense* produces a spore lytic compound against spore settlement of most seaweeds, little lysis occurred in the spores of *L. catenata* and *D. dichotoma*. No inhibition of settlement or germination occurred in spores of *D. dichotoma* (Kim *et al.,* 2004). Thus the restoration of algal whitening areas might be possible by transplanting these species first, and then inducing the recruitment of other seaweeds.

In conclusion, we isolated an odd-numbered carbon atom fatty acid of HDTA from the crustose coralline *L. yessoense* as a spore lytic compound against diverse seaweeds and microalgae. Our results support claims that crustose coralline algae may use PUFAs as a chemical defense mechanism against competing algae.

Acknowledgements

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Table III-1 Lysis activity of the purified HDTA against seaweed spores and microalgal cells. Each concentration of HDTA (μg mL^{-1}) was added to PES with spores or cells for 4 h. The activity is expressed as the relative rate of spore lysis (%). Values represent the mean ± SD ($n \geq 3$). Statistical significance is *$p < 0.001$ as compared to controls

	Concentration of HDTA (μg mL^{-1})		
	25	5	1
CHLOROPHYTA			
Enteromorpha compressa	100 ± 0*	100 ± 0*	76 ± 3*
Enteromorpha linza	100 ± 0*	100 ± 0*	72 ± 2*
Ulva pertusa	92 ± 2*	51 ± 3*	28 ± 5
RHODOPHYTA			
Carpopeltis affinis	90 ± 2*	68 ± 2*	13 ± 3
Carpopeltis prolifera	100 ± 0*	84 ± 2*	2 ± 4
Chondrus ocellatus	94 ± 1*	74 ± 4*	14 ± 2
Gracilaria verrucosa	100 ± 0*	100 ± 0*	6 ± 3
Gymnogongrus flabelliformis	100 ± 0*	68 ± 5*	2 ± 4
Lomentaria catenata	16 ± 8	8 ± 3	0 ± 3
Porphyra suborbiculata	100 ± 0*	97 ± 1*	10 ± 2
Porphyra yezoensis	100 ± 0*	96 ± 2*	14 ± 9
Symphyocladia latiuscula	100 ± 0*	71 ± 2*	14 ± 3
PHAEOPHYTA			
Dictyota dichotoma	22 ± 5	18 ± 9	5 ± 9
Sargassum horneri	100 ± 0*	87 ± 3*	44 ± 6
Undaria pinnatifida	88 ± 3*	66 ± 3*	2 ± 4
MICROALGAE			
Alexandrium catenella	32 ± 9	7 ± 9	6 ± 6
Chattonella sp. RA005	100 ± 0*	100 ± 0*	77 ± 5
Cochlodinium polykrikoides	100 ± 0*	90 ± 2*	16 ± 7
Heterosigma akashiwo	100 ± 0*	100 ± 0*	89 ± 6*

Table III-2 Lysis activity of C16–C22 fatty acids on monospores of *P. suborbiculata.* The LC_{50} was determined from dose-response curves

Fatty acids	LC_{50} (μg/mL)
Palmitic acid (C16:0)	>150
Oleic acid (C18:1 n-9)	>100
Linoleic acid (C18:2 n-6)	40.1
α-Linolenic acid (C18:3 n-3)	35.4
HDTA (C17:3 n-6)	3.1
Arachidonic acid (C20:4 n-6)	1.8
Eicosapentaenoic acid (C20:5 n-3)	2.1
Docosahexaenoic acid (C22:6 n-3)	0.8

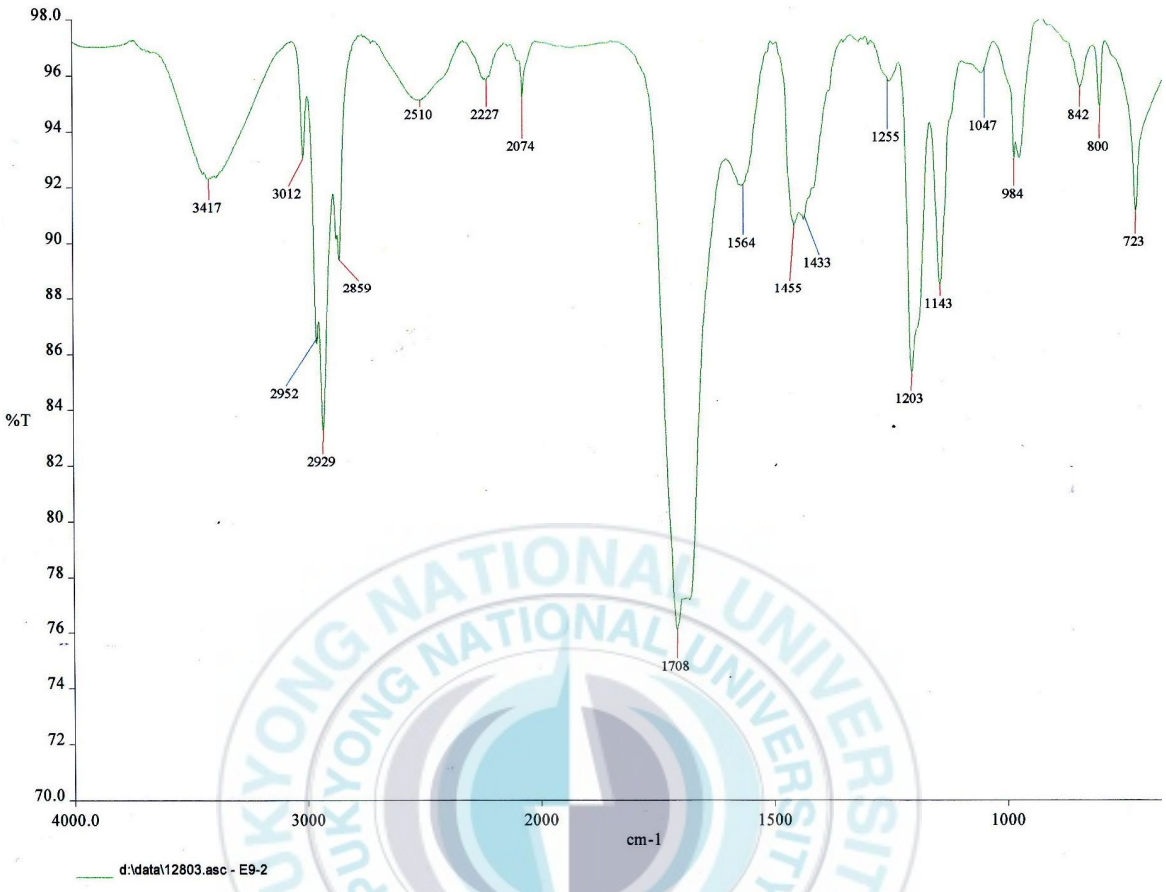

Figure III-1. IR spectrum of HDTA

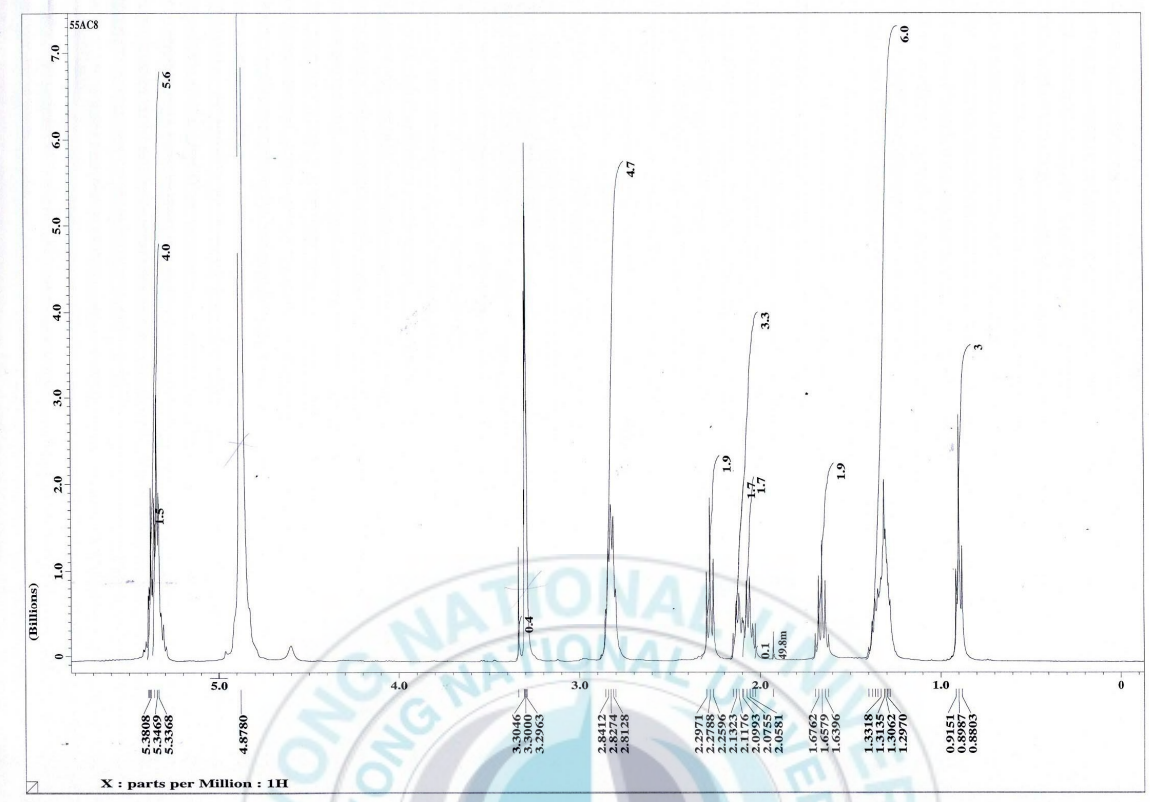

Figure III-2. ^{1}H NMR spectrum of HDTA

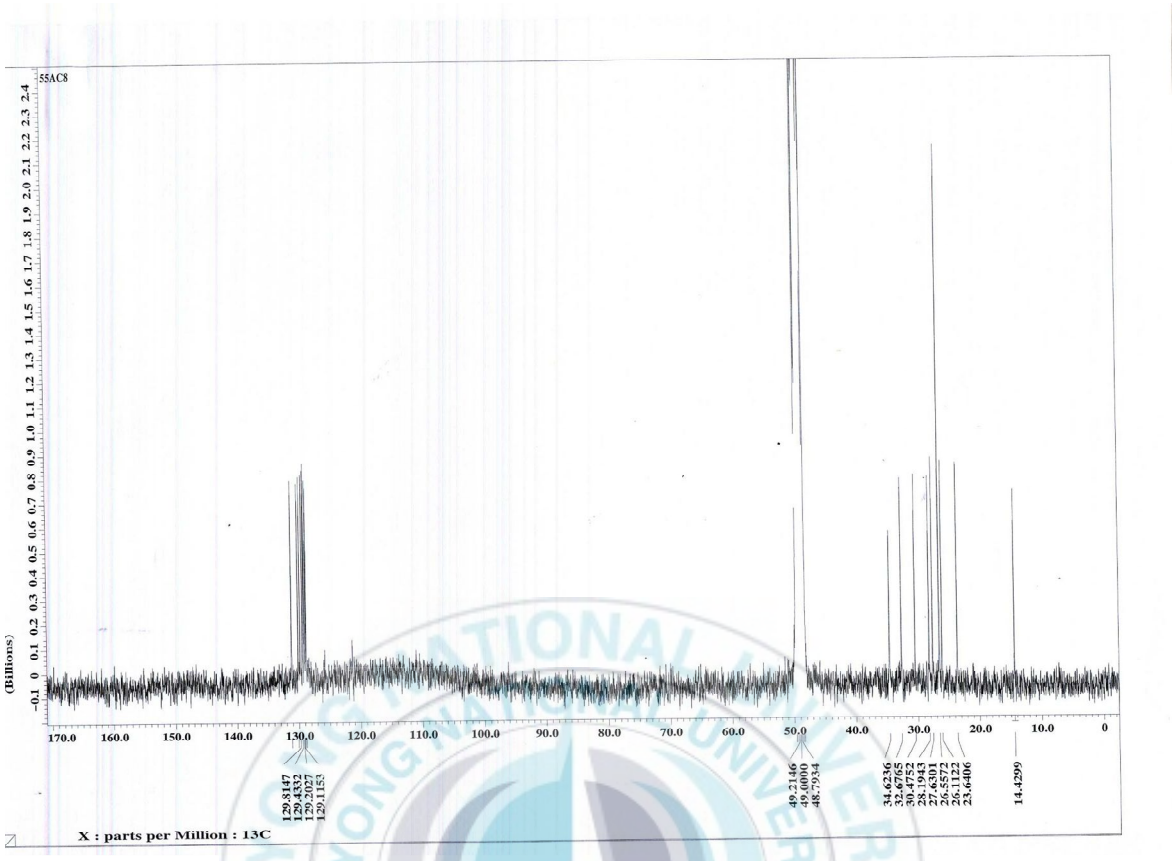

Figure III-3. ^{13}C NMR spectrum of HDTA

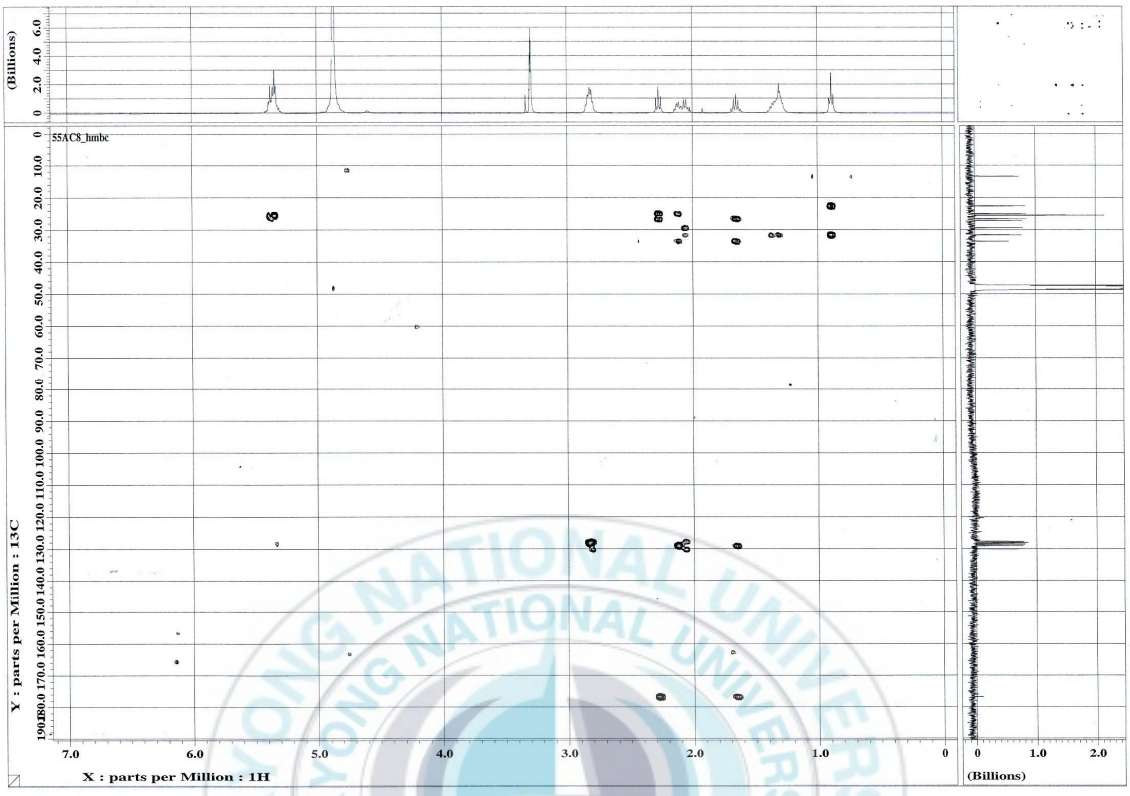

Figure III-4. HMBC spectrum of HDTA

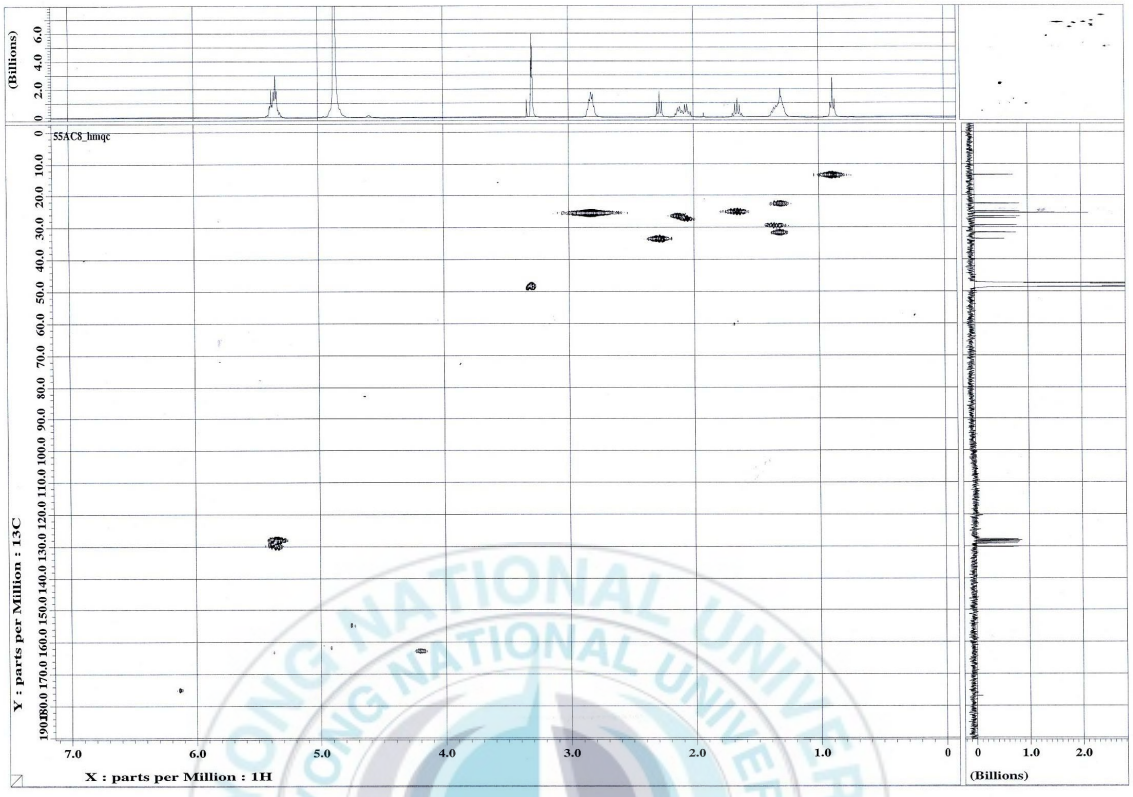

Figure III-5. HMQC spectrum of HDTA

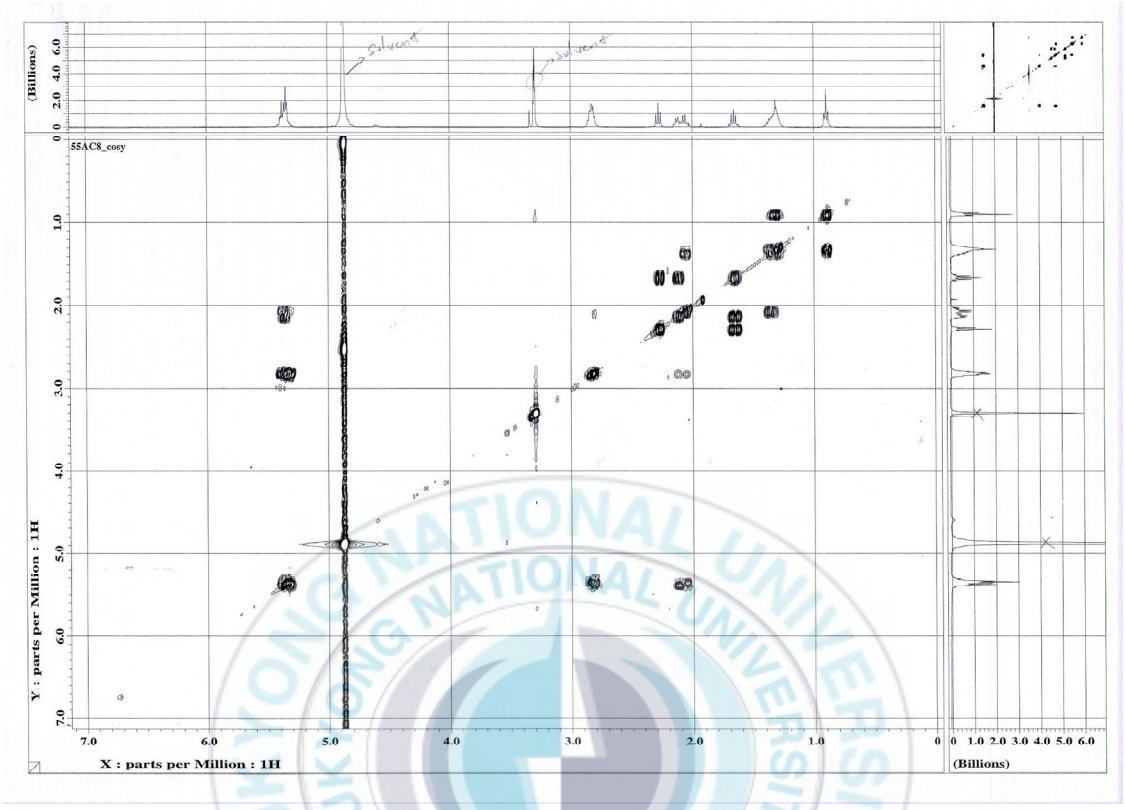

Figure III-6. COSY spectrum of HDTA

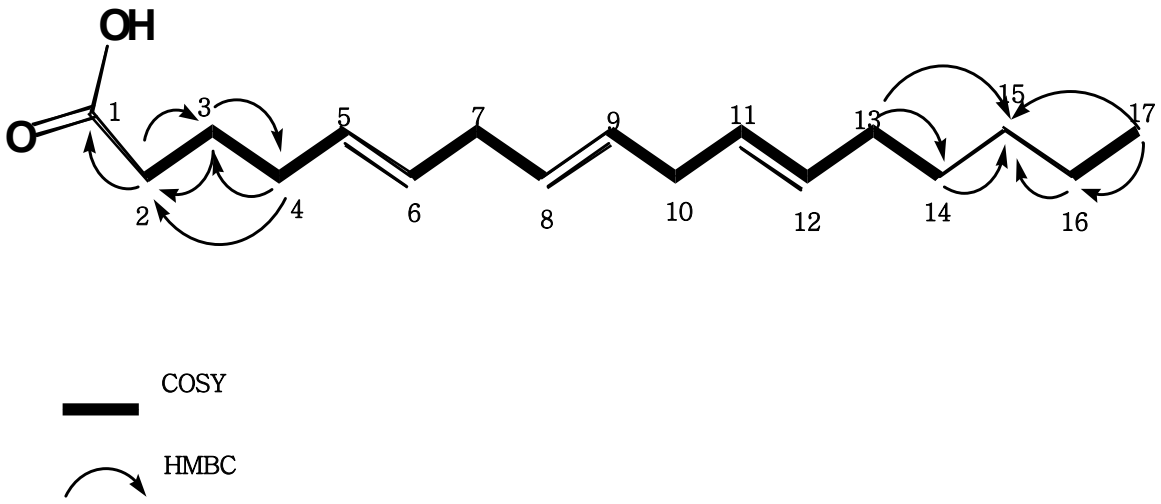

Figure III-7. Structure of heptadeca-5,8,11-trienoic acid (C17:3 n-6) isolated from the crustose coralline seaweed *Lithophyllum yessoense* Foslie

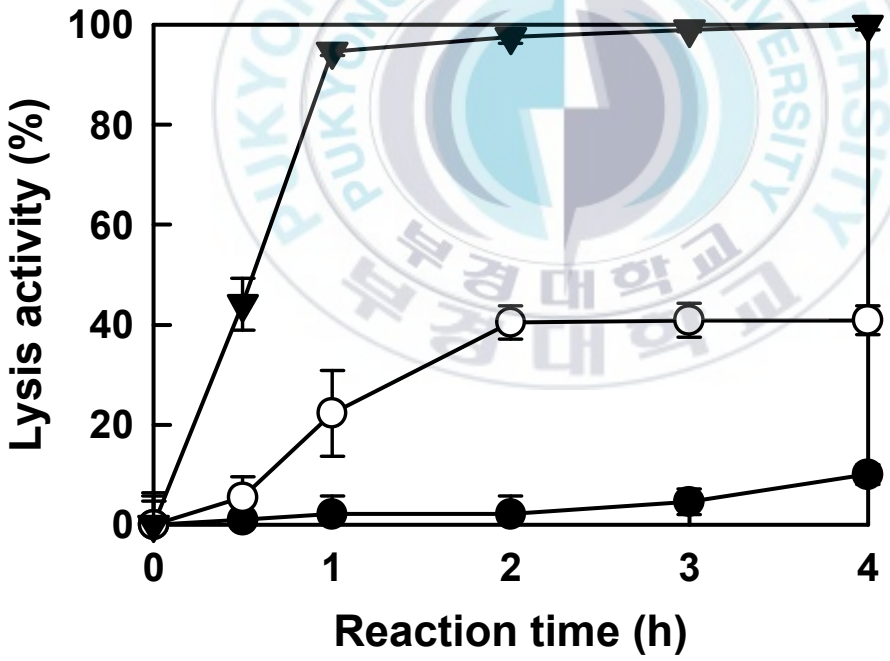

Figure III-8. Lysis activity of the isolated HDTA on monospores of *P. suborbiculata*. Concentrations of 1 μg/mL (●), 2.5 μg/mL (○), and 5 μg/mL (▼) of HDTA were added to PES (200 μL) containing approximately 200 monospores. Values represent the mean ± SD ($n \geq 3$)

Chapter 4

Anti-inflammatory activity of polyunsaturated fatty acids derived from *Codium fragile* (Suringar) Hariot in mice

Abstract

The large, green macroalga *Codium fragile* (Suringar) Hariot is known as a source of bioactive compounds. In this chapter we summarize our study on the anti-inflammatory effects of *C. fragile* involving isolation and structure determination of active substances. The main active compound was isolated by acetonitrile extraction, fractionating by polarity, silica gel column chromatography, and reverse-phase HPLC to give pure compounds CF-2, CF-6 and CF-7. The structure of compound CF-2 was identified by 1D and 2D of ^{1}H and ^{13}C NMR spectroscopy and GC-MS data and suggested as eicosa-5,8,11,14,17- pentaenoic acid (EPA) (C20:5 ω-3). The structure of compound CF-6 and CF-7 were also studied by the NMR and MS experiments and its structures will be constructed. Purified compounds of EPA, CF-6 and CF-7 were tested for anti-inflammatory activities against the PMA-induced mouse ear inflammation symptoms of edema and erythema. The inhibitory effects of different concentrations of EPA, CF-6 and CF-7 topically applied to mouse ears were dose-dependent. The EPA concentrations producing IC_{50} were 230 and 462 μg per ear for edema and erythema, respectively.

Introduction

One of the principal aims of medicinal research is to seek new active principles from species whose natural growing area coincides with that of the population that needs them as a remedy. Marine sources are being explored for possible pharmaceutical products, and reports of useful compounds identified are occurring with increasing frequency in the literature (Glombitza *et al.,* 1985; Fahy *et al.,* 1988,). Marine pharmacology has begun to focus on several new areas of pharmaceutical development, with an emphasis on inflammatory diseases (Kernan and Faulkner, 1987; Glaser *et al.,* 1988) and cancer (Crews *et al.,* 1984; Tringali *et al.,* 1984). Thus, marine research may offer a means of providing new biomedical leads. For this reason, we have studied the anti-inflammatory effects of several seaweed species in the hope of providing new leads for an alternative treatment of inflammation. In our previous study (Khan *et al.,* 2005), a methanol extract of *Codium fragile* was demonstrated to display potent anti-inflammatory activities against phorbol myristate acetate (PMA)-induced mouse ear inflammation.

The marine green alga, *Codium fragile* (Suringar) Hariot, is a research model for many fields from algal physiology and heavy metal accumulation to invasion ecology, algal genetics, and natural products. In different parts of the species' broad geographic range, *C. fragile* is consumed by humans, used as invertebrate food by the mariculture industry, is a pest of natural and ultivated shellfish beds, and is a source of bioactive compounds. It is widely distributed in temperature areas throughout the world (Silva and Womersley, 1956; Yoshida, 1998; Trowbridge, 1998), and is eaten in Korea, China and Japan (Abbott, 1988). In Korea *C. fragile* is an attractive seaweed species for cultivation because it commands a high market value compared to species such as *Porphyra* and *Undaria* (Sohn, 1998). The alga is an additive of Kimchi, a traditional fermented

vegetable. Anti-inflammatory activity has been reported from *C. fragile* so far, but it has not been documented for clearly active principles (Paya *et al.,* 1993).

In this chapter we summarize our study on the anti-inflammatory effects of *Codium fragile* involving isolation and structure determination of active substances against the inflammatory symptoms of edema and erythema.

Materials and Methods

Algal Material

The green seaweed *Codium fragile* (Suringar) Hariot (common names include sea staghorn, oyster thief, and dead man's fingers or Chonggak in Korea) was harvested from Wando Aquaculture Farm (34°17′N, 126°42′E) from October 2007 to March 2008, with the voucher specimen deposited in our laboratory (Y. K. Hong). This is one of the most active seaweed culture areas around the southwestern sea of Korea. For convenience, the seaweed tissue was completely dried for 1 week at room temperature, and then ground to powder for 5 min using a coffee grinder. The powder was stored at -20 ^{o}C until use.

Isolation of Anti-inflammatory Compounds

To isolate the anti-inflammatory compounds from *C. fragile* thalli, the algal powder (200 g) was extracted three times with 2 L acetonitrile, and the crude extract was evaporated under vacuum to give a dark brown residue (2.2 g). The acetonitrile extract was chromatographed on a silica gel column (70-230 mesh, 22 g, Ø 4.5 cm x 40 cm) and successively eluted with 90 mL each of *n*-hexane, methylene chloride, acetonitrile, and

methanol. The active methylene chloride eluent (1.3 g) was dried and dissolved in 4 mL of methanol for RP-HPLC. Each 300-μL (37.5 mg) aliquot was separated on a C18 column (10 mm i.d. x 25 cm) (Ultrasphere; Beckman Coulter, Fullerton, CA). The analysis was performed on a Waters 600 gradient liquid chromatograph (Waters, Milford, MA) monitored at 213 nm. The mobile phase consisted of two solvent systems: acetonitrile with 0.1% TFA and distilled water with 0.1% TFA. Elution was performed with a linear gradient of 0 to 100% v/v, and with 100% v/v acetonitrile over 30 min for compounds CF-2 (eicosapentanoic acid: EPA), CF-6 and CF-7, at a flow rate of 2 mL/min. Each eluted compound was dried under a stream of nitrogen gas.

Analytical Methods

The purified compounds were analyzed on a GC-MS-QP5050A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and compared to the spectral data from the database. EIMS and HR-FABMS data were obtained from a JMS-700 spectrometer (JEOL, Tokyo, Japan) and a JMS HX 110 Tandem mass spectrometer (JEOL), respectively. The 1-D NMR (^{1}H, ^{13}C, and DEPT) and 2-D NMR (HMQC, HMBC, and COSY) spectra were taken on a JNM-ECP 400 NMR spectrometer (JEOL), using methanol-*d* (CD_3OD) for CF-2, CF-6 and CF-7. The structure of the CF-2 compound was identified and confirmed to be identical to the spectral data in Fu et al. 2004.

Inflammatory Bioassays

BALB/c mice (8-10 weeks old; 20-25 g body weight) were used for inflammatory assays. The animals were housed at 24 ± 1^{0}C on a 12-h light/dark cycle, with free access

to food and water. Animal experiments were performed in accordance with the U.S. Institutional Animal Care and Use Committee Guidelines and national regulations concerning animal experiments, clinical studies, and biodiversity rights. Various concentrations of the purified compounds and indomethacin as a reference were prepared in 10 μL of 100% ethanol and applied topically to the whole inner side of the mouse ear. Phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO; 0.2 μg in 10 μL of acetone) was applied topically to the same side of the ear 30 min later to allow absorption of anti-inflammatory compounds. Ear edema (swelling) was measured 10 h after the PMA application, using a spring-loaded micrometer (Mitutoyo Corp., Tokyo, Japan). The edema value was expressed as $(S_{10} - S_0)/S_0$, where S_{10} is the ear thickness 10 h after PMA application and S_0 is the ear thickness at 0 h. The edema value was 0.81 ± 0.04 with the ethanol vehicle. Ear erythema (redness) was determined at 10 h, using digital photography adjusted to balance white and Photoshop 7.0 (Adobe, San Jose, CA) to measure the magenta value. The erythema value was expressed as $(R_{10} - R_0)/R_0$, where R_{10} is ear redness 10 h after PMA application and R_0 is ear redness at 0 h.

Quantification of Anti-inflammatory Compounds

To measure the amounts of anti-inflammatory compounds in *C fragile*, the thalli were completely dried in shade at room temperature for a week and then ground for 5 min to powder. The powder (0.4 g) was extracted with 8 mL of dichloromethane on a rotator for 1 h at 30 rpm. After centrifugation at 2000g for 5 min, 4 mL of the clean supernatant was evaporated to 5 mg/mL for RP-HPLC. Each 100 μL aliquot was separated on an Ultrasphere C18 column, using the same isolation procedure as that for CF-2, CF-6 and CF-7. Each isolated compound was reconfirmed by ^{1}H and ^{13}C NMR. The amount of each

compound was assessed by measuring the dimensions of HPLC peaks, using the standard curve of each pure compound.

Statistical Analysis

The experiments were replicated at least seven times for each independent assay, and the highest and lowest values were discarded. The mean values of the indices were compared to the control using Student's *t*-test.

Results

Identification of Compounds

Of the three anti-inflammatories, the CF-2, CF-6 and CF-7 compounds were eluted at 98% (on 31.68 min, 36.56 min and 37.39 min, respectively) acetonitrile by RP-HPLC. They appeared as oily compounds, weighing 2.4 mg, 3 mg and 2.9 mg, respectively, and yielding 12 x 10^{-4} %, 15 x 10^{-4} % and 14.5 x 10^{-4} % (Fig. IV-1), respectively, from the seaweed powder. We identified the molecular composition of CF-2 as $C_{20}H_{30}O_2$ from the HR-FABMS (negative mode, [M - H]- at *m/z* 301.2168), which indicated that CF-2 contained six double-bond equivalents, comprising five carbon-carbon double bonds and one carbonyl carbon. IR (dry film) absorptions for OH (3000– 2500 cm^{-1}) and carbonyl function (1704 cm^{-1}) were observed; ^{1}H-NMR (CD_3OD, 500 MHz) δ 0.97 (3H, t, J = 7.7, H-20), 1.66 (2H, m, J = 7.3; 7.3, H-3), 2.08 (2H, m, H-19), 2.13 (2H, m, H-4), 2.29 (2H, t, J = 7.3; H-2), 2.86–2.81 (8H, m, H7, H-10, H-13, H-16), 5.40–5.30 (10H, m, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15, H-17, H-18), the proton of carboxyl-group is exchanged against deuterium from the solvent; ^{13}C-NMR (CD_3OD, 100 MHz) δ 14.6 (C-20), 21.5 (C-19), 26.0 (C- 3), 26.4 (C-16), 26.5 (C-7, C-10, C-13), 27.6 (C-4), 34.4 (C- 2), 128.2 (C-17), 128.9, 129.1, 129.1, 129.2, 129.2, 129.5 (C-8, C-9, C-11, C-12, C-14, C-15), 129.8, 130.1 (C-5, C-6), 132.8 (C-18), 177.5 (C-1). Assignments were made by analysis of the HMQC, HMBC and COSY spectra (Fig. IV-2~7). The above data prove the structural identity of CF-2 with the polyunsaturated fatty acid 5,8,11,14,17-eicosapentaenoic acid (C20:5ω3, EPA) and confirmed to be identical to data for authentic EPA (Fig IV-8).

The structure of compound CF-6 and CF-7 were also studied by the NMR (Fig. IV-9 & 10) and MS experiments and its structures will be constructed.

Anti-inflammatory Activities

Purified compounds of EPA, CF-6 and CF-7 were tested for anti-inflammatory activities against the PMA-induced mouse ear inflammation symptoms of edema and erythema. The inhibitory effects of different concentrations of EPA, CF-6 and CF-7 topically applied to mouse ears were dose-dependent. The EPA concentrations producing IC_{50} were 230 and 462 μg per ear for edema and erythema, respectively (Fig. IV-11). The CF-6 and CF-7 concentrations producing IC_{50} for edema and erythema is now in progress. The topical application of indomethacin as a positive control significantly decreased the PMA induced inflammation and yielded IC_{50} of 90 and 172 μg per ear for edema and erythema, respectively. Thus, pure EPA showed almost half the anti-inflammatory activity of indomethacin.

Amount of Anti-inflammatory Compounds in Thalli

We examined the amounts of EPA of *C. fragile* thalli by measuring the dimensions of HPLC peaks, using the standard curve of pure compound. A mature thallus of *C. fragile* contained the amount of EPA (135.9 mg per 1 kg dry powder) lower than the amount of EPA of *U. pinnatifida* thallus (193 mg per 1 kg dry powder).

The amounts of CF-6 and CF-7 of *C. fragile* are now in progress.

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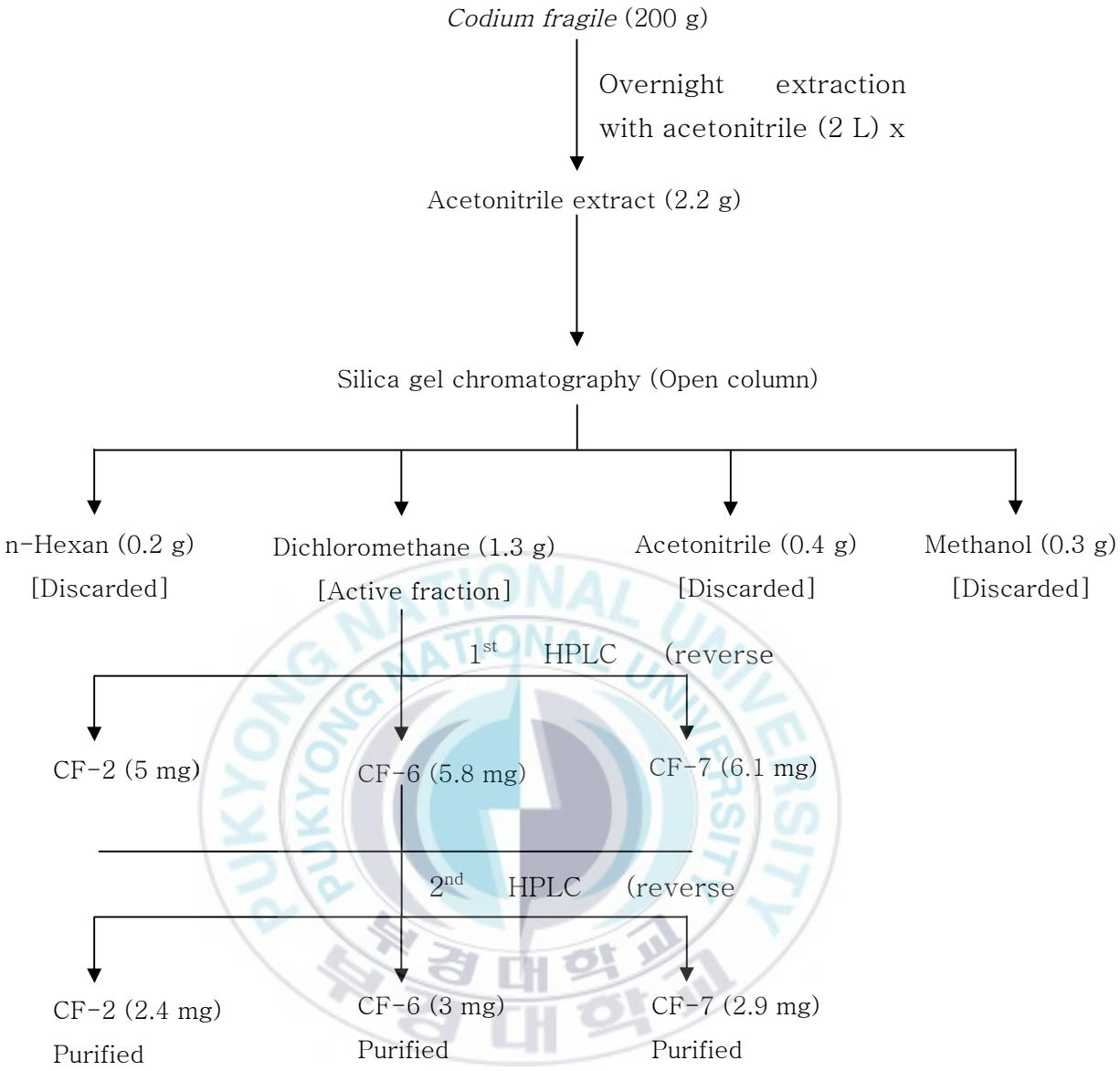

Figure IV-1. Purification procedure of anti-inflammatory compounds from *Codium fragile*

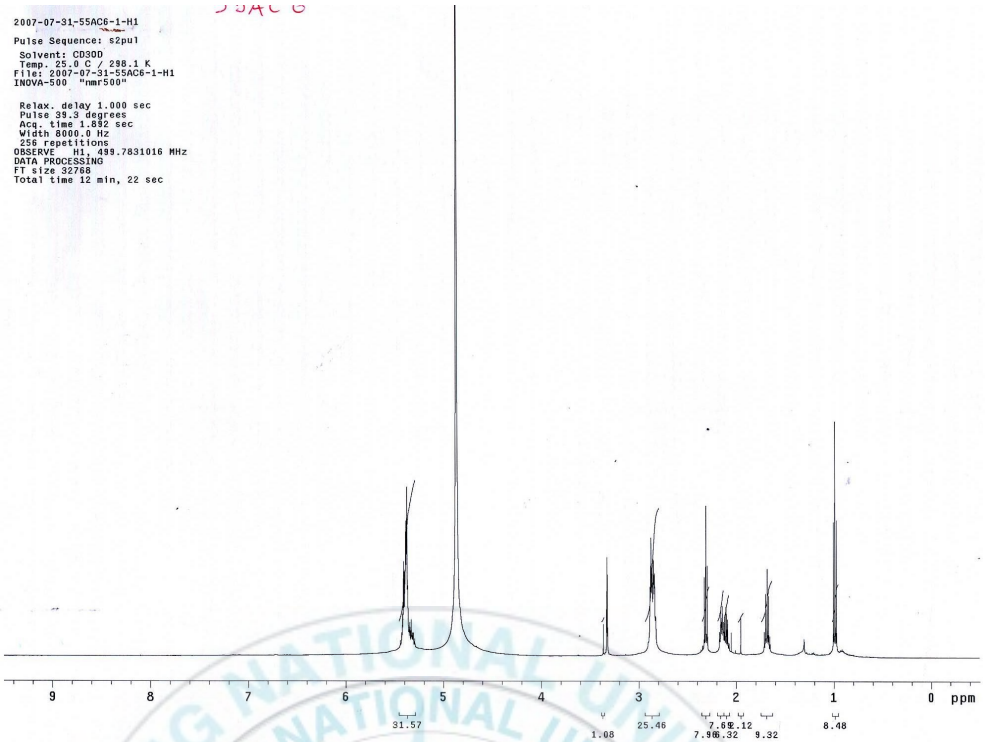

Figure IV-2. ^{1}H NMR spectrum of EPA

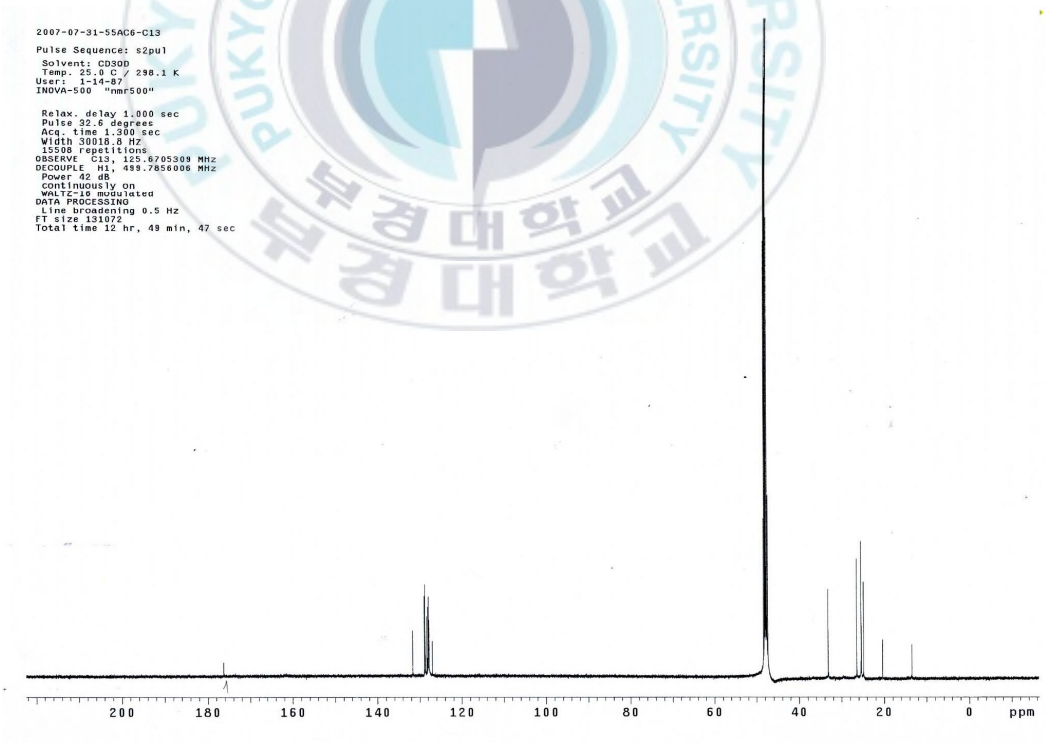

Figure IV-3. ^{13}C NMR spectrum of EPA

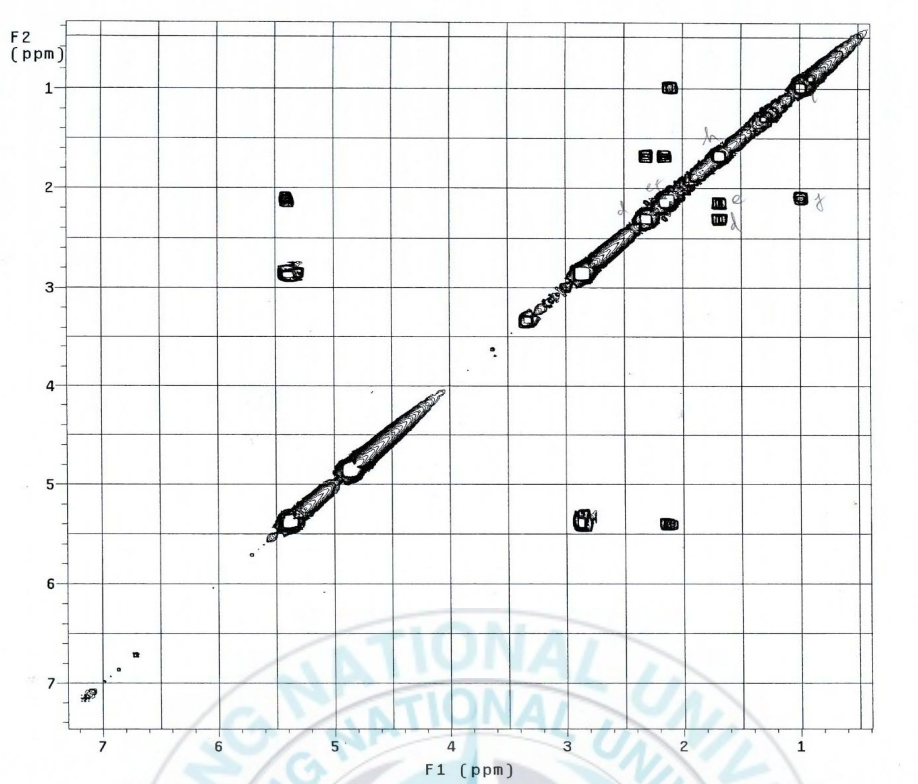

Figure IV-4. COSY spectrum of EPA

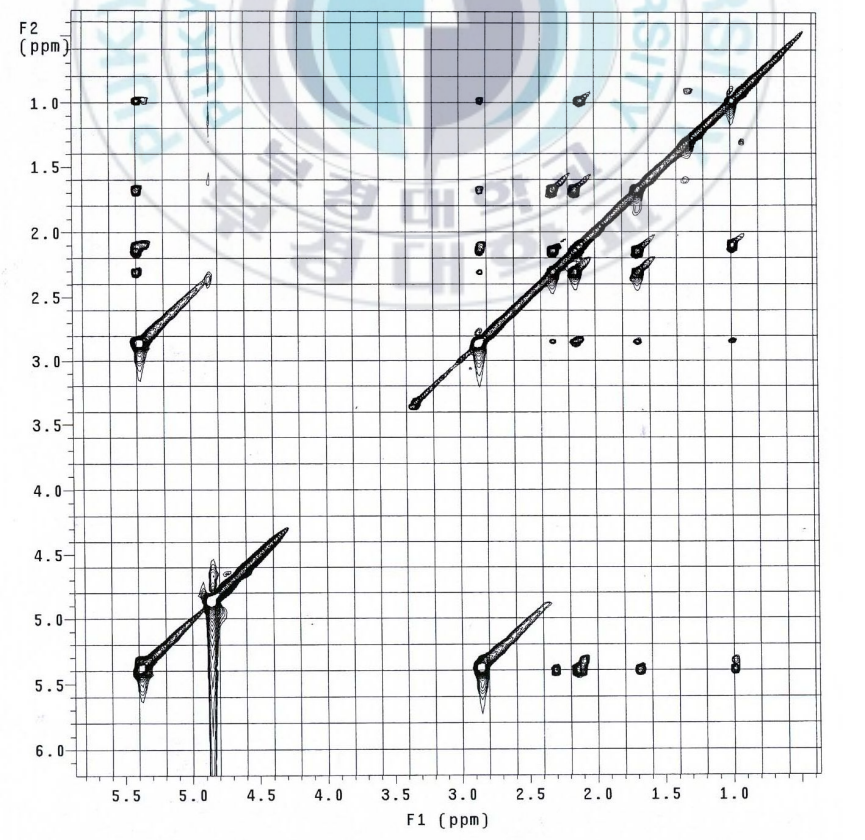

Figure IV-5. TOCSY Spectrum of EPA

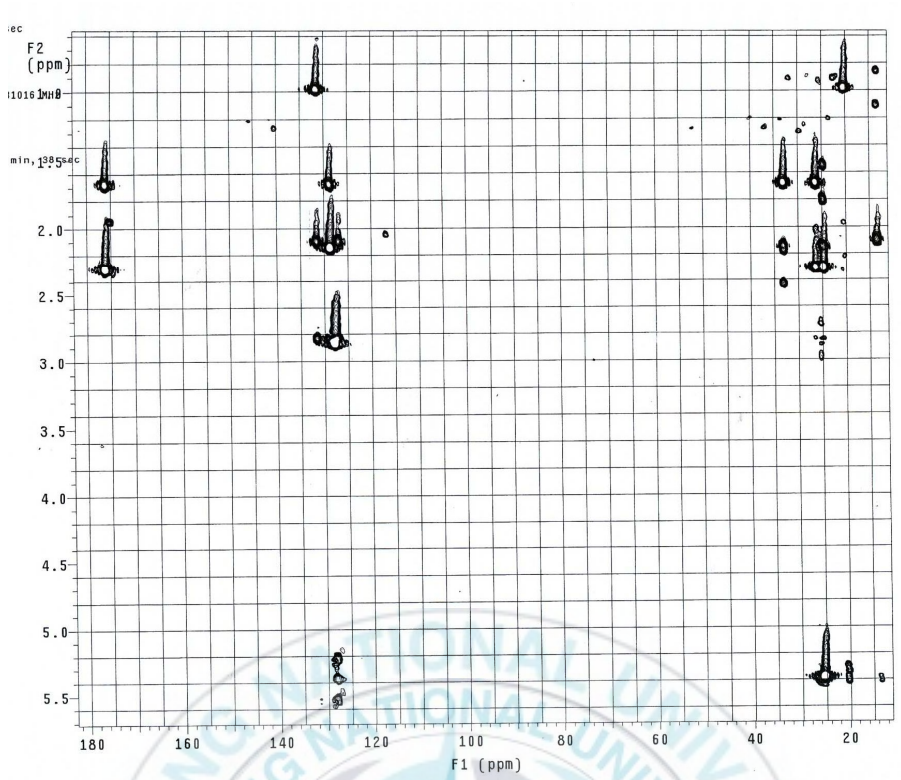

Figure IV-6. HMBC Spectrum of EPA

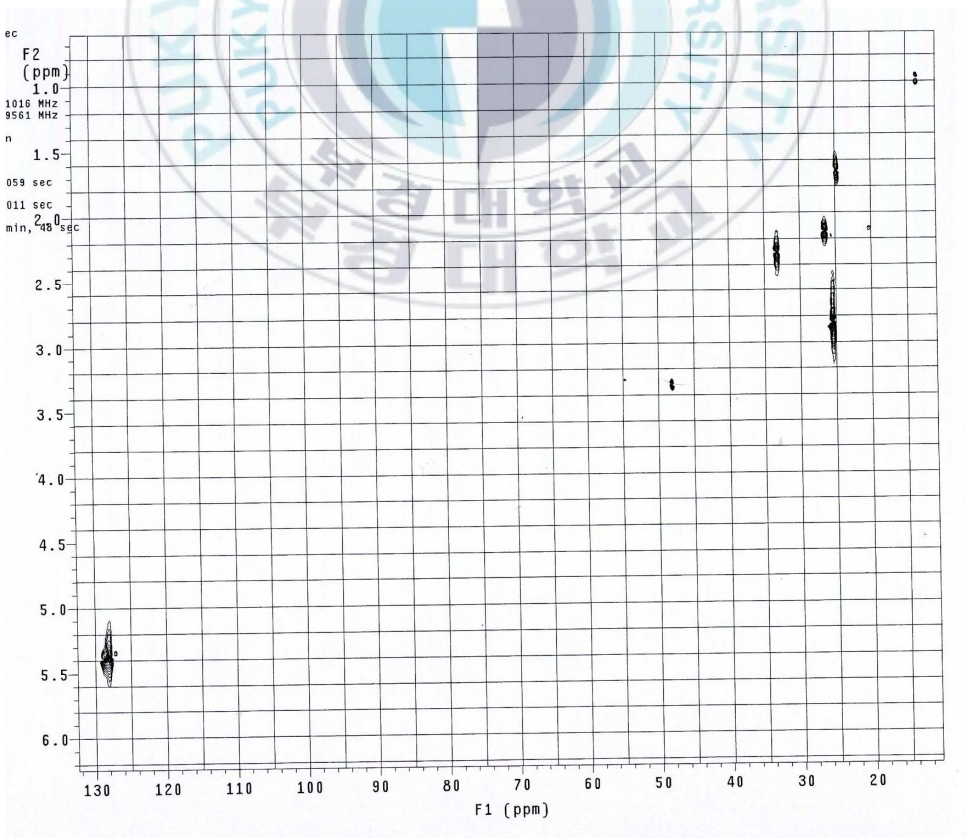

Figure IV-7. HMQC Spectrum of EPA

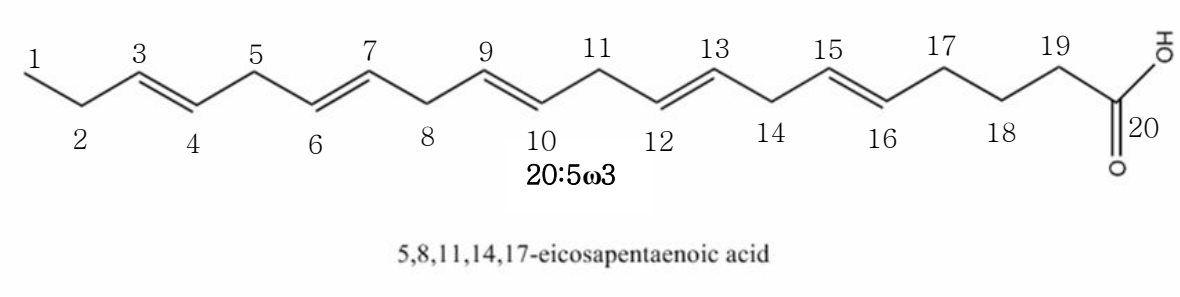

Figure IV-8. Chemical structures of 5,8,11,14,17-eicosapentaenoic acid (20:5ω3) isolated from *Codium fragile*.

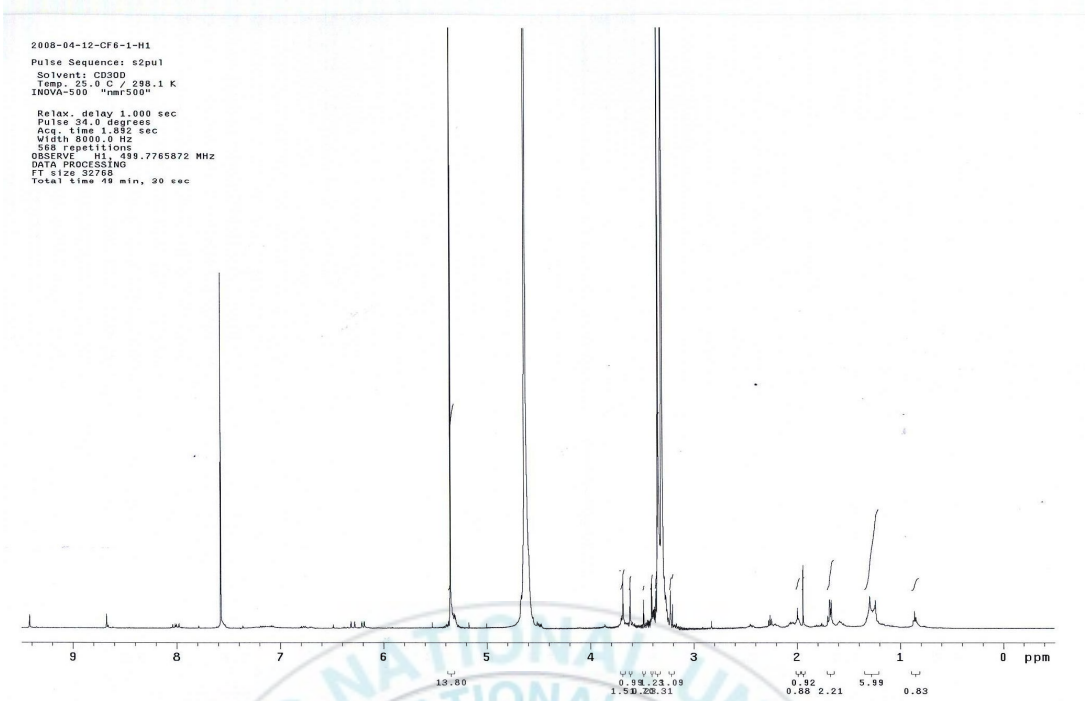

Figure IV-9. ^{1}H NMR (100 MHz, $CDCl_3$) spectrum of CF-6

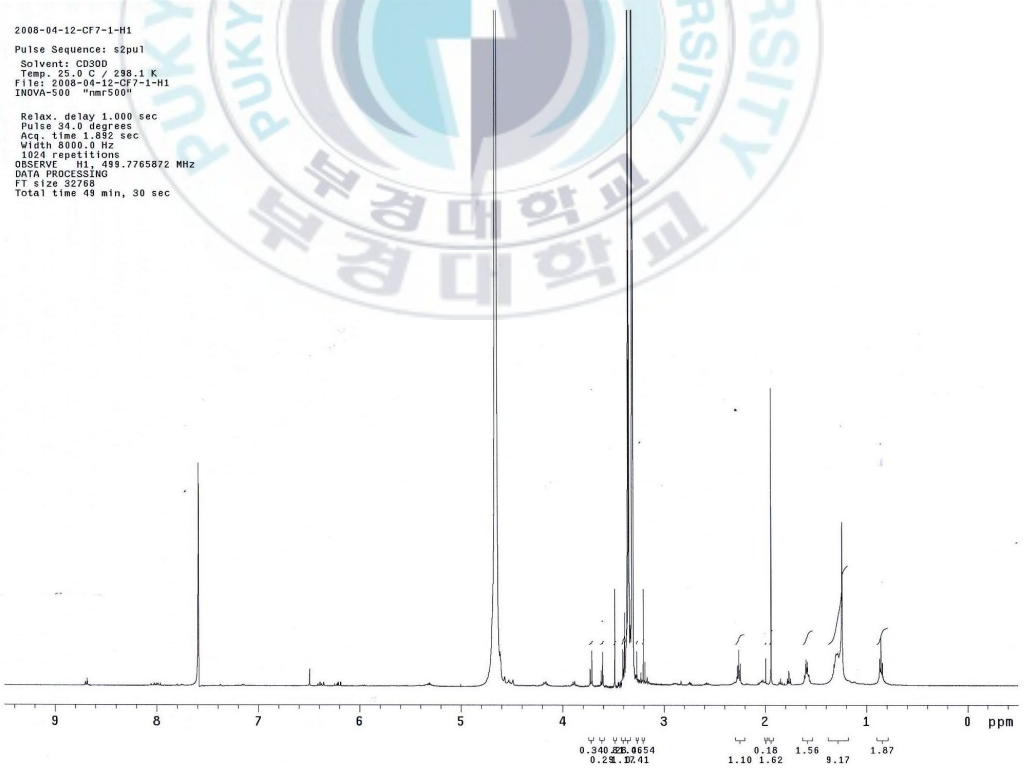

Figure IV-10. ^{1}H NMR (100 MHz, $CDCl_3$) spectrum of CF-7

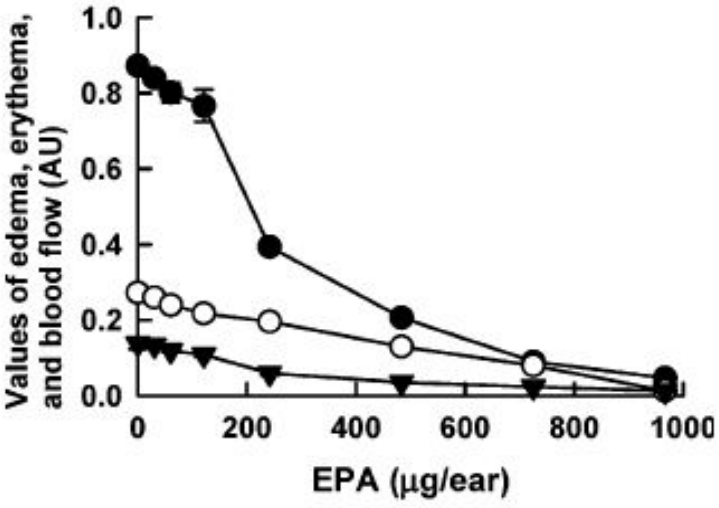

Figure IV-11. Anti-inflammatory activity of eicosapentaenoic acid (EPA) isolated from *Codium fragile*. Edema (●) and erythema (O) on mouse ear were measured with different concentrations of EPA per ear. The indomethacin as a positive control yielded IC_{50} f 90, 172, and 179 μg/ear for edema, erythema, and blood flow, respectively. Values represent the mean ± SE (n ≥ 5).

Chapter 5

Increase of the Yields of L-Lysine and Tryptophan Amino Acids by the *Porphyra suborbiculata* Following Random Mutagenesis

Abstract

A strain improvement program was initiated based on mutagenesis with the goal of increasing the content of tryptophan and lysine (essential amino acids) of *Porphyra*. Three rounds of ultraviolet radiation and selected mutagenic agents (DL-5-methyltryptophan and aminoethyl-L-cysteine) were conducted using monospores of *Porphyra* as the parent strain. When analyzing the IC_{100} of selected monospores of the first stage, value of 8 mM and 116 mM, which is higher than original stage, were obtained for DL-5-methyltryptophan and aminoethyl-L-cysteine, respectively. When analyzing the IC_{100} of selected monospores of the final stage, value of 9.3 mM and 135 mM were obtained for DL-5-methyltryptophan and aminoethyl-L-cysteine, respectively. After amino acid analysis of AEC resistant mutants, an increase of 174% of lysine over that of the parent strain was obtained. After amino acid analysis of DMT resistant mutants, it was found that the almost amino acid productions of the DMT resistant mutants were higher than the parent strain. Although this is DMT resistant mutant but an increase of 305% of lysine free amino acid over that of the parental strain was obtained. Our results appear promising that it is possible to increase the volumetric productivity of both essential amino acids using this method.

Introduction

Porphyra (Bangiales, Rhodophyta) is one of the world's most valued maricultured seaweeds, and is primarily used as food in many oriental countries. It is highly prized for its flavour and as a health food as it is rich in proteins and vitamins. The dried *Porphyra* contains various biologically active substances beneficial to human health (Hiroyuki Noda, 1993). Nearly 17 types of free amino acids, including taurine, which controls blood cholesterol levels (Tsujii et al., 1983) can be found within the genus, which has an annual value of over US$ 1.8 billion (Yarish et al., 1999). In addition, certain species of *Porphyra* also serve as important commercial sources of the red pigment r-phycoerythrin, which is utilized as a fluorescent "tag" for immunofluorescent studies and can cost as much as $360 per mg. The biology and ecology of *Porphyra* has been studied more thoroughly than that of any other red algal genus (Tseng & Sun, 1989; Cole, 1990; Hawkes, 1990). Recently, it has been reported that *Porphyra* has much more potential and can be used as an experimental system for modern biological research, like *Arabidopsis thaliana* (Sahoo et al., 2002).

Porphyra suborbiculata is not considered to have high commercial value as food compared to the cultivated strains of *P. yezoensis* and *P. tenera*. However, in areas where it is present, other potential uses have been reported. In northern Philippines for example, dried *Porphyra* spp. are utilized as fertilizer and for lowering plasma cholesterol (Trono 1999).

As has been demonstrated repeatedly with agricultural crops and other types of cultivation, genetic improvement of cultured species is generally crucial for maximizing yield and developing cost-effective cultivation programs. Seaweeds, including *Porphyra*, are no exception. However, unlike land plants, seaweed strain improvement techniques

have generally been restricted to classical breeding methods, particularly strain selection.

The most successful method of producing new strains of *Porphyra* to date has been through repeated strain selection. However, this approach has a number of disadvantages and limitations. In particular, repeated strain selection usually requires many years of intensive effort and is very labor intensive. In addition, the existing genetic variability in one or more populations of interest may not be sufficient for strain selection purposes. Furthermore, desirable and agronomically-beneficial traits that may be found in other species can not be taken advantage of by applying the methods that have been used in *Porphyra* in the past. Future improvements in the production of nori, both within and outside of the United States, will therefore most likely depend on the production of new strains that will have to be developed by new strain improvement methods.

Two methods of strain improvement that permits the rapid development of new strains and the transfer of genes and traits between species is somatic hybridization via protoplast fusion and induced mutation. Protoplast fusion is a well-developed technique in land plants and is just beginning to be successfully applied to seaweeds. Another, genetic engineering and mutant selection have been suggested as appropriate methods (Baltz, 1986) for strain improvement (de la Noue and de Paw, 1988; Brown et al., 1990); however, genetic engineering can only be applied to systems where the genetic fundamentals are well known (Baltz, 1986). Mutation, on the other hand, has advantage of simplicity. It requires little knowledge of the pathways involved in the biosynthesis of the desired product and minimum technical manipulation (Rowlands 1984; Baltz, 1986; Crueger and Crueger, 1990). Strain improvement by induced mutation is indeed broadly applied in biotechnological industries; e.g., the yield of penicillin was stepwise increased from 0.06 to 26 $mg.mL^{-1}$ via application of this method (Queener and Lively, 1986). Mutation could therefore be a successful method for *Porphyra* strain improvement. UV light was chosen

as mutagenic agent, because it is considered as one of the simplest ways to obtain a wide variety of mutant strains from bacteria (Carlton and Brown, 1981) and microalgae (Williams et al., 1979)

Free and bound amino acids have been identified in *Porphyra* (Korean name: Kim) (Kagawa, 1983). Kim is rich in alanine, aspartic acid, glutamic acid and glycine, although the ratios among these bound and free amino acids vary widely (Noda et al., 1975). However, Kim is very poor lysine and tryptophan which are two of the essential amino acids required in the diets of animals (Hiroyuki Noda, 1993). Therefore, there is considerable interest in increasing the content of these essential amino acids of Kim.

In the present study, we attempted to enhance tryptophan and lysine by induced mutagenesis in *Porphyra suborbiculata*.

Materials and methods

Monospore culture

Juvenile blades of *Porphyra suborbiculata* were collected from a rocky shore at Cheongsapo, Korea. The fresh blades were rinsed, sonicated (60 kHz) twice for 1 min in autoclaved seawater, and immersed in 1% Betadine solution with 2% Triton X-100 for 1 min to eliminate epiphytes. To liberate the monospores, the blades were cultured in Provasoli enriched seawater (PES) medium (Provasoli, 1968) under 40 μmol/m^2/s light intensity (10L:14D) at 20^{o}C. Monospores were then grown to juvenile blades under the same conditions.

Experimental Design

The mutation program used ultraviolet radiation (UV) as mutagenic agent. The UV source was a 254-nm wavelength lamp and power 30 W placed 20 cm above the culture. The first step of the mutation program was determination of the appropriate times for UV exposure of the parent culture, so as to guarantee a successful strain improvement methodology; for that purpose, death curves under UV light were obtained. Volumes of 500 μL of culture containing ca. 500 monospores per well in 24 well plate were exposed to UV light for 5 min, with continuous agitation; a sample was removed every 30 s. Exposed monospores were then stored in darkness for 1 d to avoid photoreactivation (Carlton and Brown, 1981), and counted regenerated juvenile blades after a week culture.

The second step of the mutation program used the tryptophan analog (DL-5-methyltryptophan) (DMT) and the lysine analog (aminoethyl-L-cysteine) (AEC) as selected mutagenic agents. To determine of the appropriate concentrations for amino acid analog exposure of the monospore parent, dose-response curves under different concentrations were obtained. Volumes of 1000 μL of culture containing ca. 500

monospores per well in 24 well plate were exposed to different concentrations of amino acid analogs for 1 week and then counted regenerated juvenile blades.

To isolate DMT-resistant and AEC-resistant mutants, the first round, monospores of *P. suborbiculata* after 1 d of UV exposure were spread on the PES medium containing 6 mM and 120 mM of DMT and AEC, respectively. After 1 week, we will change to normal PES medium and cultured until juvenile blades release new monospores. Survival rate (%) was calculated by dividing the live monospores by monospores in the control culture. These monospores will use for continuous rounds at higher concentrations of selected mutagenic agents. Mutants with a highest tryptophan and L-lysine productions were assayed for amino acid analysis (Fig. V-1).

Growth rates

Growth experiments of *Porphyra suborbiculata* were performed in temperature-controlled incubator and cultured under 70 μmol m^{-2} s^{-1} light intensity at 20°C for 7 days. Cells were counted under a microscope using a hemocytometer. Specific growth rate (λ) was calculated as a cell growth against the cultured day: $\log N - \log N_0 = \lambda(T - T_0)/2.303$, where N = cell numbers at day T, and N_0 = cell numbers at day T_0.

Analysis of gross biochemical composition

Biomass dry weight was measured after washing with 0.5 M ammonium bicarbonate (pH 7.5) and drying at 90 ^{o}C for 1 d. Total carbohydrate expressed as glucose was determined by the phenol-sulfuric acid method (Kochert, 1978), using glucose as a standard. Total lipid was extracted by the use of hexane and isopropanol (3:2) as a solvent (Radin, 1981) and quantified gravimetrically. The amount of soluble protein in the cell was estimated according to the method of Lowry et al. (1951) after heating the cell suspension at 100 ^{o}C in 1 N NaOH for 2 h to obtain complete solubilization of protein. Bovine serum albumin was used as the standard for protein determination.

Structural amino acid analysis

Sample (100 mg) → Add 3 ml of 6N- HCl (for normal analysis) or add 3ml of 3N-NaOH (for Tryptophan analysis)→ Sealing → Incubation at 110^{o}C heating block for 24 hrs → Filtration with Whatman No. 6 → Evaporate at 100^{o}C water bath for remove chlorine gas → Dissolve in 2 ml of Na-citrate buffer (pH 2.2) → Filtration with membrane (0.2 μm) → Analysis with amino acid analyzer (Biochrom 20).

Free amino acid analysis

Sample (100 mg) → add 5 ml of water → Place at 60^{o}C heating block for 1hr → add 100mg of sulfosalicylic acid → Place at refrigerator (4^{o}C) for 2 hrs → Centrifuge (15,000 rpm, 4^{o}C, 15min) → Transfer the supernatant in evaporating flask → evaporate with rotary vacuum evaporator → Dissolve in 2ml of Li-Citrate buffer (pH 2.2) (Filtration with membrane (0.2 μm) → Analysis with amino acid analyzer (Biochrom 20).

Alkaline hydrolysis for tryptophan analysis

Dried sample powder (20-50 mg as protein contents) → Suspended in 20 mL 4M NaOH → Sealed under Ar or N_2 gas → Hydrolysed for 17 hr at 110^{0}C → Centrifuge for 10 min at 16,000 g → Achieve the supernatant and then dilute 50 folds with water → Directly injected (20 μl) for analysis with amino acid analyzer (Biochrom 20).

Results and Discussions

Isolation of monospore

The genus *Porphyra* is one of the most important edible seaweeds commercially cultivated in Korea, Japan and China. However, *Porphyra* is very poor lysine and tryptophan which are two of the essential amino acids required in the diets of animals (Hiroyuki Noda, 1993). Therefore, in order to increase its production, a new screening procedure was tried.

First of all, to isolate monospore use for strain improvement from a culture of juvenile blades of *Porphyra*, cleaned juvenile blades were cultured in PES at 20°C for 20 d and when they started to produce monospores, we changed the medium and recycled the culture every 20 d. Approximately 40 monospores (average size, 15μm) were produced from an average 100 μm-long juvenile blade about every 20 days. Monospores were separated from juvenile blades by filtering with a 20-μm-mesh nylon membrane and then used for strain improvements.

Strain improvement by mutation

The survival data obtained on UV treatment for varying time periods are presented in Fig. V-2. Preliminary analysis of several death curves indicated that UV doses corresponding to irradiation for 30 and 60 s, with associated survival rates of ca. 90% and 70%, respectively, were appropriate, so they were selected for further consideration.

After checked the appropriate concentrations for amino acid analog exposure of the monospore parent, the dose-response curves indicated that amino acid analog concentrations corresponding to IC_{50} and IC_{100} of DMT and AEC were 4.7 mM, 7.5mM and 57 mM, 110 mM, respectively (Fig. V-3 & V-4). The results indicated that

monospores are susceptible to DMT and AEC and that when the initial concentration of these agents in PES medium is 7.5 mM and 110 mM, respectively, the inhibitory ratio for cell growth is 100%. The above experimental results explained that a high concentration of these agents in the medium was harmful for cell growth; and that therefore, tryptophan and lysine synthesizes would certainly be affected. It is necessary to screen for strains resistant to these agents. These IC_{100} concentrations were selected for further experiments.

The mutagenic agent used (UV light) is a practical, but not very efficient way to induce mutagenesis; hence, it is preferable to use long exposures, with concomitant high killing rates to increase the probability of mutation (Carlton and Brown, 1981). However, when working with monospore or microalgae, it is difficult to recover clones; as the highest possible number is essential, several continuously rounds of UV and selected mutagenic agents exposure were chosen, with corresponding killing rates between 85 % and 92 % (Fig. V-5 & Fig. V-6). These times of exposure either facilitate recovery of a high number of monospores (as the former), or increase the mutation probability (as the latter). When analyzing the IC_{100} of selected monospores of the first stage, value of 8 mM and 116 mM, which is higher than original stage, were obtained for DMT and AEC, respectively (Fig V-3 & V-4); however, this selection was based on analysis of a single culture, which does not take into account the intrinsic variation in performance of individual strains. This procedure may lead to errors, yet note that the major goal of the second stage was to isolate the highest possible number of putative mutants. Considering the ultimate goal of this work - to increase tryptophan and lysine contents – we were tried with second and third rounds to increase and maintain mutants. When analyzing the IC_{100} of selected monospores of the final stage, value of 9.3 mM mM and 135 mM were obtained for DMT and AEC, respectively (Fig V-3 & V-4). Our results appear promising that it is possible to increase the volumetric productivity of both amino acids using this

method.

Growth rate of parent and mutant strains

The specific growth rate of the monospores of parent strain, lysine mutant and tryptophan mutant in the early growth phase between 6 and 7 days were 0.423, 0.420 and 0.432, respectively, and almost similar in photoperiods for the first 4 weeks (Table V-2). The spore (approximately 20 μm in diameter) and blade shapes were similar between parent strain and mutant strains (Fig. V-7).

Gross biochemical composition

Amounts of gross biochemical compositions of carbohydrate and lipid were similar in the blades (Table V-1). Amounts of protein were highest in the blade of tryptophan mutant strain (43.15 mg), followed by lysine mutant strain (40.76 mg) and normal strain (38.2 mg) (Table V-1).

Amino acid analysis

After amino acid analysis of AEC resistant mutants, it was found that the lysine productions of the AEC resistant mutants were higher than the parent strain. An increase of 174% of lysine over that of the parental strain was obtained (Table V-3). The results also showed that almost the other amino acids similar with the parent strain in both free amino acid and structure amino acid.

After amino acid analysis of DMT resistant mutants, it was found that the almost amino acid productions of the DMT resistant mutants were higher than the parent strain. Although this is DMT resistant mutant but an increase of 305% of lysine free amino acid over that of the parental strain was obtained (Table 3). The results also showed that almost the other amino acids higher than the parent strain in both free amino acid and structure amino acid.

Because of the stability of tryptophan, tryptophan is a difficult amino acid to

determine in protein and peptides because it chemically decomposes during acid hydrolysis. Consequently, many procedures have been developed for the independent preservation and determination of this amino acid (Friedman M., J.W. Finley, 1971). In this study, we attempted to alkaline hydrolysis method for tryptophan analysis. The results were not clearly. In other hand, according to the biosynthetic pathway of amino acid, chlorismate is the central branch-point metabolite in the biosynthesis of aromatic amino acids. By one pathway it is converted to phenylalanine and tyrosine; by another it leads to tryptophan (Rawn 1983). Tryptophan, its precursors, and its catabolic products, are a source of many important biosynthetic products. Table V-3 & V-4 showed that the amount of phenylalanine and tyrosine of both of mutant strains were increased with very high level compared with parent strain. Therefore, amount of tryptophan also increase because of the amount of chlorismate increase. To make sure, we are searching another method for tryptophan analysis.

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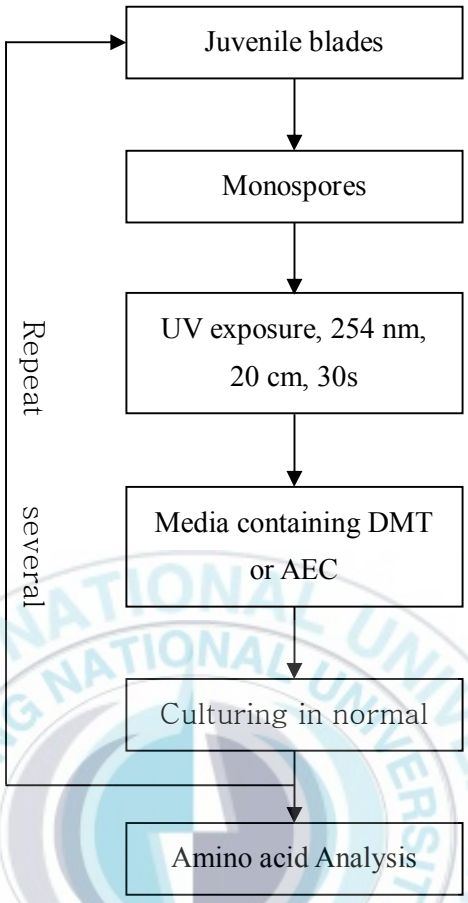


Figure V-1. Protocol used for a mutation-selection program for improving the tryptophan and lysine contents in *Porphyra suborbiculata*.

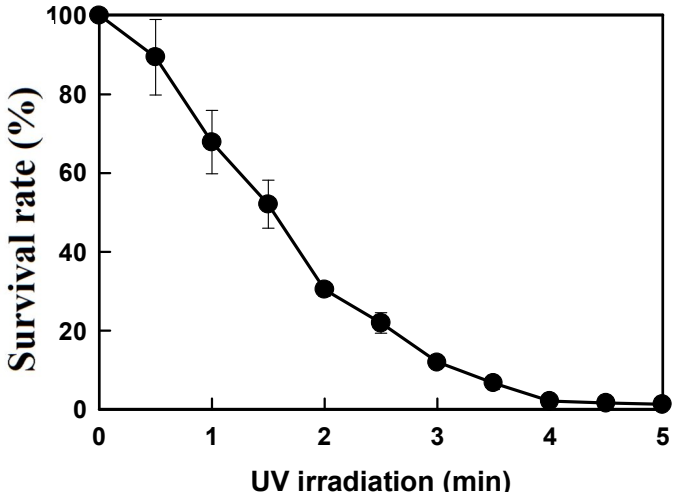


Figure V-2. Survival pattern of the parent monospores (Cheongsapo, Korea) by UV irradiation using a germicidal lamp.

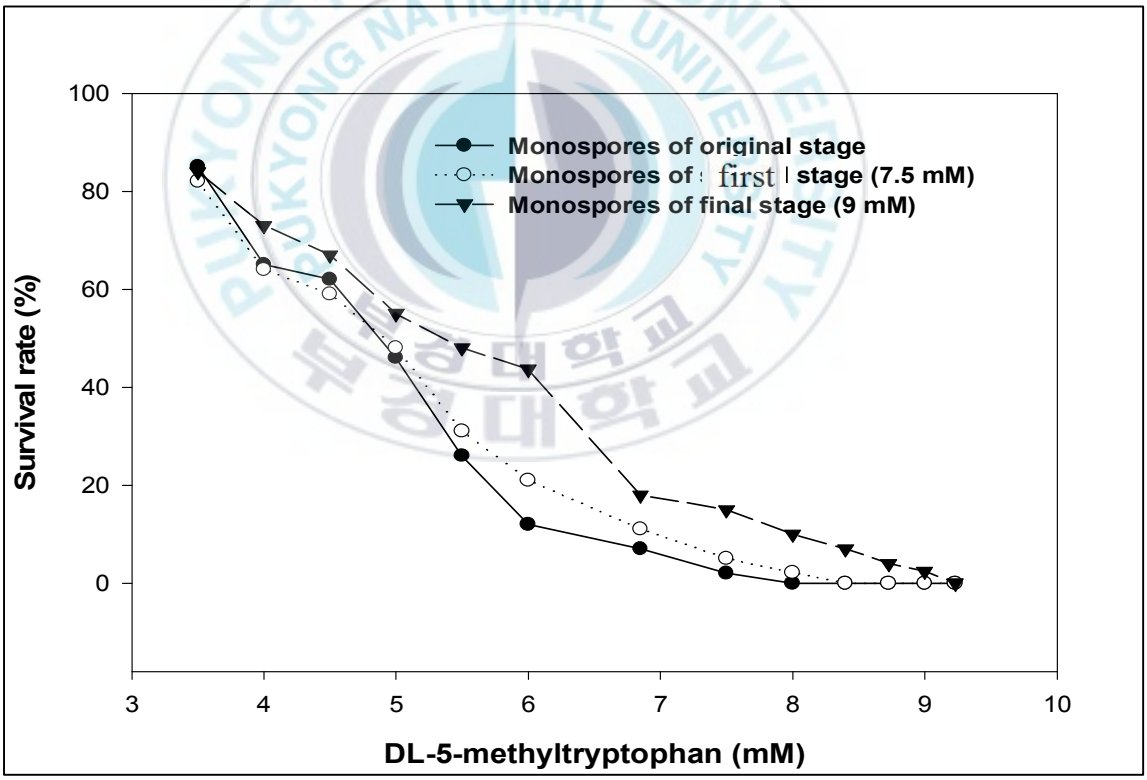


Figure V-3. Dose-response curve for effects of the tryptophan analog DL-5-methyltryptophan on *Porphyra suborbiculata* monospores. Survival rate (%) was calculated by dividing the live monospores by monospores in the control culture.

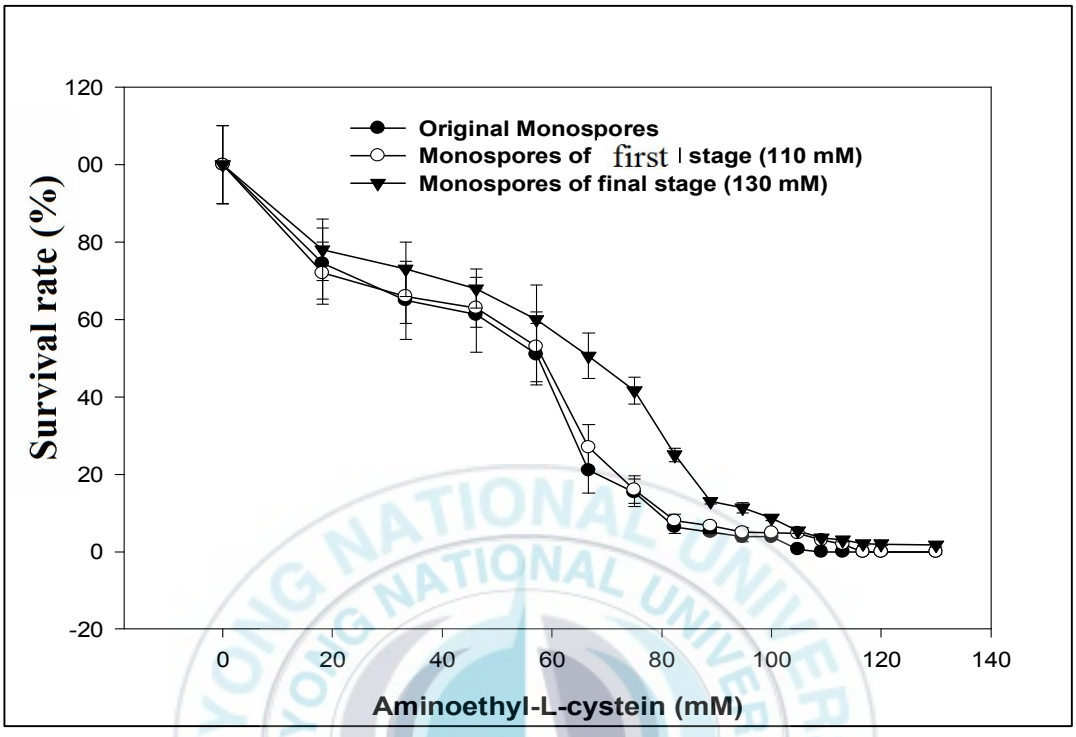


Figure V-4. Dose-response curve for effects of the lysine analog aminoethyl-L-cysteine on *Porphyra suborbiculata* monospores. Survival rate (%) was calculated by dividing the live monospores by monospores in the control culture.

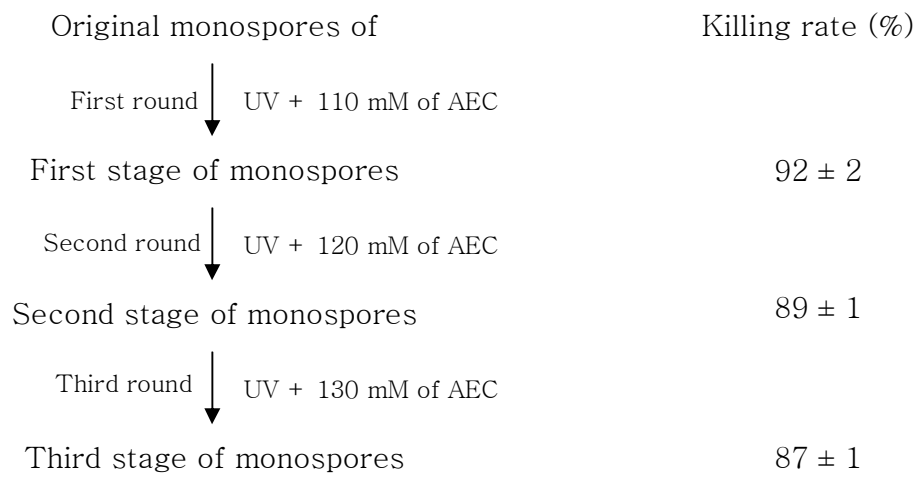


Figure V-5. The genealogical tree of monospores of *Porphyra suborbiculata* to improve the quality of L-lysine.

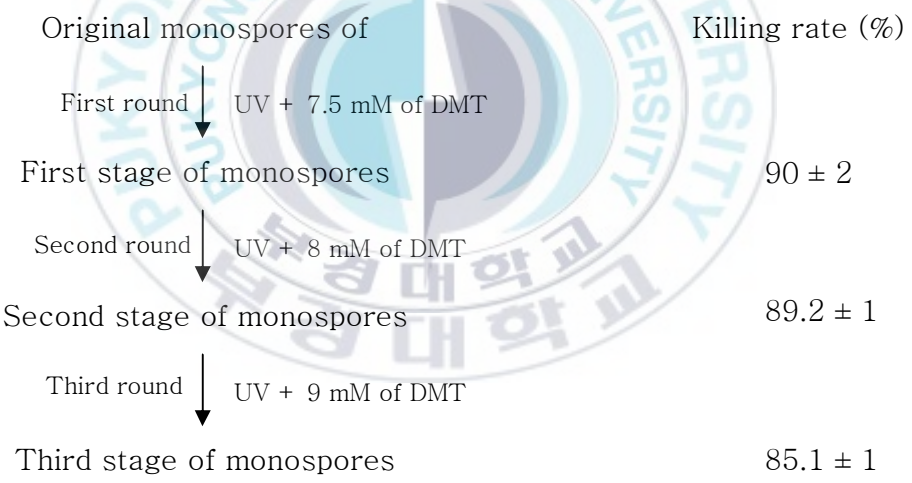
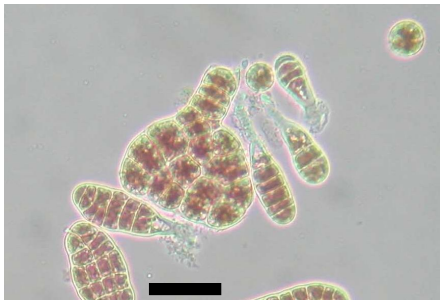
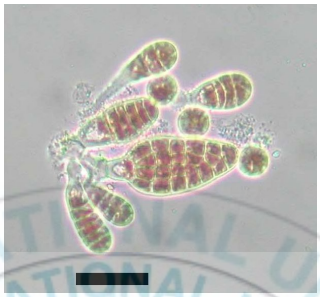


Figure V-6. The genealogical tree of monospores of *Porphyra suborbiculata* to improve the quality of tryptophan.

A



B



C

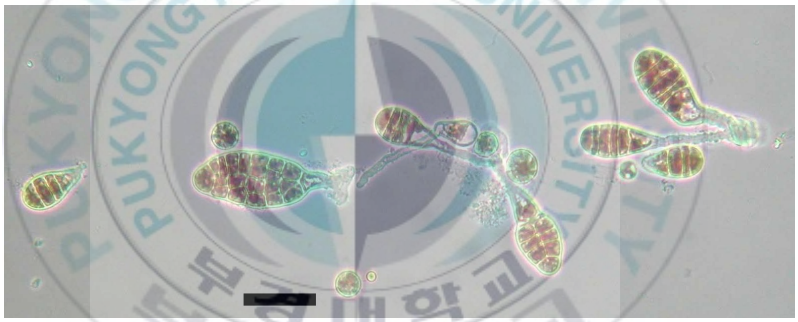


Figure V-7: Surface view of the early growth phase of the strains of Porphyra suborbiculata in culture. (A) Original strain. (B) Lysine mutant strain. (C) Tryptophan mutant strain. Scale bars = 60 μm.

Table V-1: Gross biochemiscal composition of *Porphyra suborbiculata*. Values are expressed on a dry weight basis, mg per 100 mg sample**.**

	Parent	Lysine Mutant	Tryptophan mutant
Carbohydrate	40.5	39.68	40
Lipid	1.33	1.35	1.32
Protein	38.2	40.76	43.15

Table V-2: The daily growth rate of the strains of *Porphyra suborbiculata***.**

Strains	The daily growth rate
Parent	0.423 ± 0.05
Lysine mutant	0.420 ± 0.02
Tryptophan mutant	0.432 ± 0.04

Values represent the mean ±SE (n≥3)

Table V-3: Amount and percentage of free amino acids of mutant strains compared with parent strain on dry weight basis.

No.	Amino acids	Amount of amino acid (ng/100mg)			Enhancement (%)	
		Parent	Lysine mutant	Trytophan mutant	Lysine Mutant	Tryptophan Mutant
1	Alanine	9580.69	9617.74	10878.63	100	114
2	Ammonia	2090.31	2080.62	1428.24	100	68
3	Arginine	939.739	1110.23	1666.21	118	177
4	Aspartic acid	ND	ND	ND	ND	ND
5	Cystein	ND	ND	ND	ND	ND
6	Glutamic acid	3274.96	3827.58	5780.90	117	177
7	Glycine	1598.27	1998.14	2215.86	125	139
8	Histidine	ND	ND	ND	ND	ND
9	Isoleucine	253.975	258.73	583.66	102	230
10	Leucine	1854.28	1366.31	2500.26	74	135
11	Lysine	378.35	657.66	1154.63	174	305
12	Methionine	ND	ND	ND	ND	ND
13	Ornithine	619.605	651.85	746.11	105	120
14	Phenylalanine	401.463	721.14	765.23	180	191
15	Phospho-ethanolamine	1465.23	1294.00	2190.48	88	149
16	Phospho Serine	6565.09	3932.86	3572.32	60	54
17	Proline	ND	ND	ND	ND	ND
18	Serine	1892.24	1472.12	2418.15	78	128
19	Taurine	15504.6	13602.44	8723.14	88	56
20	Threonine	745.906	942.03	1687.77	126	226
21	Tyrosine	ND	ND	ND	ND	ND
22	Valine	501.952	682.46	1033.13	136	206

ND, not detectable

Table V-4: Amount and percentage of structure amino acids of mutant strains compared with parent strain on dry weight basis.

No.	Amino acids	Amount of amino acid (ng/100mg)			Enhancement (%)	
		Parent	Lysine mutant	Trytophan mutant	Lysine Mutant	Tryptophan Mutant
1	Alanine	65671.17	63203.95	102820.56	96	157
2	Ammonia	13308.32	12783.93	19019.10	96	143
3	Arginine	44248.09	39313.01	61057.39	89	138
4	Aspartic acid	68745.37	58846.30	86193.03	86	125
5	Cystein	2593.71	4170.91	5605.56	161	216
6	Glutamic acid	75666.89	57268.07	95142.29	76	126
7	Glycine	45374.11	37580.74	55460.63	83	122
8	Histidine	9047.78	8251.32	12352.89	91	137
9	Isoleucine	22978.93	24094.66	34665.57	105	151
10	Leucine	43153.43	44170.13	62713.91	102	145
11	Lysine	47775.26	41295.11	59217.00	86	124
12	Methionine	4604.11	6843.06	10065.38	149	219
13	Ornithine	ND	ND	ND	ND	ND
14	Phenylalanine	23550.07	25181.96	35081.52	107	149
15	Phospho-ethanolamine	ND	ND	ND	ND	ND
16	Phospho Serine	ND	ND	ND	ND	ND
17	Proline	37793.68	28550.89	61579.26	76	163
18	Serine	35085.87	25410.32	42297.09	72	121
19	Taurine	ND	ND	ND	ND	ND
20	Threonine	37229.77	29346.20	42643.20	79	115
21	Tyrosine	11017.96	21760.83	27824.62	198	253
22	Valine	34686.21	42603.34	59553.61	123	172

ND, not detectable

국문요약

해조생물공학은 해조류 자원으로부터 식품, 약학, 화학, 환경개선물질 등을 생산하기 위한 종합학문분야이다. 수세기 동안, 해조류는 식물학적, 산업적, 약학적 중요성이 대두되어지고 있다. 해조류로부터 추출된 물질들은 제약 산업에서 중요하게 여겨진다. 특히, 이 분야에서 본 연구는 (1) 미세조류 성장촉진제, 방오물질, 항염증물질 등의 생리활성물질들의 screening, 분리, 생물학적 분석, (2) 유용물질의 대향 생산을 위한 조직배양과 돌연변이 선발의 두 가지 주제를 다뤘다. 이러한 연구는 매우 학제적이고 해양생물공학에서 다양한 방법론을 이끌어낸다.

첫째로, 미세조류 성장 촉진제 동정에서 녹조류 *Monostroma nitidum* (홑파래)로부터 다양한 배양액에서 여러 미세조류의 세표성장을 촉진하는 levoglucosan 성분을 분리하였다. 해조류 분말로부터 얻은 물질의 수율은 5×10^{-3} % (w/w)였다. 10mM 농도에서 levoglucosan 은 세포성장을 촉진하였고 모든 미세조류 종의 세포 성장률이 대부분의 배양액에서 거의 150%를 나타내었다. 세포의 지방산 특성과 세포크기는 levoglucosan 의 유무에 따라 배양액 사이에 약간 차이가 있었다.

둘째는, 무절 석회 조류 *Lithophyllum yessoense* Foslie 로부터 해조류 포자 용해성 지방산 heptadeca-5,8,11-trienoic acid (HDTA: C17:3) 를 분리하였다. 홀수개의 탄소원자를 가진 지방산, HDTA 는 녹조류 3 종, 홍조류 8 종, 갈조류 2 종, 미세조류 3 종에 대하여 5 μg/mL 에서 50%이상의 용해활성을 보였다. 지방산의 이중결합과 탄소원자 수가 증가할수록 용해활성도 증가된다. HDTA 는 LC_{50} 이 3.1 μg/mL 의 농도로 α-linolenic acid (C18:3) 보다 10 배 강한 활성을 보인다.

셋째는, 활성물질의 분리와 구조분석과 함께 청각의 항염증 효과에 대하여 연구하였다. 주 활성물질은 acetonitrile 추출, 극성에 따른 분류, 실리카 겔 크로마토그래피, 역상 HPLC 를 통하여 순수한 물질 CF-2, CF-6, CF-7 를 분리하였다. CF-2 의 구조는 1D, ^{1}H, ^{13}C NMR, GC-MS 데이터로 동정하였고, eicosa-5,8,11,14,17- pentaenoic acid (EPA)로 생각되어진다. CF-6 과 CF-7 의 구조는 NMR 과 MS 를 통하여 분석하였으며 구조결정은 진행 중에 있다. 정제된 EPA, CF-6, CF-7 은 PMA 로 유도되어진 쥐 귀의 부종과 충혈에 대하여 항염증활성을 측정하였다. EPA, CF-6, CF-7 의 다른 농도에 따른 쥐 귀에 국부적 저해 효과는 농도 의존적이었다. EPA 의 IC_{50} 은 부종과 충혈에 대하여 각각 230, 462 μg 이었다.

마지막으로, 품종개량 프로그램은 *Porphyra* 의 트립토판과 라이신 (필수아미노산) 함량증가에 대한 돌연변이 생성을 수행하였다. three round 방사선 조사와 아미노산 유도체 (DL-5-methyltryptophan, aminoethyl-L-cysteine)는 모체 strain 인 *Porphyra* 의 포자를 이용하여 수행되었다. 초기단계의 선별된 포자의 IC_{100} 는 DL-5-methyltryptophan 와 aminoethyl-L-cysteine 에 대하여 기존단계보다 높은 8 mM, 116 mM 의 값을 각각 얻었다. 최종단계의 선별된 포자의 IC_{100} 는 DL-5-methyltryptophan 와 aminoethyl-L-cysteine 에 대하여 각각 9.3 mM, 135 mM 의 값을 얻었다. 본 실험결과는 실험에서 수행되어진 방법을 이용하여 필수 아미노산의 생산성 증가가 가능함을 나타낸다.

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