UNIVERSITY OF CALIFORNIA

Santa Barbara

Physiological Chemistry of Pseudopterosin Biosynthesis in Symbiotic Dinoflagellates from the Soft Coral *Pseudopterogorgia elisabethae*

A Dissertation Submitted in Partial Satisfaction of the

Requirements for the Degree Doctor of Philosophy in Marine Science

by

Laura D. Mydlarz

Committee in Charge

Professor R.S. Jacobs, Chair
Professor B.B. Prezelin
Professor M. Brzezinski
Professor D.J. Chapman

June 2004

The dissertation of Laura D. Mydlarz is approved

Barbara B. Prezelin

Barbara B. Prezelin

Mark Brzezinski

David J. Chapman

Robert S. Jacobs, Committee Chairman

June 2004

Physiological Chemistry of Pseudopterosin Biosynthesis in Symbiotic Dinoflagellates from the Soft Coral *Pseudopterogorgia elisabethae*

By

Laura D. Mydlarz

ACKNOWLEDGEMENTS

I would like to thank the many people whose help and encouragement made this work possible. Most importantly I would like to thank my committee members, Barbara Prezelin, Mark Brzezinski, David Chapman and my advisor, Robert Jacobs for their advice, support and undivided attention. Barbara Prezelin has continually supported my research and allowed to get involved in a number of her own research programs, I am grateful for her advice and friendship. Mark Brzezsinki has always made time to meet with me to help me in evaluating my data and making sense of it all. David Chapman has always been supportive and interested in my progress. I would like to particularly thank my advisor, Dr. Robert Jacobs for being a terrific mentor and supporter of my research even when faced with doubt from our peers. I am extremely grateful for all the opportunities that Dr. Jacobs has given me to direct my research plan and present my work at meetings.

I would also like to thank my collaborator and former mentor, Russell Kerr, who has continually supported my work and allowed me time and space to work in his laboratory at Florida Atlantic University. His continual contributions and suggestions made most of the biosynthetic work stronger. At FAU, I would also like to acknowledge the contributions of Tyrone Ferns for help with organic reactions and Lory Santiago-Vasquez for help collecting and shipping corals to UCSB. Frithjof Kupper at The Scottish Association for Marine Science, and Cliff Ross

(UCSB) were instrumental in development of the oxidative burst assay and I am grateful for their support and contributions. Frithjof's enthusiasm for science and this project were very inspiring and kept me motivated. James Pavlovich (UCSB Chemistry) was instrumental in aiding me with all mass spectrometry and was available for consultation and advice on any analytical chemistry question. I am also pleased that we were able to form a relationship as colleagues and as friends. I would also like to thank the wonderful staff at the Smithsonian Marine Station at Link Port and the Keys Marine Lab, for enjoyable stays and especially Lonny Anderson at KML for coral collections.

My time at UCSB was facilitated and made enjoyable by the wonderful Biology staff, classmates and labmates that I have encountered over the years. Sue Marton, June Varbel, Melanie Abe, Stephanie Slosser, Doug Hart, Bob Fletcher, Todd Richardson and Angie Gallagher in Biology were always there to assist me and fulfill constant last minute requests. I would like to thank several of people in the Marine Science Program for their support, Amelia Shevenell, Sarah Goldthwait, Claudia Mengelt, Jim Kennett and especially Alice Alldredge whose commitment to the Marine Science program and the students was evident in every thing she does. My labmates have been instrumental in making my time in the lab enjoyable; I am grateful for the support and knowledge of Claudia Moya (who taught me everything I know about statistics, pharmacokinetics and calculating molarity), Cliff Ross (who taught me gel electrophoresis), Daniel Day, Hamta Madari, and Lory Santiago-

Vasquez. I also would like to especially thank Lori Hensic, whose assistance on Florida trips made most of the biosynthetic work possible.

I would also like to thank my parents, brothers, sister and extended family for their constant support and encouragement. Completion of this Ph.D. would not have been possible without my husband, my companion and my best friend, Brian Norton. Brian tried to keep me sane and take my mind off work, when things got challenging and demanding, I am grateful for his constant patience and serenity.

CURRICULUM VITAE

EDUCATION

2004 Doctorate in Marine Science
Interdepartmental Marine Science Program and
Department of Evolution, Ecology and Marine Biology
University of California at Santa Barbara, CA 93106
Advisor: Robert S. Jacobs

1998 Master of Science Degree
Department of Biology and Center for Molecular Biology and
Biotechnology, Florida Atlantic University, Boca Raton, FL 33431
Advisor: Russell G. Kerr

1996 Bachelor of Science Degree in Marine Biology
Department of Biology
Florida Atlantic University
Boca Raton, FL 33431

ACADEMIC TRAINING

1998-2004 Marine Pharmacology Laboratory, University of California, Santa Barbara
1996-2002 Keys Marine Laboratory, Long Key, Florida
1996-2002 Marine Natural Products Laboratories, Florida Atlantic University

AWARDS/HONORS

2004 Fiona Goodchild Award for Excellence as a Graduate Student Mentor of Undergraduate Research
2004 Graduate Division Dissertation Fellowship
2003 Graduate Division Travel Award
2003 Worster Award for summer research and mentoring of undergraduate student
2002 Worster Award for summer research and mentoring of undergraduate student

GRANTS/FUNDING

2003-2004 Graduate Researcher, Toxic Phytoplankton along California Coast: a 500 Mile Perspective. University of California Office of the President Project

2002-2003 Graduate Researcher, Advancing Marine Biotechnology: Use of OCS Oil Platforms as Sustainable Sources of Marine Natural Products, U.S. Dept. of Interior, Mineral Management Services, MMS-17609

1999-2001 Sea Grant Traineeship, University of California, Santa Barbara. National Sea Grant Biotechnology Initiative. Pharmacological Adaptation of Protoctista as Novel Models to Study Inflammation. Grant # R/MP-85

PUBLICATIONS

Mydlarz, L.D. and R.S Jacobs. 2004. Hydrogen peroxide production in gorgonian corals in response to injury and heat stress. (Manuscript in Preparation).

Mydlarz, L.D. and R.S Jacobs.2004.Comparison of an Inducible Oxidative Burst in Free-Living and Symbiotic Dinoflagellates Reveal Properties of the Pseudopterosins. **Phytochemistry**. (Manuscript Submitted)

Santiago-Vázquez L, L.D. Mydlarz, R. S. Jacobs and J.G. Pavlovich. 2004. Identification of hydroxy fatty acids by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry in *Euglena gracilis*. **Journal of Chromatography B, Anl.Tech. in the Biomed. and Life Sci,** Vol.. 803 (2) pp. 233-236

Mydlarz, L.D, Jacobs R.S., Bohnlein, J and Kerr, R.G. 2003. Pseudopterosin biosynthesis in *Symbiodinium* sp., the dinoflagellate symbiont of *Pseudopterogorgia elisabethae.* **Chemistry and Biology.** Vol. 10, No. 11. pp 1051-1056, November, 2003

Jacobs, R.S, Mydlarz, L, and Kerr, R. 2002. Pseudopterosins of *Symbiodinium microadriaticum* Isolated From *Pseudopterogorgia elisabethae*. **US Patent # P67104US1.**

Coleman, A.C, Mydlarz, L.D. and Kerr, R.G. 1999. *In Vivo* and *In Vitro* investigations into the biosynthetic relatedness of the Pseudopterosins. **Organic Letters**, Vol. 1 (13) pp 2173-2175.

Mydlarz, L.D. 1998. Biosynthetic production of Pseudopterosin C: An anti-inflammatory agent from a marine soft coral. **Masters Thesis**. Boca Raton, FL 33431

OUTREACH, MENTORSHIP AND SERVICE

2003-2004 Mentor for High School Students Science Projects
2002-2003 Worster Mentor for Summer Research Projects for Undergraduate Students, University of California, Santa Barbara
2001-2002 Participant, UCSB Scienceline, online outreach to K-12 school children
2001-2003 Panel Speaker for Student/Parent Orientation, University of California, Santa Barbara.
2001-2002 Docent for Marine Science Institute, University of California, Santa Barbara. Guided tours and marine science education for community, middle school students and parents of students
2001 Graduate Women's Survival Guide Committee. Writer/Editor for campus wide publication.
2000-2001 Career Choices in the Life Sciences Committee. Organized panel workshops for graduate students. University of California, Santa Barbara.

2000	Mentor for College of Letters and Science, University of California, Santa Barbara
1999-2000	Mentor for California Alliance Minority Program,College of Letters and Science, University of California, Santa Barbara

TEACHING EXPERIENCE

2002-2003	Teaching Assistant for Marine Pharmacology Lab Designed and developed teaching protocols University of California, Santa Barbara
1998-2002	Teaching Assistant for General Biology Laboratories University of California, Santa Barbara
1999-2000	Student Coordinator for "TA Teaching Techniques" Class University of California, Santa Barbara
1996-1998	Teaching Assistant for Organic Chemistry and Microbiology Laboratories, Florida Atlantic University

PRESENTATIONS

Mydlarz, L.D. 2004. Studies into the Origins and Endogenous Function of the Marine Natural Products, the Pseudopterosins. **Invited Speaker**. University of California, Santa Barbara Pharmacology and Biotechnology Colloquia. Santa Barbara, California.

Mydlarz, L.D. and Jacobs R.S. 2003. Physiological chemistry of pseudopterosin biosynthesis in dinoflagellates: New insights into endogenous function. **Oral Presentation**, International Symposium of Chemistry and Biology of Marine Organisms, Kolympari, Crete, Greece

Mydlarz, L.D. and Jacobs R.S. 2003. Physiological and ecological significance of terpene synthesis in the symbiotic association of the gorgonian coral Pseudopterogorgia elisabethae. **Poster Presentation.** American Society of Limnology and Oceanography, Aquatic Sciences Meeting, Salt Lake City, Utah

Mydlarz, L.D., Bohnlein, J, Kerr, R and Jacobs, R.S. 2002. Pseudopterosin biosynthesis in Symbiodinium isolated from Pseudopterogorgia elisabethae. **Poster Presentation**. Marine Natural Products Gordon Research Conference, Ventura, California.

Santiago-Vázquez L, L.D. Mydlarz, J.G. Pavlovich and R. S. Jacobs. 2001.Identification of Hydroxyeicosatetraenoic Acids (HETE's) in two Species of Algae using High-Performance Liquid Chromatography and APCI Mass Spectrometry. Regional American Chemical Society. Santa Barbara, California

Jacobs, R.S., L. Santiago-Vazquez, and L. Mydlarz. 2000. Physiologically distinct protist models for studying the function and biosynthesis of eicosanoids. International Symposium on Non-mammalian Eicosanoids and Bioactive Lipids, Berlin, Germany.

Mydlarz, L. and Jacobs, R.S. 2000. Physiologically distinct protist models for studying the function and biosynthesis of eicosanoids and related compounds. **Poster Presentation.** Marine Natural Products Gordon Research Conference, Ventura, California.

Biosynthesis of the Pseudopterosins: Potent anti-inflammatory agents from *Pseudopterogorgia elisabethae*. **Poster Presentation.** National Meeting of the American society of Pharmacognosy, 1998. Orlando, Florida

The biosynthesis of the Pseudopterosins: Anti-inflammatory agents from a marine soft coral. 1998. **Oral Presentation**. South Florida Meeting of the American Chemical Society, Orlando, Florida

RESEARCH EXPEDITIONS

2003 Near Shore Ground fish Tagging Project, California Department of Fish and Game

2001 R/V Bellows, Florida Institute of Oceanography

1998 R/V Bellows, Florida Institute of Oceanography

1997 R/V Bellows, Florida Institute of Oceanography

ABSTRACT

Physiological Chemistry of Pseudopterosin Biosynthesis in Symbiotic Dinoflagellates from the Soft Coral *Pseudopterogorgia elisabethae*

by

Laura D. Mydlarz

The pseudopterosins (Ps) are unique diterpene glycosides with potent anti-inflammatory properties which were isolated from the gorgonian *Pseudopterogorgia elisabethae.* The relationship of the anti-inflammatory effects of Ps to their endogenous function in the coral-symbiont association is not known. In this study the biosynthetic origins and the potential endogenous physiological function of the Ps within the coral-symbiont complex are investigated.

Significant levels of endogenous Ps A, B, C and D were identified within the dinoflagellate symbiont, *Symbiodinium* sp, isolated from *P. elisabethae*. Biosynthetic studies using tritiated geranylgeranyl bisphosphate, yielded radiochemically pure Ps A, B, C and D and the first committed intermediate, elisabethatriene. The photosynthetic production of organic carbon in *Symbiodinium* sp. was traced with the aid of radiolabelled $^{14}CO_2$ uptake studies. Results indicated that photosynthate is a significant carbon source for the production of the pseudopterosins and their

intermediates. Ps constitute 10-15% of the intracellular lipids of *Symbiodinium* sp. and are biosynthesized at a low daily rate. This is the first report of Ps biosynthesis occurs within the algal symbiont of *P. elisabethae*, and thus prompted further study of the physiological implications of this biosynthesis.

In comparative physiological studies of *Symbiodinium* sp. isolated from *P. elisabethae* and in the free-living dinoflagellate *Heterocapsa pygmaea,* physical sonic injury induced a significant oxidative burst of highly reactive oxygen species (ROS). *Symbiodinium* sp. cells from *P. elisabethae* had an attenuated oxidative burst in response to these injuries when compared to *H. pygmaea* and other related *Symbiodinium* species. Exogenously added Ps inhibited ROS release in a dose-dependant manner in physically stressed cells of *H. pygmaea* and *Symbiodinium* sp. isolated from *P. americana*. The reductions in ROS by Ps were not due to direct anti-oxidant effect indicating that they may provide a protective role to the cell membrane.

In studies with gorgonian corals, hydrogen peroxide was identified as signal molecule in response to physical and heat stress. In these experiments *P. elisabethae* also exhibited a muted stress response when compared to related gorgonian corals. Overall this study demonstrates that the biosynthetic production of Ps in *Symbiodinium* sp. cells from *P. elisabethae* occurs in sufficient quantities to confer beneficial effects to both symbiont and host cells.

TABLE OF CONTENTS

CHAPTER FIVE: HYDROGEN PEROXIDE RELEASE AS A STRESS SIGNAL IN GORGONIAN CORALS IN RESPONSE TO PHYSICAL INJURY AND HEAT STRESS

CHAPTER SIX: GENERAL CONCLUSIONS AND HYPOTHETICAL MODEL OF SIGNAL TRANSDUCTION PATHWAYS LEADING TO AN OXIDATIVE BURST IN DINOFLGELLATES AND GORGONIAN CORALS

FIGURES **Page**

SCHEMES **Page**

TABLES **Page**

ABBREVIATIONS

A23187	calcium ionophore
CO_2	carbon dioxide
CoA	coenzyme A
DMSO	dimethylsulfoxide
DCFH-DA	dichlorofluoroscein diacetate
DPI	diphenyleneiodonium chloride
EtOH	ethanol
E. fusca	*Eunicea fusca*
H_2O_2	hydrogen peroxide
GGPP	geranylgeranyl bisphophate
g	gram
L. chilensis	*Lophogorgia chilensis*
ml	milliliter
mg	milligram
mM	millimolar
MVA	mevalonic acid
$NaHCO_3$	Sodium bicarbonate
NADPH	α-nicotiniamide adenine dinucleotide phosphate
N_2	nitrogen gas
nmol	nanomole
NP-HPLC	normal phase high performance liquid chromatography
M	molarity
PAGE	polyacrylimide gel electrophoresis
PA	*Pseudopterogorgia americana*
P. americana	*Pseudopterogorgia americana*
P. elisabethae	*Pseudopterogorgia elisabethae*
PE	*Pseudopterogorgia elisabethae*
pmol	picomole
Ps	pseudopterosins
PsA	pseudopterosin A
PsB	pseudopterosin B
PsC	pseudopterosin C
PsD	pseudopterosin D
ROS	reactive oxygen species
RP-HPLC	reverse phase high performance liquid chromatography
µl	microliter
µg	microgram
µM	micromolar
µmol	micromole

CHAPTER ONE

INTRODUCTION AND BACKGROUND

1. 1 MARINE NATURAL PRODUCTS

The search for novel pharmaceuticals has led to the exploration of new marine habitats and an extensive study of marine organisms. The ocean is a source of a vast number of organisms that produce biologically active and structurally unique compounds (Faulkner, 1984). Historically, marine natural products have been defined as secondary metabolites produced by marine organisms that are not necessary to sustain the life of the organism but do offer an adaptive advantage. Until recently, the possibility that many of these natural products have important physiological functions has been largely ignored, while their potential as new pharmaceutical agents has been of paramount importance (Fenical, 1987). Many marine natural products exhibit promising anti-viral, anti-tumor and anti-inflammatory properties. Among them, the pseudopterosins have received considerable attention from chemists and pharmacologists because of their potent anti-inflammatory activity and their growing commercial market.

1. 2 INTRODUCTION TO THE PSEUDOPTEROSINS

The pseudopterosins are D-xylose pentosides of a structurally rare class of *o*-catechol diterpenes (Fenical, 1987). The pseudopterosins were first isolated from *Pseudopterogorgia elisabethae*, a deep-water inhabitant of Bahamian reefs in the early 1980's. There are presently fifteen derivatives of the compounds that are named pseudopterosin A through O (Figure 1). They all contain the same diterpene aglycone but differ in their glycosidic moiety and glycosidic bond placement. Pseudopterosins A-D are the pseudopterosins found in *P. elisabethae* collected from the Bahamas, specifically Sweetigs Cay, Chub Cay and Crooked Island. The organic extract of these organisms contained 7.5% pseudopterosin C and less than 1% of each pseudopterosin A, B and D (Look and Fenical, 1986). Pseudopterosins E , F , G, H, I and J were isolated from *P. elisabethae* collected in Bermuda, pseudopterosin E and F made up the major portions of these extractions. Pseudopterosins K and L were the only pseudopterosins found in organisms obtained from sites in Great Abaco Island (Roussis and Fenical, 1990). Pseudopterosins M and N and O were isolated from a collection of *P.elisabethae* collected at 25 meters depth off of Long Key, Florida (Ata et al., 2003). This expanding group of structurally related diterpenoids has the potential of becoming a distinctive class of anti-inflammatory compounds with pharmacological potential that has not yet been uncovered.

In general, the pseudopterosins were isolated from organic extracts of the coral-symbiont complex using rapid-elution chromatography with TLC grade florisil,

and subsequently purified by high performance liquid chromatography (Look and Fenical, 1986). Results from high resolution mass and ^{13}C NMR spectrometry revealed the molecular formula of pseudopterosin C to be $C_{27}H_{38}O_7$, which indicated that the compound was highly unsaturated (Look and Fenical, 1986). The five methyl resonances on ^{1}H NMR suggested a diterpenoid structure. ^{13}C NMR revealed an acetal group and numerous hydroxyl groups, along with an extra 5-carbon unit, indicative of a pentose sugar complexed to the diterpene (Look and Fenical, 1986).

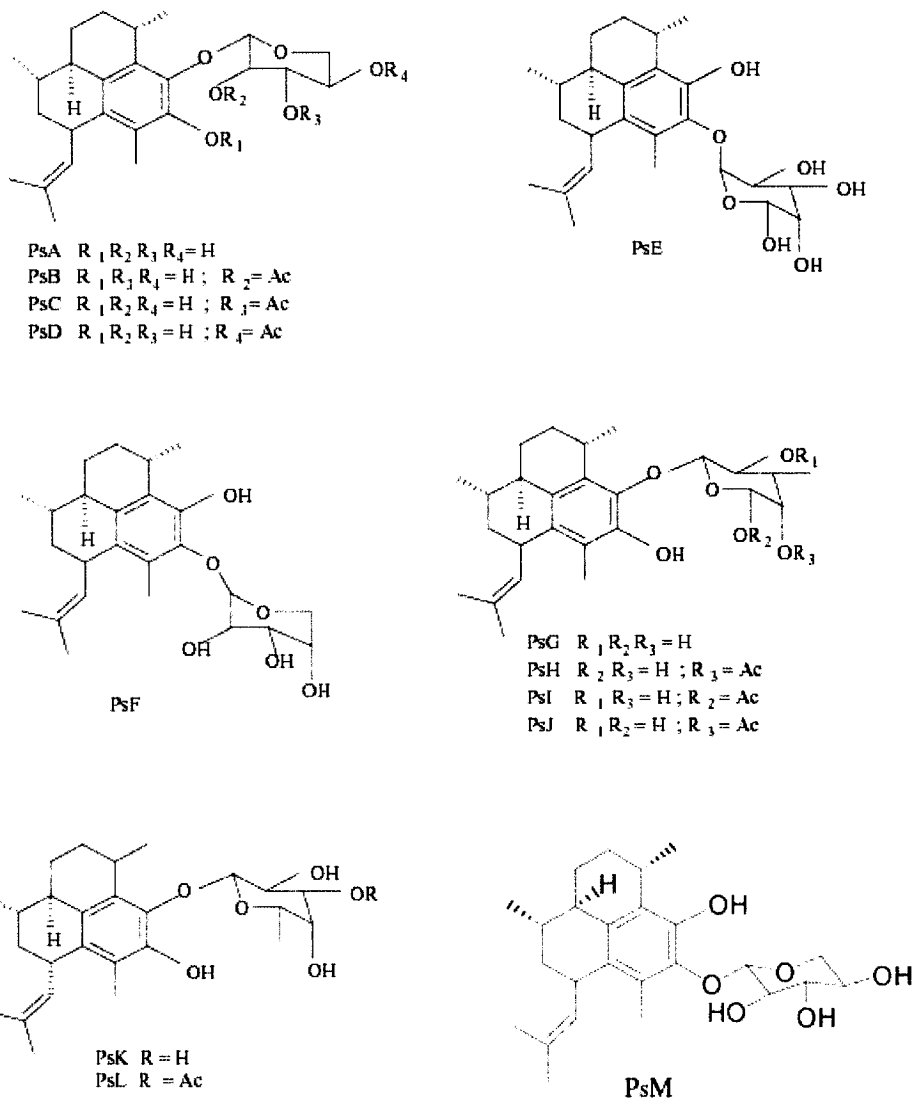


Figure 1: Structures of the pseudopterosins

1.3 PHARMACOLOGICAL BACKGROUND OF THE PSEUDOPTEROSINS

The pseudopterosins are compounds with potent anti-inflammatory and analgesic properties. Initial laboratory experiments showed the inhibition of sea urchin embryo cleavage by pseudopterosin A, the IC_{50} was 2.5 x 10^{-5}M. These effects were irreversible and concentration dependant (Ettouati and Jacobs, 1987). It inhibited 100% of first mitosis but showed cytotoxicity. Pseudopterosin A blocks mitosis in a time dependant and cell cycle dependant manner and must be in contact with the cell prior to prophase to perform its inhibitory action (Ettouati and Jacobs, 1987). Cells exposed to pseudopterosin A inhibited phenylanaline uptake and thymidine incorporation into DNA during S_2, therefore inhibiting S_2 phase DNA synthesis (Ettouati and Jacobs, 1987).

In anti-inflammatory assays pseudopterosins A and E have shown unique and unprecedented mechanisms of action, which still remain unresolved. The pseudopterosins were significantly more potent than the industry standard non-steroidal anti-inflammatory indomethacin in preventing phorbol myristate acetate (PMA) induced topical inflammation. As an analgesic Ps administered subcutaneously blocked phenyl-quinone writhing (ED_{50}=3.12 mg/kg). Ps treatment prevents delayed skin sensitivity responses to pro-inflammatory compounds in guinea pigs and in mice. Pseudopterosin A has been shown to inhibit pancreatic phospholipase A_2 (IC_{50} between 3.0 μM and 80 μM). Pseudopterosin E inhibited the synthesis of the pro-inflammatory leukotrienes and showed no toxicity in acute assays at levels higher than 300 mg/kg (Roussis et al., 1990).

Ps may mediate their anti-inflammatory effects by preferentially inhibiting eicosanoid release from neutrophils and other pro-inflammatory cells. Pseudopterosin A inhibited prostaglandin E and leukotriene C4 production in zymosan stimulated mouse macrophages (Mayer et al., 1998). The pseudopterosins were also inactive in inhibiting phospholipase A2, cyclooxygenase and cytokine release (Mayer et al., 1998). The pseudopterosins are currently being used in over 80 commercial cosmetic formulations as a cosmaceutical to inhibit retinol inflammation in face creams. The international the cosmetic company Estée Lauder uses them in their line of Resilience face creams. (Rouhi, 1995).

1.4 INTRODUCTION TO GORGONIAN CORALS

The phylum Cnideria contains a diverse group of aquatic organisms. The phylum can be divided into three classes. The classes Hydrozoa and Scyphozoa contain the hydrocorals, fire corals and various jellyfish. These organisms are carnivorous and use nematocysts or stinging cells to deter predators. Thus far, few structurally or pharmacologically interesting natural products have been found in this class (Faulkner, 1984). Class Anthozoa includes the subclasses Octocorallia that includes true soft corals and gorgonians, and Hexacorallia which includes the stoney and the black corals. The stoney corals do not produce any compounds of interest to natural product chemists presumably because of their calcium carbonate skeleton and built in means of protection (Faulkner, 1984). In contrast, much progress has been made in the chemistry of the octocorals because they are easy to collect and they contain a large amount of extractable material (Scheuer, 1978). The order

Alcyonacea is abundant in the Indo-Pacific and the order Gorgonacea is dominant on tropical-Atlantic reefs. Both corals are the major source of reef biomass and grow in large colonies in relatively shallow waters (Scheuer, 1978). It is therefore, not surprising that these soft corals have been the subject of much of the chemical investigation conducted on Cniderians.

Caribbean gorgonians make up 38% of known octocorals and there are over 195 species recorded (Fenical, 1987). Gorgonians include organisms commonly known as sea plumes, sea rods, flat sea whips and sea fans. Each has a central skeleton or axis that is surrounded by a gelatinous material, known as the rind. The polyps are contained in the rind and extend themselves through the apertures for feeding. The shape of the polyp apertures or calyces are taxonomically distinct, which aides in genus and species identification (Humann, 1993). Colonies of the gorgonian corals tend to branch out from a single stem that is attached to the substrate with a holdfast. The different branching patterns define different genera and species and can be planar three dimensional (Humann, 1993).

P. elisabethae is the gorgonian used in this study and contains the pseudopterosins The coral is deep purple in color and grows in short bushy colonies (Humann, 1993) (Figure 2). The colony is comprised of a main branch with many secondary branches extending pinnately in one plane. Some of the secondary branches grow into each other, creating attachments and overlaps of the branchlets. The polyps are arranged in rows and only present on one side of the branchlets and the calyces are indistinguishable, if not absent (Humann, 1993). *P. elisabethae* is

morphologically similar to other gorgonians that populate the reefs, therefore microscopic examination of the spicules or chemotyping of the lipid extract for presence of the pseudopterosins is necessary for positive identification (Look et al., 1986).

Figure 2: *P. elisabethae* from Sweetings Cay, Bahamas

1.5 NATURAL PRODUCTS AND DITERPENES FROM GORGONIAN CORALS

Early chemical investigations of the gorgonian corals in the late 1960's led to the finding of prostaglandins in *Plexaura homomalla*, a common Caribbean Sea rod (Weinheimer and Spraggins, 1969). Both 15-epi-prostaglandin A_2 (PGA_2) and its diester were discovered in large quantities in the dried coral. Structural analysis indicated that they were epimeric with the prostaglandin mammalian hormone at the allylic hydroxyl center (Weinheimer and Spraggins, 1969). The high yield of 15-epi-prostaglandin A_2 (PGA_2) and its diester was large benefit for research community, especially the exploration of their utility as a synthetic precursor to the mammalian prostaglandins (Fenical, 1987; Weinheimer and Spraggins, 1969).

The majority of natural products isolated from gorgonians are diterpenes (Rodriguez et al., 1995). In the past, the dominant diterpene compounds isolated from gorgonians species were of the cembranoid class. Cembranoid diterpenes contain a 14-membered carbocycle and are believed to originate from geranylgeranyl pyrophosphate (Rodriguez et al., 1995). Some cembranoids and cembrene derivatives exhibit anti-inflammatory and anti-tumor activities (Fenical, 1987). Diterpenes with the cembranoid structural backbone have been found in species of algae and plants (Shigamori et al., 2002; Ayaad et al., 2001) which may imply that the biosynthetic source of the diterpenes are algal in nature.

Diterpene glycosides are found in the gorgonian corals *Eunicea fusca* and *Pseudopterogorgia elisabethae* (Fenical, 1984). Until their detection the glycosides

characterized in marine organisms were generally steroids or triterpenes. The fuscosides are a group of diterpene glycosides from *Eunicea fusca*, which have fuscol as their diterpene skeleton (Shin and Fenical, 1990). Fuscoside A is bicyclic, while fuscoside B-D have fuscol aglycons. The compounds have been found to be selective inhibitors of leukotriene synthesis and neutrophil infiltration into phorbol myristate-acetate induced regions of inflammation (Jacobson and Jacobs, 1992). Effects of the fuscosides are irreversible and dependent on the concentration of cells/proteins. They were the first marine natural products shown to regulate the lipoxygenase pathway of arachidonic acid metabolism in mammalian models (Jacobs et al., 1993).

The abundance of diterpenes in gorgonian corals begs the question of their function and intracellular origin. A general hypothesis is that these compounds act as a predator defense mechanism (Fenical, 1987) since many of the gorgonian lipid extracts are distasteful and unpalatable to fish (Pawlik et al., 1987). Due to the large amounts of these compounds usually present within the coral- algal complex, it is believed that these compounds may act as secondary messengers or signaling molecules. The function of diterpenoids in the cell biology and ecology of gorgonians are largely unkown, in part because the intracellular biosynthetic origin and localization of the diterpenes have yet to be defined.

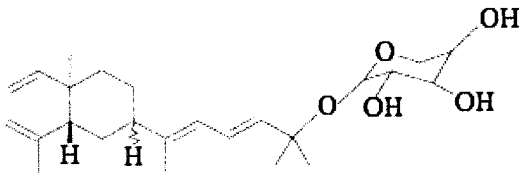


Figure 3: Structure of Fuscoside B

1.6 SYMBIOTIC DINOFLAGLELLATES

Dinoflagellate symbionts of the species *Symbiodinium* spp. are one of the most important algal and protist groups in the reef environment (Figure 4). They are the most common photosynthetic endosymbiont of tropical invertebrates and as intracellular symbionts they contribute to the survival and productivity of their hosts. *Symbiodinium* spp. may reach levels as high as 2 million cells/ cm^2 of host tissue, much higher than would naturally exist in the nutrient poor tropical waters. *Symbiodinium* spp. are classified by clades, which differ in a genetic marker in the ITS 2 region (LaJeunesse, 2001; 2002). There are currently 5 clades, named A,B,C,D and E, although with current phylogenetic studies these clades are increasing in number and subunits. These symbiotic dinoflagellates are photosynthetic, e.g. they synthesize new organic carbon from the uptake of inorganic carbon in the form of carbon dioxide and/or bicarbonate from their surroundings. The organic matter they synthesize is translocated to the animal host as glycerol, glycolate, lactate and succinate making them major primary producers in reef habitats (Gates et al., 1999).

In sea anemones, a mixture of free amino acids termed the "host factor" increases the amount of newly fixed carbon translocated to the host (Gates et al., 1999).

Gorgonian corals contain dinoflagellate symbionts that are typically of the B1 type sub-clade (LaJeunesse, 2002). There has been some debate in the literature regarding the general contributions of symbiont and host to diterpene biosynthesis and to this date the involvement of symbionts in terpene production remains unresolved (Kokke et al., 1984; Cieresko, 1989; Michalek-Wagner et al., 2001; Papastephanou and Anderson, 1982).

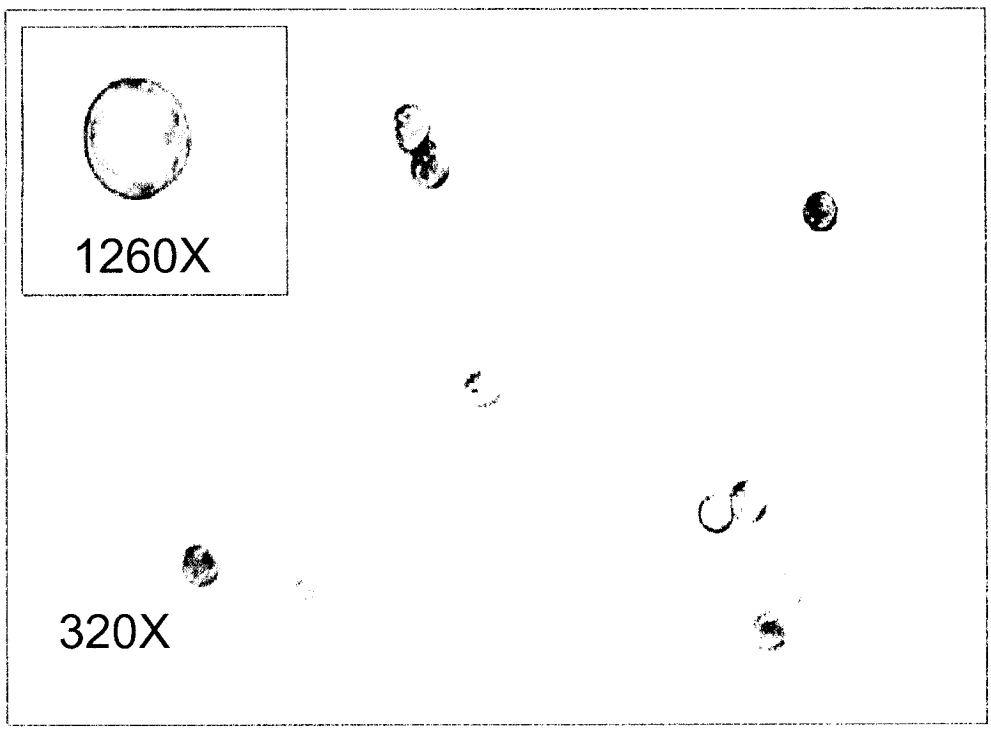


Figure 4: Symbiotic dinoflagellates under light microscopy

1.7 PSEUDOPTEROSIN RESORCE PROBLEMS AND SOLUTIONS

Broader applications for the therapeutic use of the pseudopterosins have been supply limited, even though field harvesting has reached the tonnage level in recent years (Fenical, 1997). The rate of coral replacement does not match the rate of coral harvesting and ecological and environmental concerns for the sensitive reef habitats in the Caribbean have initiated numerous efforts aimed at the development of viable production methods for sustainable pseudopterosin supplies (Broka et al., 1988; Coleman et al., 1999). These production methods range from pure *in vitro* synthetic production to *in vivo* biosynthesis methods rather than by 'farming' the reefs (Broka et al., 1988; Coleman et al., 1999).

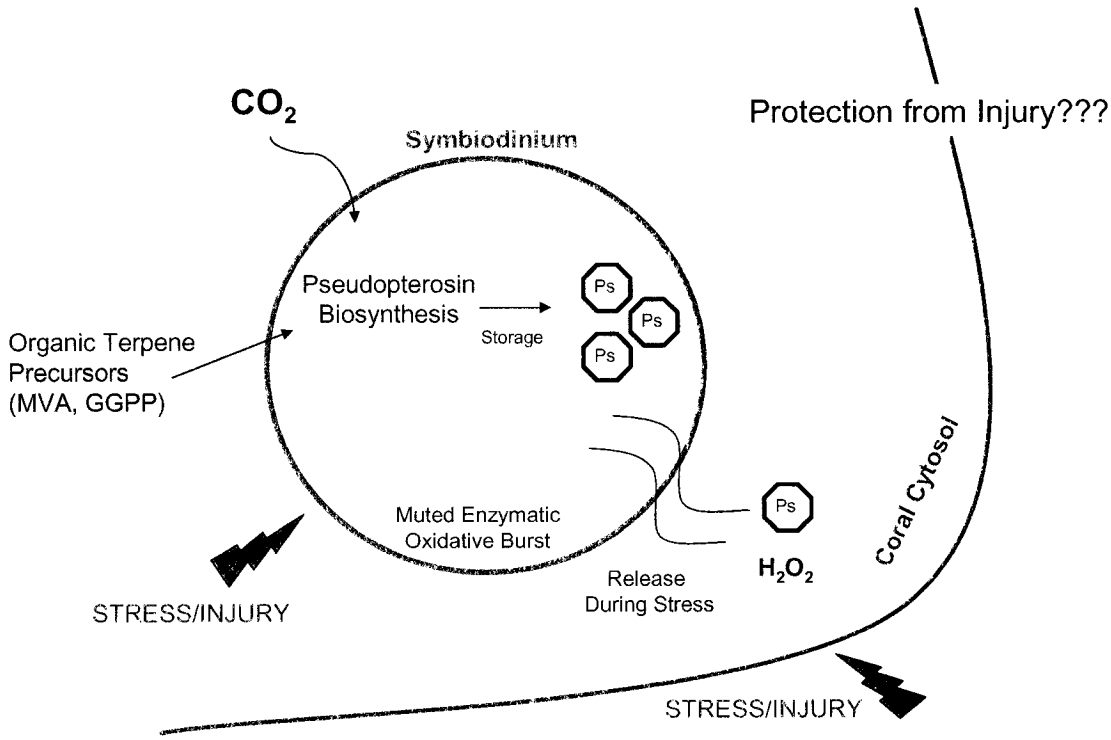
Total organic synthesis of the pseudopterosins has not yet proven to be efficient or cost effective, in part because the stereochemical integrity of the compound is difficult to conserve (Buszek and Bixby, 1995; Broka et al., 1988). Mariculture of has proven difficult as the corals are sensitive to environmental changes and their growth requirements are not well known. Biosynthetic studies of the compound are promising as they can produce the compound through enzymatic methods. Biosynthetic studies of the compound can lead to the elucidation of its metabolic origin as well as identifying the primary building blocks necessary for the development of a viable chemoenzymatic synthesis (Coleman and Kerr, 2000). Experiments with crude enzyme preparations and purified enzymes can more completely define the pathway by which the organism produces the metabolite, and

can eventually lead to the isolation of the enzyme(s) responsible for pseudopterosin production (Kohl et al., 2003). Through subsequent recombinant DNA techniques the protein can be expressed in bacteria or yeast, this can eventually lead to a large-scale production of the compound in fermentation tanks. The end result of this sequence may provide a suitable and practical solution to the supply problem of many marine natural products, especially the pseudopterosins. In order for the chemoenzymatic synthesis to be practical and comprehensive, the origins of pseudopterosin biosynthesis within *P. elisabethae* must be elucidated from within the symbiotic complex. This could lead to another feasible solution to the supply issue which is the culturing of symbiotic dinoflagellates, cyanobacteria or bacteria which may actually be the biosynthetic source of the compounds.

1.8 PROJECT GOALS

The overall goals of this project are to examine the origin of pseudopterosin biosynthesis in the algal-host relationship of *Pseudopterogorgia elisabethae,* to elucidate physiological aspects of the biosynthesis and to examine the physiological role of the compounds in the cell biology and signaling pathways of the symbionts and in the symbiotic-host association. This study was the first confirmed report of pseudopterosin biosynthesis in dinoflagellate symbionts of *P. elisabethae* (Chapter 2). Carbon dioxide was fixed through photosynthesis and determined to be a precursor for the pseudopterosins. Also, the biosynthetic rate of pseudopterosin biosynthesis was found to be slow, while the compounds are stored in large amounts within the algal cell (Chapter 3).

To examine the physiological role of the pseudopterosins an approach was developed that used oxidative stress as an index of changes in the physiological state of the symbiotic cells and corals. It was discovered that both symbionts and corals were capable of producing an enzymatic oxidative burst (Chapter 4 and 5). In comparative studies with similar coral and symbiont species, the oxidative burst in *P. elisabethae* whole coral and cells was muted when compared to the other species tested. These results led us to deduce that the pseudopterosins may be imparting a protective feature to *P. elisabethae* and further experiments were designed to prove or disprove this theory. Scheme 1 is a graphical model representation of some of the concepts discussed in this dissertation.



Scheme 1: Working model for the localization, biosynthesis and cellular function of the pseudopterosins (Ps) in the algal-host relationship of the gorgonian coral *P. elisabethae* and its dinoflagellate symbiont (see text for more details).

CHAPTER TWO

PSEUDOPTEROSIN BIOSYNTHESIS IN DINOFLAGELLATE SYMBIONTS OF *P. ELISABETHAE* FROM TERPENE PRECURSORS

2.1 RATIONALE FOR SYMBIODINIUM AS SOURCE OF PSEUDOPTEROSIN BIOSYNTHESIS

The role of symbiotic dinoflagellates in the production of marine natural products, especially terpenoids and sterols has been disputed without resolution (Cieresko, 1989). We hypothesized that the symbiont known as *Symbiodinium* sp. was the source of the pseudopterosins being harvested from gorgonian corals for a number of reasons which pertain to the physiology of dinoflagellate symbionts and the biosynthesis of terpenoid compounds.

First, symbiotic dinoflagellates are major primary producers of the tropical reef system, they contribute to the growth and maintenance of reef building corals, soft corals, anemones and sponges in these nutrient deplete areas. They are source of all newly synthesized organic matter in the algal-host relationship while carnivorous grazing would supply organic matter in the same form found in the plankton. They utilize the dissolved inorganic nutrients supplied from the host which are then

recycled within the symbiotic association (Muscatine, 1989). The symbionts are extremely metabolically active whereas their invertebrate hosts have a much lower metabolism which makes it unlikely that they would be able to produce terpenoid natural products which are energetically costly and involve complex and distinct enzymatic systems.

Second, the concentrations of natural products from marine invertebrates with dinoflagellate symbionts are unusually high and can vary widely within a single coral sampled from different tropical locations. The patchy distribution of diterpenoids within a single invertebrate species can be attributed to the difference in symbiont composition. *Symbiodinium* as a species have been genetically divided into clades and subspecies that may occur in one location but absent from another. In the case of the pseudopterosins, corals collected from different location in the Caribbean contain differing concentrations of pseudopterosins as well as pseudopterosins that are structurally related (see section 1.2) (Roussis et al.,1990). Some octocorals that lack symbionts do contain diterpenoids, but in much lower concentrations than in corals that have symbionts. One explanation to this may be that the asymbiotic corals are getting the small amounts of terpenoids from their diets (Ciereszko, 1989). Alternatively, one might suggest that the presence of symbionts somehow stimulates diterpene biosynthesis in the host coral while producing little or none itself.

Third, terpenoid synthesases are a highly specific class of enzymes that regulate and catalyze the first committed steps leading to various terpene class structures. Diterpenoids are widespread in plants, with over 3000 structures

identified (Bohlmann et al., 1998) and are copious in brown algae (Culioli et al., 1999; Soto et al., 2003; Soares et al., 2003). The biosynthetic precursor in most of these cases appears to be geranylgeranyl bisphosphate. The most familiar acyclic ditepene is the phytol side chain of chlorophyll. To date two types of cyclic diterpene structures are recognized the macrocyclic diterpene, such as cembrene, and the tricyclic or tetracyclic diterpenoid such as kaurene, which involves the generation of the intermediate coplyl bisphosphate (Bohlmann et al., 1998). Both diterpenoid structural types have been isolated from plants and marine invertebrates. Cembrenoid diterpenes are ubiquitous diterpenoids isolated from a variety of soft corals (Gray et al., 2000; Wei and Rodriguez , 2003; Rodriguez et al., 1995) this structural class has also been found in plant species such *Eremophila* and *Echinodorus* (Ghilsaberti et al., 1994; Shigemori et al., 2002). The tricyclic carbon backbone of the pseudopterosins has also been found in the Australian resin-producing higher plant, *Eremophila serrulata* (Ghilsaberti, 1992). This compound, a tricyclic diterpene phenolic acid was shown to possess a carbon skeleton of the C-3 epimer of pseudopterosin K (Roussis et al., 1990). The similarity in plant, algal and gorgonian diterpenoid structures is hard to ignore and leads to a possible conclusion that photosynthetic organisms have evolved these biosynthetic pathways over time.

2.2 PSEUDOPTEROSIN AND TERPENOID BIOSYNTHESIS

In order to begin biosynthetic studies of the pseudopterosins, the biosynthetic pathway by which the compounds are produced was examined. This hypothetical retrosynthetic assessment can aid in choosing precursors to use in biosynthetic

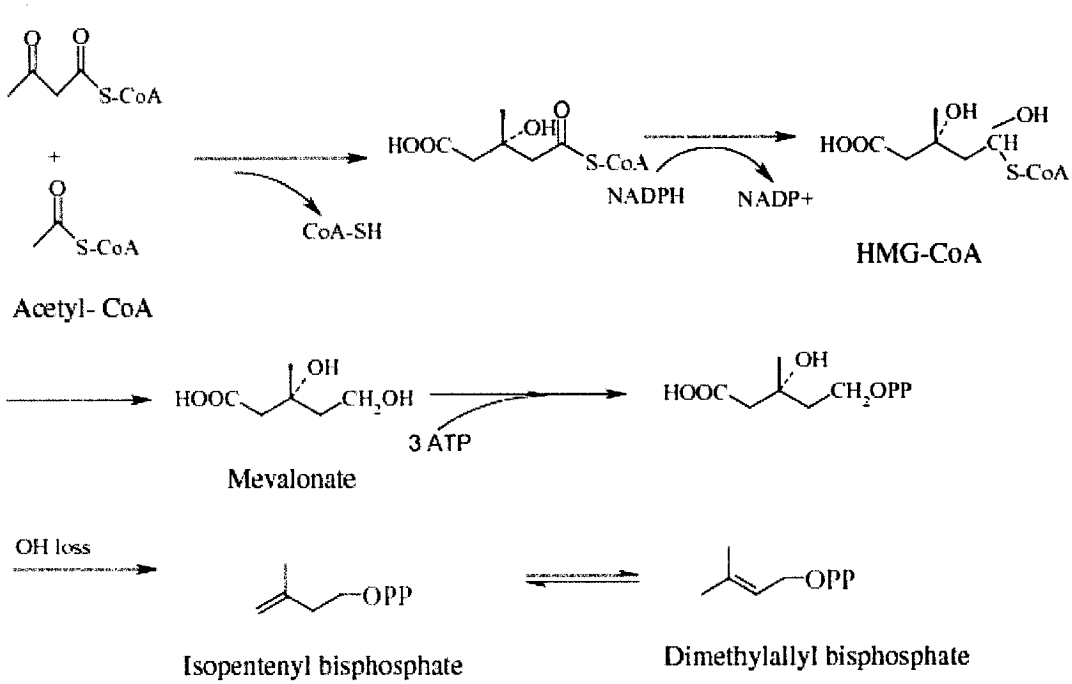
feeding experiments and provide useful information on the relationship of the pathway to known biosynthetic pathways. Upon structural examination of the pseudopterosins, two biosynthetically distinct moieties are evident; the tricyclic terpene and the sugar moiety.

The methyl groups on the rings indicate a diterpene origin and the biosynthesisis hypothesized to follow standard terpene chemistry. If so, then mevalonic acid is formed from the conversion of β- hydroxy-β-methylglutaryl-CoA (HMG-CoA) and subsequently undergoes phosphorylation and loss of water by ATP to form isopentenyl pyrophosphate (Herbert, 1989). This 5-carbon unit is isomerized to dimethylallyl pyrophosphate, which facilitates its attack on another isopentenyl pyrophosphate (Richards and Hendrickson, 1964). This interaction results in the formation of the monoterpene precursor geranyl pyrophosphate, which can continue its attack on isopentenyl pyrophosphate to produce the sesquiterpene farnesyl pyrophosphate. Farnesyl pyrophosphate becomes homologated again to geranylgeranyl pyrophosphate (GGPP), the diterpene precursor of the pseudopterosins. This pathway is illustrated in scheme 2.

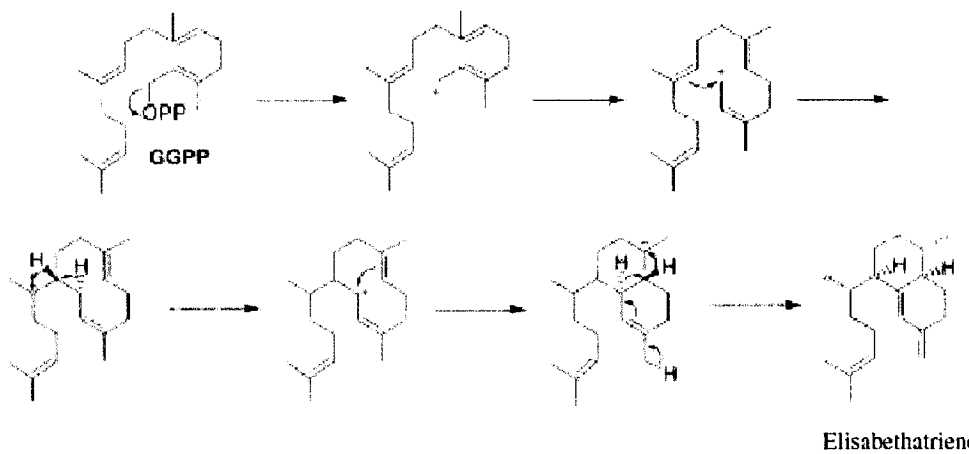
GGPP is the substrate for several carbocation rearrangements typical of terpene chemistry to produce a bicyclic intermediate that has been isolated using radio-labeled biosynthetic techniques (Coleman and Kerr, 2000). The diterpene cyclase product was isolated as a bicyclic hydrocarbon called elisabethatriene and is believed to be the only diterpene cyclase product present in enzyme extracts of the coral-algal complex (Scheme 3). Diterpene cyclases are the putative enzymes in

terpenoid biosynthesis and often can cyclize a unique chemical structure in one step. Very little homology exists between known terpene cyclases and typically different diterpene cyclases or transferases have 6-15% sequence identity (Kohl et al., 2003; Rynkiewicz et al., 2001).

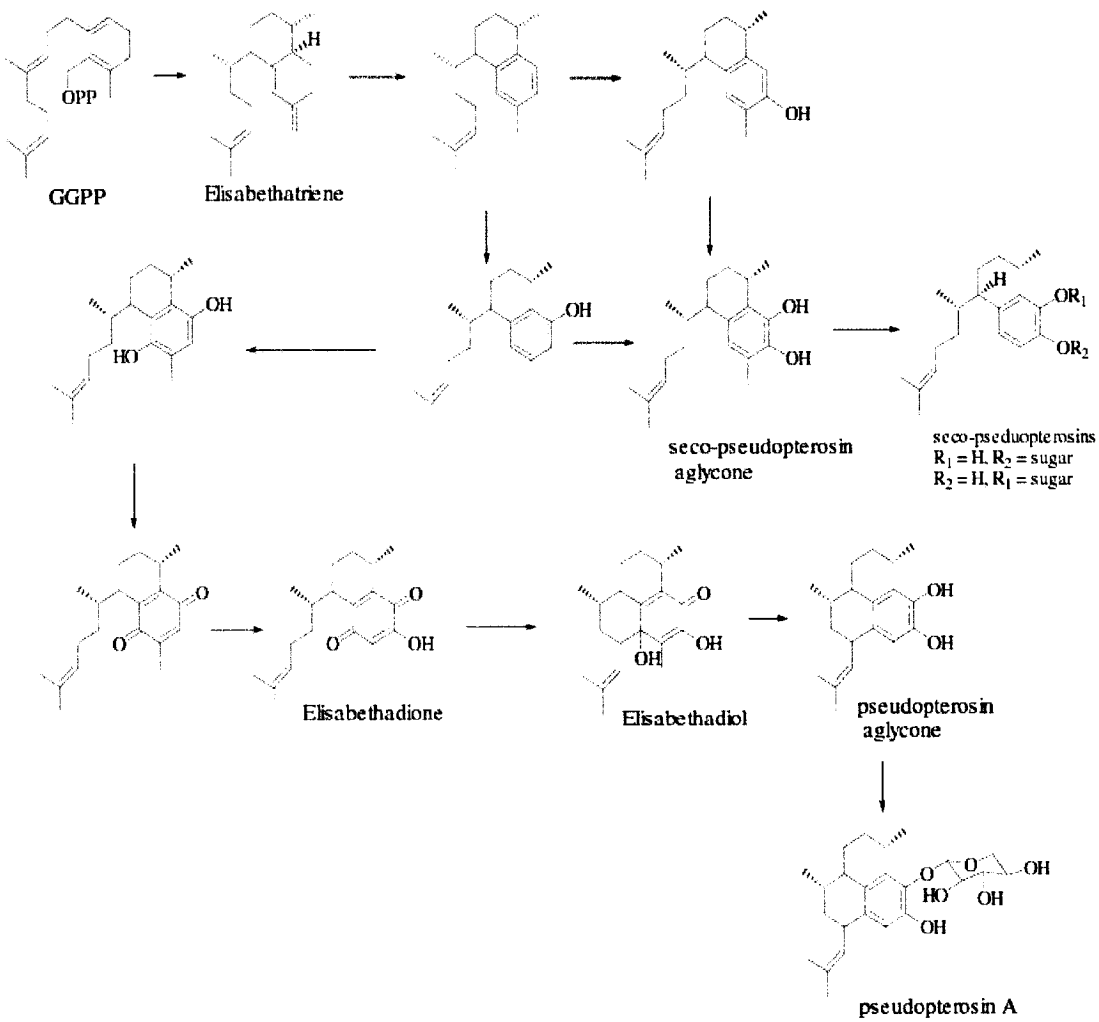
Scheme 3 illustrates how elisabethatriene undergoes several oxidation reactions to produce the seco-pseudopterosins which are bicyclic. The seco-pseudopterosin carbon structure then goes through cyclization and oxidation reactions as well as hydride shifts to form the diterpene aglycone as a diol. The alcohol then produces a glycosidic bond using a glycosidase or glycosyl transferase with the appropriate sugar moiety (Kohl et al., 2003). Earlier studies (Coleman et al., 1999) have demonstrated that pseudopterosin A is the precursor to pseudoptersosin B, C and D.



Scheme 2: Biosynthesis of isopentenyl bisphosphate, the five carbon precursor for terpene biosynthesis



Scheme 3: Suggested transformation of GGPP to elisabethatriene through carbocation rearrangements



Scheme 4: Intermediates of seco-pseudopterosin and pseudopterosin biosynthesis

2.3 RESULTS AND DISCUSSION

The study of pseudopterosin biosynthesis in *Symbiodinium* sp. from *P. elisabethae* began with the positive chemical identification of putative molecules in the pseudopterosin biosynthetic pathway in the purified symbiont cells. The probing of the terpenoid pathway in *Symbiodinium* sp. from *P. elisabethae* using radiolabeled terpene precursors was also examined.

2.3.1 *Symbiodinium* purity and species identification

The *Symbiodinium* sp. collected from *P. elisabethae* at Sweetings Cay, Bahamas was identified as the common type "B1", a symbiont also found in numerous other gorgonians and corals from the Caribbean (LaJeunesse, 2001). The ITS 2 sequence of "B1" is identical to the informally described species *Symbiodinium burmudense.*

Symbiodinium sp. cells purified by repeated washing and Percoll® gradients showed no visible impurities as determined by light microscopy. Cell viability was confirmed using trypan blue incorporation. Furthermore, DNA labeling by DAPI staining failed to detect any bacterial or coral cell contaminants (Figure 5). These findings indicate that our preparation of *Symbiodinium* sp. cells achieved a high level of purity.

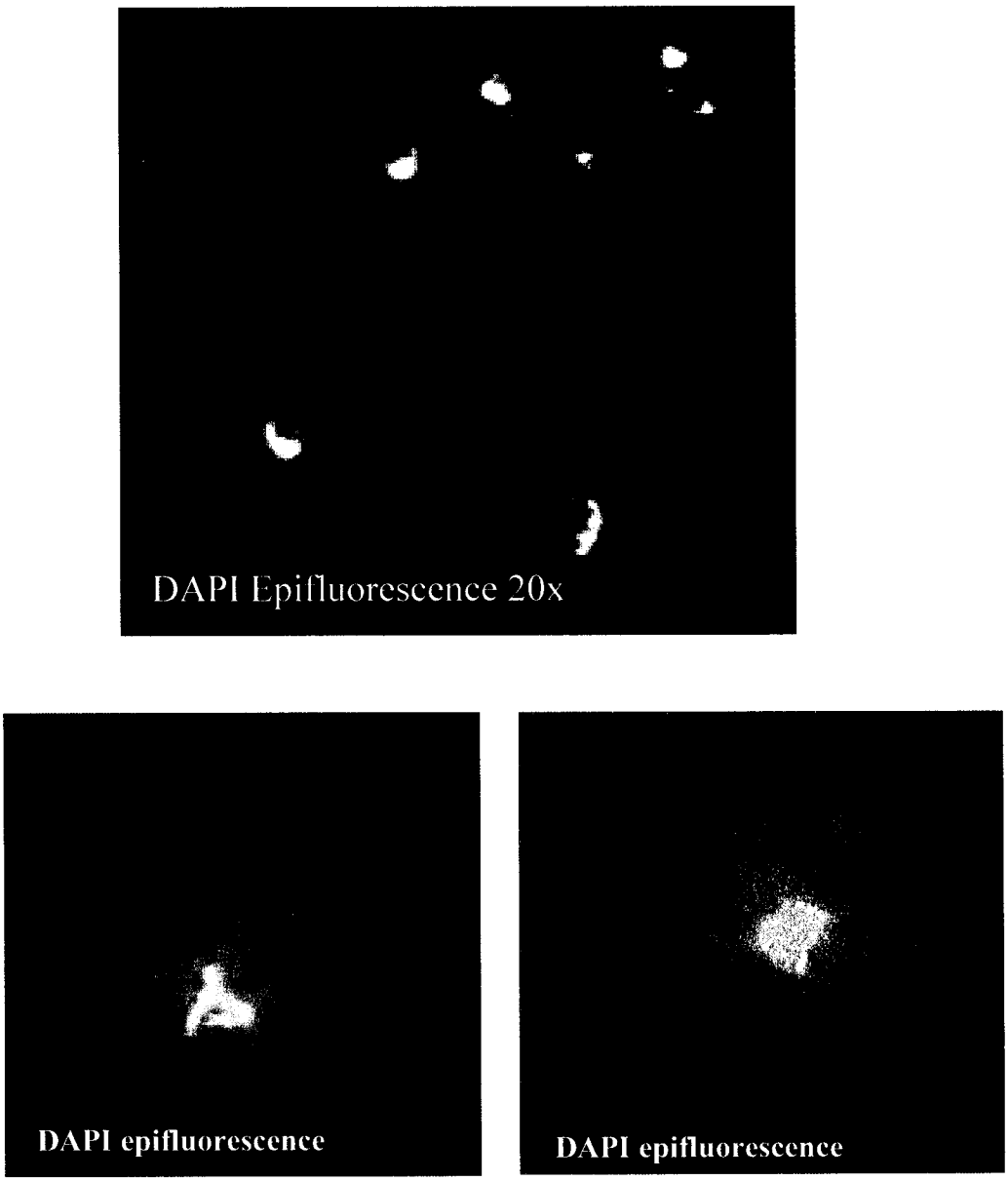


Figure 5: DAPI epifluorescence photos of *Symbiodinium* sp. from *P. elisabethae*

2.3.2 Presence of endogenous elisabethatriene in *Symbiodinium* sp.

In previous studies, elisabethatriene was identified as the diterpene cyclase product, and thus the first committed intermediate of pseudopterosin biosynthesis (Coleman and Kerr, 2000). In the current study, endogenous elisabethatriene was detected in a hexane extract of purified *Symbiodinium* sp. cells by RP-HPLC. Levels as high as 0.113μg/10^6 cells (0.034% of lipid extract) were observed. The presence of elisabethatriene in this extract was confirmed by ^{1}H-NMR. ^{1}H-NMR assignments for elisabethatriene were previously published (Coleman and Kerr, 2000).

2.3.3 Presence of endogenous pseudopterosins in *Symbiodinium* sp.

Endogenous levels of pseudopterosins A, B, C and D in purified *Symbiodinium* sp. were quantified from lipid extracts using NP-HPLC (Figure 6). Compounds were identified by comparison of retention times with those of standards and confirmed by co-injection with authentic standards. The pseudopterosin content was quantified by using previously established standard and calibration curves. The concentration of the mixture of pseudopterosin A through D in the purified algal cells was found to be 19 ± 4 μg/10^6 cells or 1 - 4 x10^{-2} pmol Ps/cell.

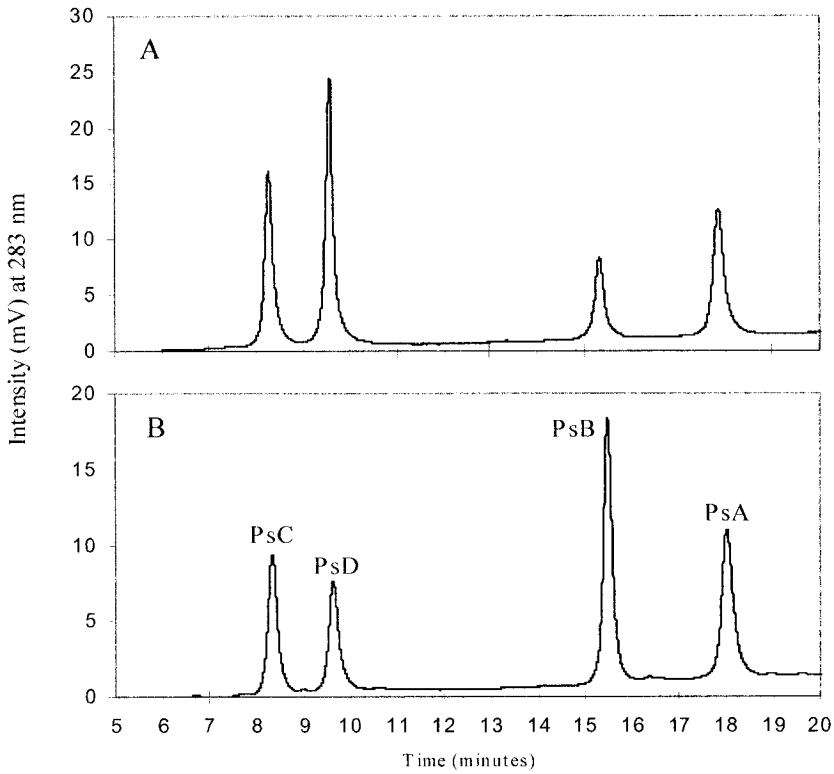


Figure 6: NP-HPLC Chromatogram of A) Pseudopterosins isolated from *Symbiodinium* sp. and B) Authentic standards

We have observed that the concentration of pseudopterosins and other biosynthetic intermediates varies between individual *P. elisabethae*. An analysis of a clipping from an individual specimen of *P. elisabethae* revealed that the pseudopterosins comprised 5% of the lipid extract, whereas an analysis of purified *Symbiodinium* sp. cells from the same individual indicated that these compounds comprised 11% of the algal lipid extract.

Cell lysates of both pure symbiont tissues and the entire coral-algal complex tissue were prepared for biosynthetic experiments. Protein concentrations of each enzyme extract were measured using the Bradford protein assay. The lipids of each cell free extracts were extracted using chloroform and the concentrations of pseudopterosins in extract measured using NP-HPLC. The coral extract contained 5.07×10^{-6} mmol Ps/ mg protein while the symbiodinium extract contained 2.56×10^{-5} mmol Ps/ mg protein (Figure 7). These data indicate that the ratio of protein to metabolites is higher in the *Symbiodinium* sp. cells and that they are the likely source of production. From this increased concentration of pseudopterosins in both the lipid and enzyme extracts of the symbiont we can deduce that the compounds are diluted in the entire coral-symbiont complex and that the production is contained within the symbionts.

The analytical work described above clearly indicates that pseudopterosins are present in the *Symbiodinium* sp. cells, in fact, at a much higher concentration than in the coral tissue. Further, the presence of elisabethatriene in the *Symbiodinium* sp. extract suggests that this dinoflagellate is the biosynthetic source of these potent anti-

inflammatory agents. To confirm the algal origin of pseudopterosin biosynthesis, a number of biosynthetic experiments were conducted.

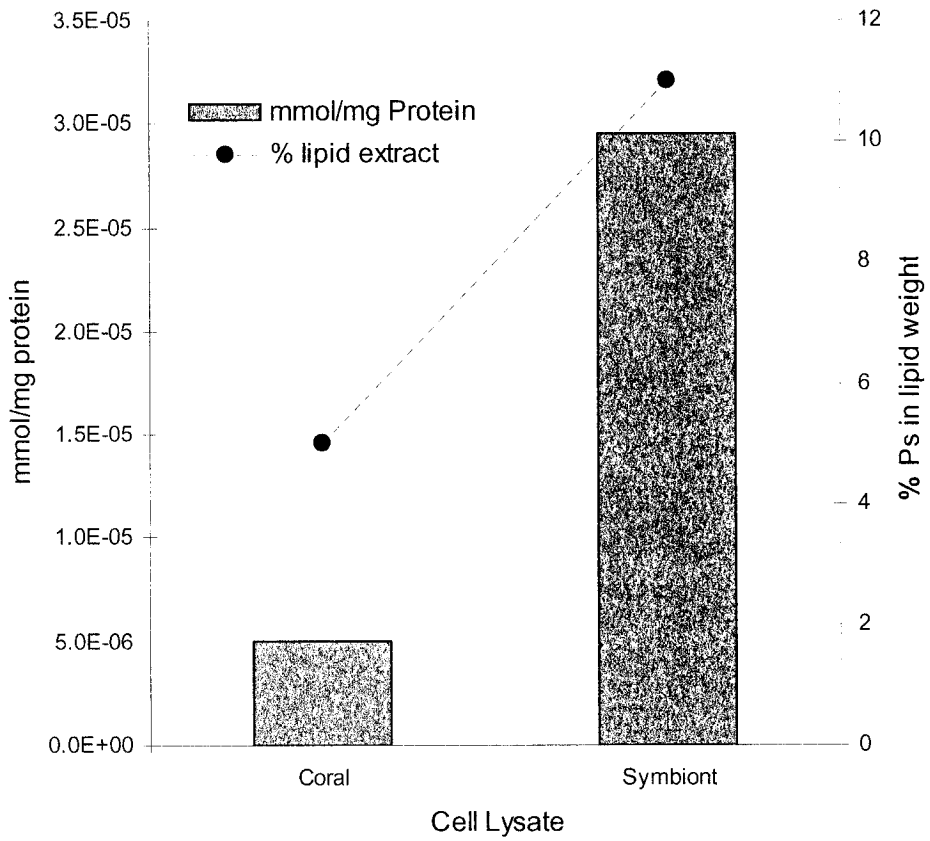


Figure 7: Comparison of pseudopterosin content in coral and symbiont extracts.

2.3.4 Biosynthesis of pseudopterosins from terpene precursors

The capability of purified *Symbiodinium* sp. cells to metabolize terpene precursors was tested using the five carbon mevalonic acid and the 20 carbon geranylgeranyl bispohosphate. 10 uCi of mevalonic acid was added to 5 ml of cell lysate prepared with a phosphate buffer (pH 7.7) and 5mM DTT, the mixture was incubated for 24 hours. Following extraction and purification by NP-HPLC, the pseudopterosins were found to be radioactive (2.4 x 10^3dpm and specific activity of 2.44 x 10^8 dpm/mmol). This represented a yield of only 0.011% of the biosynthetic transformation of mevalonic acid to the pseudopterosins. This meager incorporation can be explained by the fact that a small molecule such as the five carbon mevalonic acid will get diluted in the precursor pool of endogenous mevalonic acid and enter many primary and secondary biosynthetic pathways in the cell. Therefore an early precursor in the desired biosynthetic pathway is not ideally suited for these types of biosynthetic experiments. For this purpose a precursor later in the terpenoid pathway (20 carbons) was chosen.

Geranylgeranyl bisphoshate (GGPP) was tested in two systems: an *in vivo* system using intact cells and an *in vitro* system using a cell lysate. In the *in vivo* system, purified cells in filtered seawater were incubated with (0.72 μCi) ^{3}H-GGPP for 48 hours. Purification of the pseudopterosins by NP- HPLC, and analysis by scintillation counting, indicated that pseudopterosins A-D collectively were radioactive (3.7 x10^3 dpm, 5.4 x 10^7dpm/mmol). This represented a yield of only 0.23 % for the biosynthetic transformation of GGPP to the pseudopterosins

suggesting that GGPP transport into intact cells may not be optimal. Elisabethatriene was also found to be radioactive (640 dpm). The transport of precursors into lipid pools is limited when the lipid is sequestered in subcellular compartments, and for this reason cell lysates were developed to increase substrate bioavailability.

In the *in vitro* experiment, a cell lysate was prepared with a phosphate buffer (pH 7.7) was incubated with (5 μCi) ^{3}H-GGPP and 5mM DTT for 24 hours. Following extraction and purification by NP-HPLC, the pseudopterosins were found to be radioactive (6.8 x 10^{3} dpm and specific activity of 4.62 x 10^{9} dpm/mmol). In this experiment a cell lysate of partially purified coral cells was also prepared and incubated with radiolabeled GGPP under the same conditions. When comparing the specific activity of the radiolabeled pseudopterosins (combined with elisabethatriene radioactivity) for the symbiont and semi-pure coral cell lysates, the symbiont has greater specific activity (Figure 8). This indicates that there was a higher ratio of protein to biosynthetically produced pseudopterosins in the symbiont and that more molecules of GGPP/mg protein were channeled into the pseudopterosin synthesis pathway. The radioactivity in the coral extract indicates some pseudopterosin biosynthetic activity, this can be attributed to the initial preparation of the coral lysate where the entire coral symbiont- complex is homogenized in a blender prior to cell separation. It is extremely likely that *Symbiodinium* cells were lysed in this process and their cell contents ended up in the coral cellular fraction.

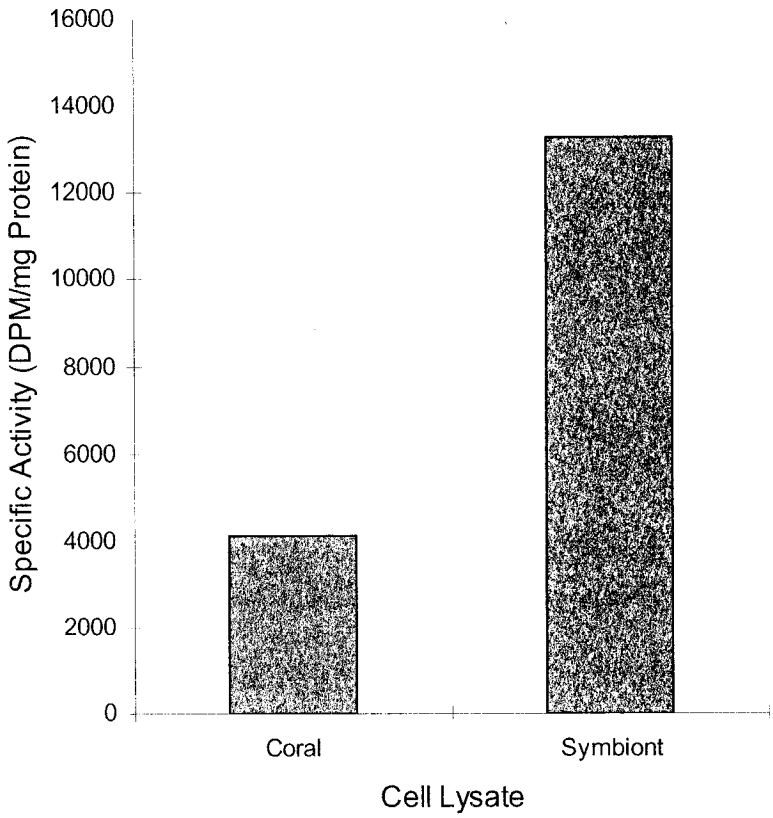


Figure 8: Comparison of radioactivity of Ps metabolites produced from coral and symbiont cell lysates. Normalized to mg protein determined by Bradford protein assay.

Another cell lysate was prepared with different buffer conditions to optimize the incubations and achieve better radioactive counts and increase substrate availability. The pure symbiont cells were homogenized with 20 mM TRIS-HCl buffer (Ph 7.7) and incubated with (5 μCi) ^{3}H-GGPP, $MgCl_2$ and 1% Triton X-100 for 24 hours. Triton X-100 was added to help solubilize GGPP and to optimize substrate micelle concentration. Both substrate availability and yield of the pseudopterosins were increased by 10 fold in the presence of Triton X-100. Following extraction and purification by HPLC, the pseudopterosins were found to be radioactive (6.9×10^4 dpm, specific activity of 4.23×10^9 dpm/mmol).

This represents a yield of 0.62% for the conversion of GGPP into the pseudopterosins, a marked increase from the intact cell yields and the cell lysate prepared with phosphate buffer. In a 45 minute incubation, elisabethatriene was also found to be radioactive (1,016 dpm). The pseudopterosins and elisabethatriene were not radiolabeled when the cell free extract was boiled for 1 hour prior to incubation. A summary of all the terpenoid precursor radiolabeled experiments is presented in Table 1.

Precursor	Radioactivity	Cell Preparation	Ps radioactivity (DPM)	% Incorporation
MVA	10uCi	Lysate Phosphate Buffer	2400	0.011%
GGPP	5uCi	Lysate Phosphate Buffer	6800	0.06%
GGPP	5uCi	Lysate Tris Buffer	69000	0.62%
GGPP	0.72uCi	Live	3700	0.23%

Table 1: Summary of results from labeled terpene precursor studies

2.4 GENERAL CONCLUSIONS

These results provide the first direct evidence of the presence of a pseudopterosin biosynthetic pathway in *Symbiodinium* sp. cells. The chemical analysis of the purified *Symbiodinium* sp. from the gorgonian *P. elisabethae* indicated a high level of pseudopterosins (11% of lipid content) and the presence of the diterpene cyclase product, elisabethatriene. Pseudopterosin biosynthesis in the symbiont cell also utilizes organic diterpene precursors as shown in the biosynthetic experiments with tritium labeled GGPP. Under our experimental conditions, this synthesis may follow the mevalonic acid pathway as suggested by the incorporations of mevalonic acid into pseudopterosin biosynthesis.

2.5 MATERIALS AND METHODS

P. elisabethae was collected in May 2001 at Sweetings Cay in the Bahamas at a depth of 10 m. All trans geranylgeranyl diphosphate [1-^{3}H] triammonium salt (60 Ci/mmol) and DL- Mevalonic acid [2-^{14}C] DBED salt (55mCi/mol) were purchased from American Radiolabeled Chemicals, St. Louis, MO. 0.22 μm Millipore Steritop filters were purchased from Fisher Scientific. Percoll® and Triton® X-100 were purchased from Sigma-Aldrich Chemicals. DNeasy plant mini prep kit was purchased from Qiagen Corporation, Santa Clarita, CA.

HPLC purifications were performed using either a Perkin Elmer Series 400 pump connected to a PE 235 diode array detector with Vydac C18 or silica columns, or a Hitachi L-6200A Intelligent Pump connected to a L-4200 UV-Vis detector with Varian Chrompack C18 or silica columns. ^{1}H-NMR spectra were recorded in C_6D_6 on an Inova Varian 500 spectrometer at 500 MHz.

Isolation and purification of *Symbiodinium* sp. from *P. elisabethae*

Flash frozen or live coral were homogenized in a blender with 0.22μm filtered seawater and 10mM EDTA, and filtered through 4 layers of cheesecloth. Algal symbionts were pelleted out by centrifugation at 250 x g and subsequently washed 10 times with 40 ml clean filtered seawater and pelleted by centrifugation at 750 x g. *Symbiodinium* sp. cells were further purified on Percoll® step gradient of 20%, 40%, and 80% two or more times until <1% impurities were seen using light microscopy. Cell viability was confirmed using trypan blue incorporation, examined

under light microscopy. DNA staining using DAPI detected on epifluorescence microscopy was used to detect contaminants due to bacterial or coral cells. Cells isolated from live coral were diluted to final concentration of $4x10^5$ or $2x10^6$ cells/ml using a hemocytometer and maintained in filtered seawater or used.

DNA from purified symbionts was extracted using the DNeasy plant mini prep kit. As described by LaJeunesse (2001), denaturing gradient gel electrophoresis (DGGE) was then used to analyze the internal transcribed spacer 2 (ITS 2) sequences to identify the symbiont type occurring in the samples of *P. elisabethae*.

Extraction of endogenous pseudopterosins and elisabethatriene from *Symbiodinium* sp.

Purified *Symbiodinium* sp. cells were lyophilized and extracted with ethyl acetate and filtered through a 1 cm silica column. Pseudopterosins A through D were purified by normal phase HPLC with a hexane/ethyl acetate gradient (60:40 to 100% ethyl acetate in 30 minutes) using UV detection at 283 nm. Peak areas corresponding to the pseudopterosins were used to quantify amounts. Elisabethatriene was extracted from lyophilized cells using hexanes and purified on reverse phase HPLC (isocratic in methanol) at 240 nm. NMR conditions are published elsewhere (Coleman and Kerr, 2000).

Cell lysate preparation

Purified cells were re-suspended in chilled 0.02 M Tris buffer with 3mM EDTA, 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol and lysed in a French press at

1200 psi followed by addition of 1% Triton-X. The homogenate was centrifuged at 1000 x g for 10 minutes. Protein concentration was determined by Bradford assay.

In Vitro and _In Vivo_ incubation with terpenoid radioactive substrates and radioactive measurements

Cell lysates were incubated with 5 μCi GGPP or 10 μCi MVA for 24 hours at 29° C at 200 rpm. 12 ml of live cells diluted to 2 $x10^6$ cells/ml were incubated with 0.72 μCi GGPP for 48 hours at room temperature under constant artificial light source. Cell lysates and intact cells were extracted using HPLC grade chloroform and ethyl acetate. Crude extracts were partitioned between methanol/water (9:1) and hexanes, followed by partitioning between methanol/water (1:1) and chloroform. Radiochemical purity of all compounds was ensured by purification two times through HPLC. During the second HPLC run, fractions were collected at minute intervals and counted in a LKB Wallac 1219 Rackbeta liquid scintillation counter. Efficiency for ^{14}C and ^{3}H was checked using internal standards. Radioactivity (in dpm) includes subtraction of background and was, at a minimum, double background levels.

CHAPTER THREE

PSEUDOPTEROSIN BIOSYNTHESIS IN DINOFLAGELLATE SYMBIONTS FROM *P. ELISABETHAE* FROM INORGANIC CARBON

3.1 INTRODUCTION

Symbiotic dinoflagellates are the dominant primary producers of the modern coral reef. They are able to produce an average 0.6 gC/ m^2/day through photosynthetic processes using sunlight absorbed through the coral tissue. The organic carbon algal symbionts leaks or transfers to the coral tissue is a factor that allows the productivity of the reefs to be sustained (Muscatine and Porter, 1977). In exchange for protection from high sunlight intensity and predation and having a continuous source of inorganic nutrients, the symbionts provide the main source of energy-rich compounds to the coral polyp and enhance the ability of the coral to precipitate $CaCO_3$. In fact, coral symbionts showed 3 times higher rate of carbon fixation than free-living dinoflagellates in the surrounding waters (Schlichter et al., 1983). The importance of carbon fixation in the algal-coral symbiosis led us to hypothesize that inorganic carbon uptake in *Symbiodinium* sp., isolated from *P. elisabetha* would be capable of pseudoptereosin synthesis. Using radiolabeled

$NaH^{14}CO_3$, algal biosynthetic pathways and production of the pseudopterosins were monitored independent of coral metabolism.

3.2 RESULTS AND DISCUSSION

3.2.1 Biosynthesis of pseudopterosins from inorganic carbon - 24 hour experiment

Initial experiments to monitor rates of photosynthetic carbon fixation in *Symbiodinium* sp. cells were carried out using 80 ml of 4 x 10^5 cells/ml, incubated with 100 μCi $NaH^{14}CO_3$ for 24 hours under ambient light. The cells were extracted and the transformation of $NaH^{14}CO_3$ to total lipids and pseudopterosins quantified using NP-HPLC. The radioactivity of the total lipids was 91.5 ± 9 x 10^4 dpm while the radioactivity of the pseudopterosins A through D was 818 ± 89 dpm. Elisabethatriene was also radioactive with 524 dpm. Based on the radiochromatograms of elisabethatriene and the pseudopterosins (Figure 9 and 10) radiochemical purity of the compounds was determined. It was evident, however, that further optimization of these experiments was necessary to increase the absolute radioactivity of the pseudopterosins. This was accomplished by increasing the concentration of cells, increasing the concentration of labeled $NaH^{14}CO_3$ and increasing the incubation time (see section 3.2.2).

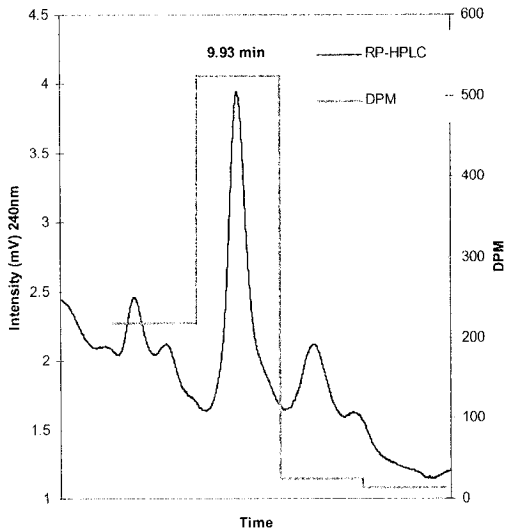


Figure 9: Radiochromatogram of elisabethriene

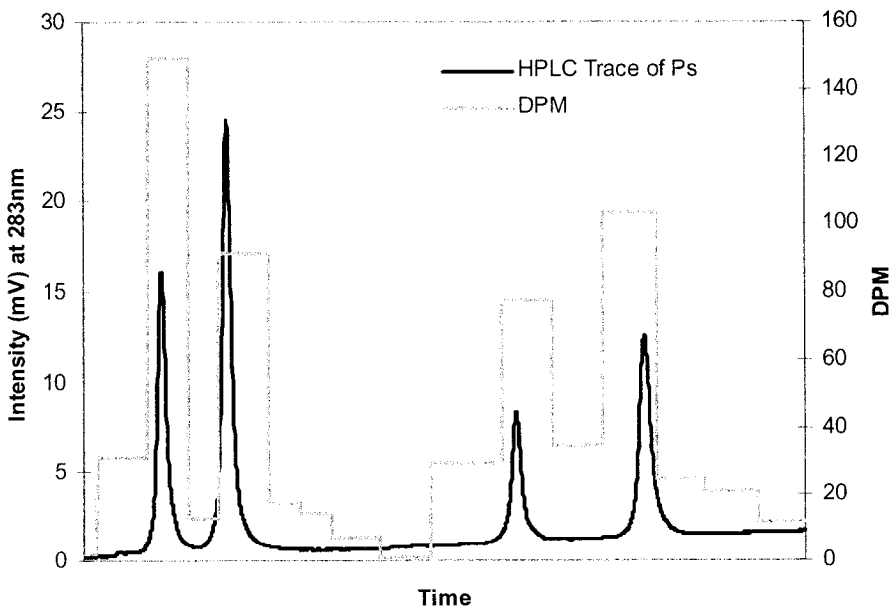


Figure 10: Representative radiochromatogram of all Ps from 24 hour experiment.

3.2.2 Biosynthesis of pseudopterosins from inorganic carbon – 48 hour experiment

Photosynthetic uptake and biosynthesis of inorganic carbon into the pseudopterosins was observed 48 hours post incubation of *Symbiodinium* sp. cells treated with ^{14}C labeled $NaH^{14}CO_3$ under ambient light. Analogous experiments were carried out in the dark or with cells treated with 100μM mevastatin to inhibit pseudopterosin biosynthesis. In all experiments, cells were extracted and the transformation of $NaH^{14}CO_3$ to total lipids and pseudopterosins quantified. Incorporation of the photosynthetic label into the lipids of the cells was clearly evidentg (5.06×10^6 dpm) indicating that photosynthesis is readily occurring. The key biosynthetic intermediate, elisabethatriene, had radioactivity of $4.3 \pm 0.54 \times 10^3$ dpm, specific activity of $3.69 \pm 0.2 \times 10^8$ dpm/mmol. Recovered radioactivity of the pseudopterosin pool (Ps A-D) was $2.6 \pm 0.2 \times 10^3$ dpm with specific activity of $5.18 \pm 0.54 \times 10^7$ dpm/mmol.

After initial experiments (data presented in section 3.2.1) it was found that increasing incubation time from 24 hours to 48 hours, increasing concentration of cells to 2×10^6 cells/ml and increasing concentration of added $NaH^{14}CO_3$ to 2.29 μCi/ml increased levels of recovered radioactivity in both total lipids and pseudopterosins relative to earlier experiments (Table 2). The labeled $NaH^{14}CO_3$ added in this experiment was equivalent to approximately 0.038 μM. The natural occurring levels of inorganic carbon in seawater range from 1800 - 3200 μM, indicating over a 40,000 fold dilution of the label in seawater. Once incorporation

occurs, further dilution of the label in the metabolic pool of compounds in the *Symbiodinium* sp. cells takes place. In spite of these dilution factors, significant labeling does occur and is evidence of *de novo* synthesis of the pseudopterosins in photosynthesizing cells.

The ability of the cells to photosynthesize and incorporate inorganic carbon into the lipid fraction in *Symbiodinium* sp. is evidence of viability of the cells after the separation procedure and their ability to survive *in vitro* for at least 48 hours. The isolation of ^{14}C labeled pseudopterosins within the lipid pool is evidence that CO_2 is a substantial carbon source for these compounds. Expressed on a per hour basis, *Symbiodinium* sp. cells produced radioactive pseudopterosins at a rate of approximately 3.6 dpm/10^6 cells/hour, which comprised only 0.05% of the total labeled lipids after 48 hours. This indicates a low turnover rate of the pseudopterosins and a sizeable reserve pool of the compounds that is also reflected in the high endogenous (unlabeled) pseudopterosin concentrations (Table 3). Under these experimental conditions, the *Symbiodinium* sp. cells produce 0.35% of the extractable pseudopterosin pool. These data imply that the *Symbiodinium* sp. cells are storing large quantities of the pseudopterosins and that the compounds may serve an important physiological function and play an important role in cell signaling and metabolism.

	24 hour Incubation	48 hour Incubation	% Increase
dpm All Ps	818 ± 89	2427 ± 355	66.2
dpm Elisabethatriene	525 ± 26	4275 ± 540	87.7
Specific Activity (dpm/mmol) all Ps	5.51×10^6	5.19×10^7	84
Specific Activity (dpm/mmol) elisabethatriene	1.8×10^6	3.69×10^8	99.5
dpmPs/day	818	1213	32.5
dpmPs/10^6 cells/hour	1.07	3.16	66.1
% CO_2 (dpm) incorporated into lipids	0.45	10.06	95.5
% CO_2 (dpm) incorporated into Ps pathway	6.05×10^{-4}	1.42×10^{-2}	96.3
Total C (mg) fixed into lipids	118	2872	95.8
Total C (mg) fixed into Ps pathway	0.159	3.85	95.8
Total C (mg) fixed into Ps pathway/10^6 cells/day	0.0049	0.107	95.3

Table 2: Comparison of radioactivities and CO_2 incorporation in 24 hour and 48 hour experiments

Biosynthetic Rate/24 hr (% Labeled Lipids)	Endogenous Ps Pool (% Total Lipids)
0.05 ± 0.007	14.3 ± 1.76

Table 3: Comparison of *deNovo* pseudopterosin biosynthesis and endogenous pseudopterosin levels.

Overall, the data presented here is supported by previous studies conducted in several algal groups. In diatoms (Cvejic and Rohmer, 2000) and in the unicellular chlorophyte, *Acetabularia* (Baeuerle et al., 1990) it has been demonstrated that inorganic carbon fixation via photosynthesis is the main carbon source for isoprenoid biosynthesis. In *Acetabularia*, 48% of the radiolabeled lipids were attributed to prenyl lipids with biosynthesis taking place in isolated chloroplasts. In various cultured phytoplankton species such as the chlorophyte *Dunaliella* and the cryptophyte *Rhodomonas* showed quantitatively similar labeling in terpenoid based pigments from $^{14}HCO_3$ incubations (Pickney et al., 1996). Rice et al. demonstrated ^{14}C carbonate uptake into intact coral sections of *Pseudoplexaura porosa*, including the labeling of the diterpene crassin acetate (Rice et al., 1970).

3.2.3 Evaluation of radiochemical purity

Radiochemical purity of the pseudopterosins was established by HPLC purification and subsequent derivatization. Following initial collection of the pseudopterosin peaks the radioactivity profile of the eluent was monitored during the second HPLC analysis and in all cases, radioactivity was only associated with the pseudopterosin fractions (Figure 11 and 12). To further confirm radiochemical purity of the pseudopterosin compounds, base hydrolysis of Ps B, C and D yielded Ps A. 400 μg of standards was added to the cell extract (Figure 13). Specific activity of Ps A, B, C, D before base hydrolysis was 2.04×10^6 dpm/mmol. Final specific activity of the Ps A was 1.85×10^6 dpm/mmol. This small change in specific activity indicates that the final Ps A is both chemically and radiochemically pure.

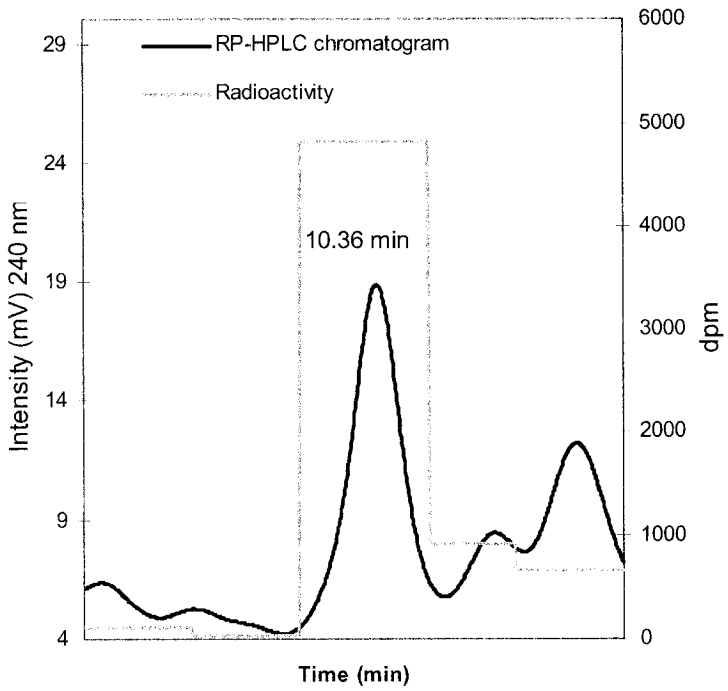


Figure 11: Radiochromatogram of elisabethatriene from 48 hour incubation with $NaH^{14}CO_3$

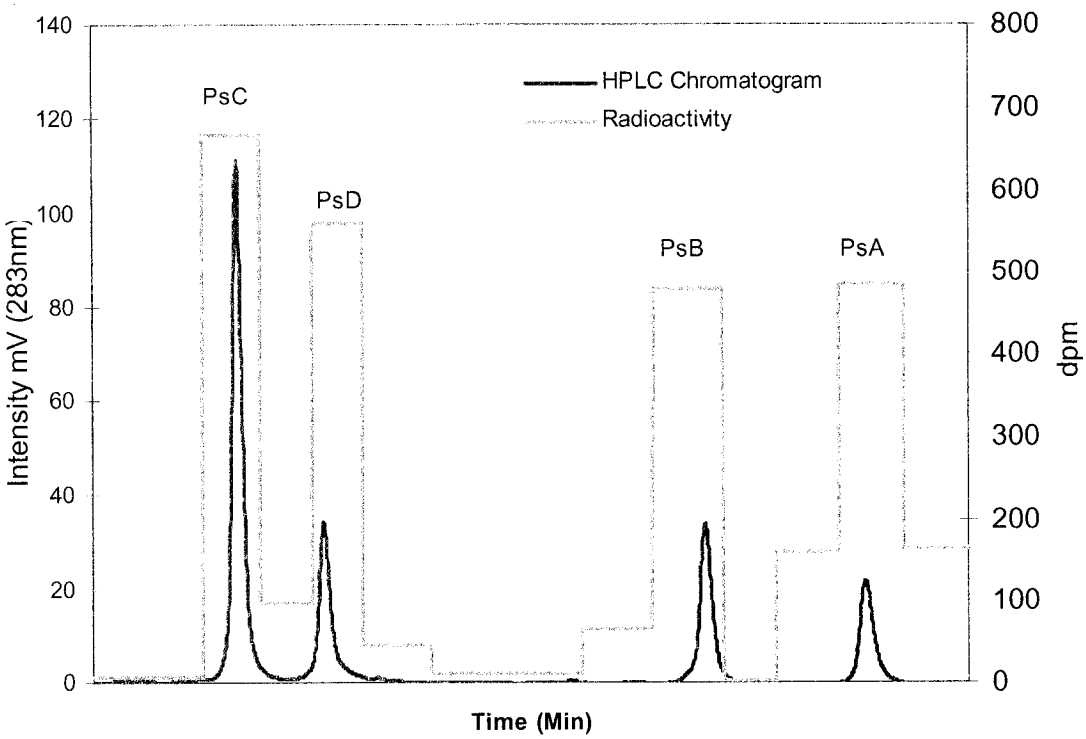


Figure 12: Radiochromatograms of pseudopterosins from 48 hour incubation with $NaH^{14}CO_3$

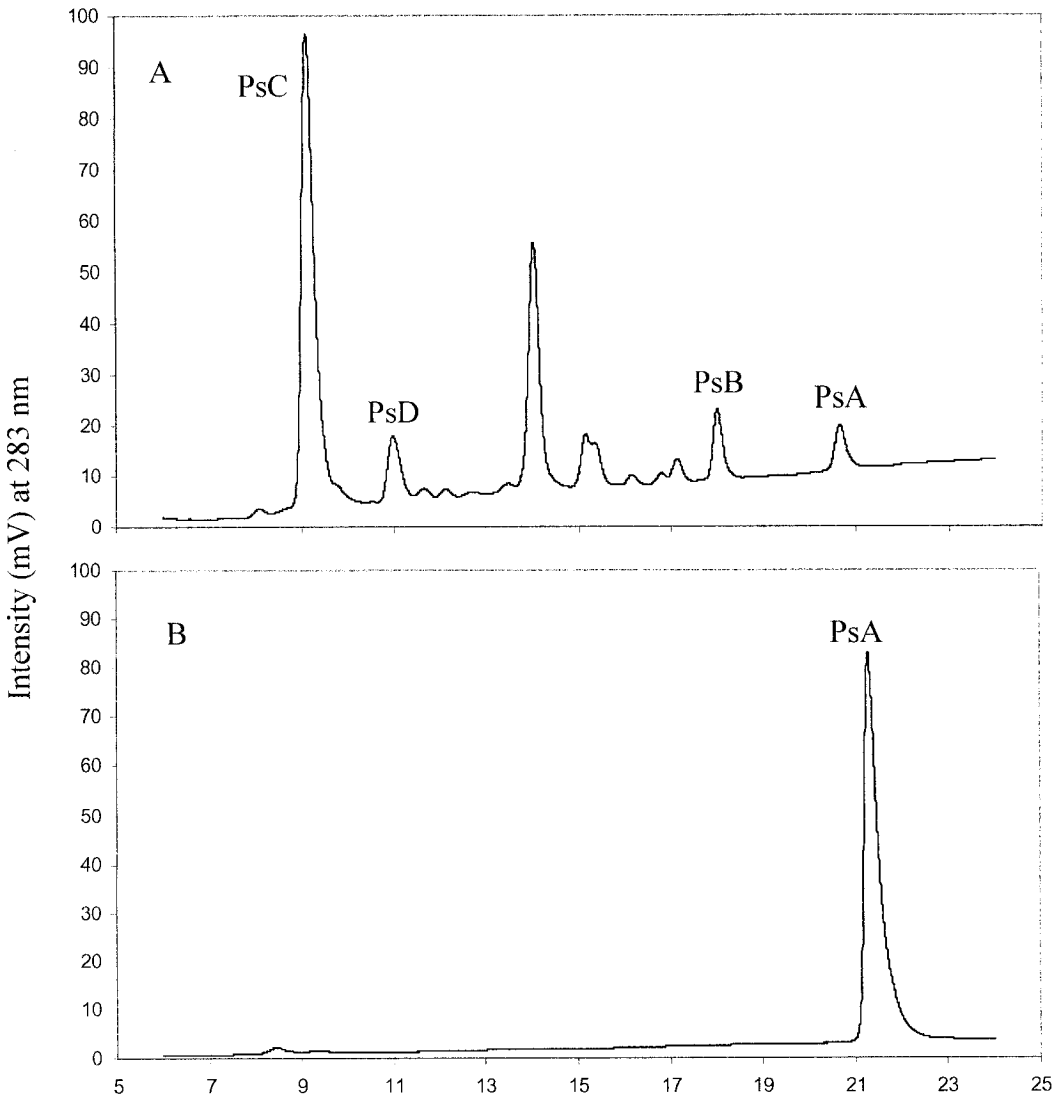


Figure 13 : NP-HPLC trace of pseudopterosins before and after base hydrolysis

A) NP-HPLC trace of pseudopterosin A through D before base hydrolysis. Only the peaks corresponding to the pseudopterosins were collected and subjected to base hydrolysis. B) Reaction mixture after base hydrolysis to yield PsA as only product.

3.2.4 Photosynthetic inhibition of pseudopterosin biosynthesis

In a separate set of experiments, inhibition of pseudopterosin biosynthesis in *Symbiodinium* sp. cells was analyzed in 24 hour dark and light adapted cells and boiled cells. There was a 98% reduction of photosynthesis as measured by inorganic ^{14}C incorporation into lipids in dark treated cells. There was corresponding reduction (100%) in inorganic ^{14}C incorporation into the pseudopterosin pool which demonstrates that the labeling phenomena seen in the light exposed cells was indeed due to photosynthetic processes and not isotopic exchange. The complete inhibition of pseudopterosin biosynthesis in boiled cells further confirms that the inorganic ^{14}C incorporation into the pseudopterosins is due to an enzymatic process.

3.2.5 Metabolic inhibition of pseudopterosin biosynthesis

Since biosynthesis was blocked in dark treated and boiled cells, we further investigated pseudopterosin biosynthesis using the mevalonic acid pathway inhibitor, mevastatin sodium. Mevastatin sodium inhibits HMG-CoA reductase and the biosynthesis of mevalonic acid which subsequently inhibits the production of cholesterol and other terpenoid products (Kumar et al., 2002). Statin compounds have been used in algae such as *Haematococcus* and various chlorophytes to discern the origins of terpenoid biosynthetic pathways (Hagen and Grunewald, 2000; Schwender et al., 2001). In *Symbiodinium* sp. cells incubated with radiolabeled $NaH^{14}CO_3$ for 48 hours, the inhibitory effects of mevastatin can be observed during pseudopterosin biosynthesis (Figure 14). Inorganic ^{14}C incorporation into the pseudopterosins was reduced significantly ($P<0.01$) by 55% compared to controls

while inorgainc ^{14}C incorporation into lipids was not effected. The effects of mevastatin suggest the involvement of the mevalonic acid pathway in pseudopterosin biosynthesis.

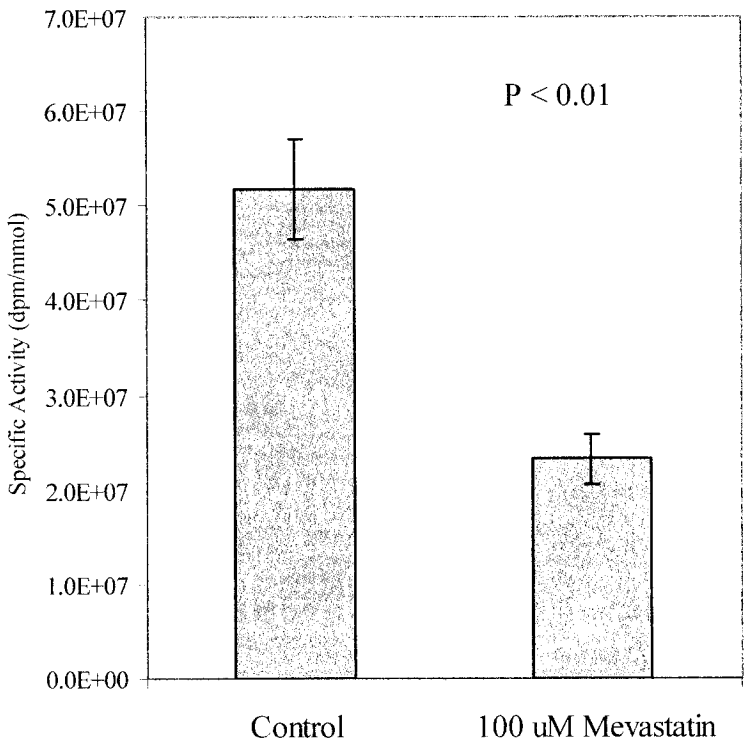


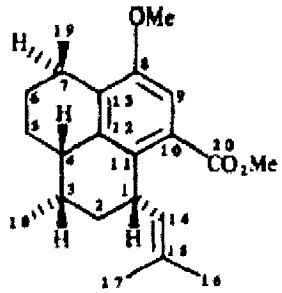
Figure 14: Inhibition of incorporation of $NaH^{14}CO_3$ into pseudopterosin biosynthesis by the HMG-CoA reductase inhibitor, mevastatin sodium measured by specific activity of Ps A, B, C and D as dpm/mmol (n=3).

3.3 COMPARISON OF DITERPENOID METABOLITES FROM *EREMOPHILA* SP. TO THE PSEUDOPTEROSINS

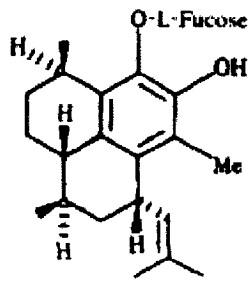
The seco-pseudopterosin diterpene backbone (see scheme 2) has frequently been referred to as a serrulatane diterpenoid because of its bicyclic structure. The seco-psedopterosins are not only precursors to the pseudopterosins (Kohl et al., 2003) but have been isolated as the main natural product in *P. elisabethae* population in the Florida Keys (Look and Fenical, 1987). The serrulatane diterpenoids were first isolated and thus named after compounds from the Australian resin-producing desert plant *Eremophila serrulata* (Ghilsaberti, 1992).

The serrulatane class of diterpenoids has repeatedly been isolated from many species of the genera *Eremophila* and in some cases the authors have noted the similarities to the pseudopterosin and seco-pseudopterosin class of compounds. Tippett and Massy-Westropp (1992) isolated serrulatane diterpenoids from *Eremophila duttonii* and noted the structural similarity to the seco-pseudopterosins. In addition, Ghilsaberti (1992) found a compound structurally identical to the tricyclic carbon backbone of the pseudopterosins in *Eremophila serrulata.* This compound, a tricyclic diterpene phenolic acid was shown to possess a carbon skeleton of the C-3 epimer of pseudopterosin K first isolated by Roussis et al. in 1990 (Figure 15). Although, the actual pseudopterosin glycosides have not been reported in any terrestrial plants to date, these reports of structurally similar compounds supports the hypothesis that the pseudopterosins are products of the algal

symbiont. In addition, these related compounds may serve as useful precursors to a chemo-enzymatic synthesis of the pseudopterosins as discussed in section 1.7.



Tricyclic diterpenoid acid from *E. serrulata*



Pseudopterosin K

Figure 15: Structures of serrulatane

3.4 GENERAL CONCLUSIONS

The results from inorganic ^{14}C uptake studies confirmed that the algal cells possess all of the biosynthetic machinery required for pseudopterosin biosynthesis. The increase in pseudopterosin biosynthesis with the increase in number of cells and incubation time with radiolabeled $NaH^{14}CO_3$, and the elimination of this biosynthesis in dark treated cells, supports the conclusion that the dinoflagellate symbionts are the biosynthetic source of the pseudopterosins in the gorgonian corals. These experiments also revealed that the biosynthetic rate of production of the pseudopterosins is low and that the products are stored within the symbiont cell in large quantities as reflected by the large intracellular standing pool of extractable

pseudopterosins. The accumulation of pseudopterosins within the symbiont cells indicates that the compounds may have a physiologically important role and may be stored as an acute signal transduction molecule.

Under our experimental conditions, this synthesis may follow the mevalonic acid pathway, as suggested by the reduction of pseudopterosin biosynthesis by the HMG-CoA reductase inhibitor mevastain. It is possible that the mevalonate and non-mevalonate pathways are both functioning within the *Symbiodinium* cell while resolving this issue requires additional studies (Hemmerlin et al., 2003).

In conclusion, the symbiotic dinoflagellate *Symbiodinium* sp. can produced sufficient pseudopterosins to account for the amounts of these compounds that can be harvested from the gorgonian coral *P. elisabethae* growing in its natural habitat. Our findings provide the important knowledge necessary to develop strategies for the production of the pseudopterosins and to define their role in the complex symbiont-coral community.

3.5 MATERIALS AND METHODS

P. elisabethae was collected in May 2001 at Sweetings Cay in the Bahamas at a depth of 10 m. $NaCO_3$ [^{14}C] 50-60mCi/mmol was purchased from American Radiolabeled Chemicals, St. Louis, MO. 0.22 μm Millipore Steritop filters were purchased from Fisher Scientific. Percoll® was purchased from Sigma-Aldrich Chemicals. Mevastatin was purchased from Biomol. HPLC purifications were performed using a Hitachi L-6200A Intelligent Pump connected to a L-4200 UV-Vis detector with Varian Chrompack C18 or silica columns.

Isolation and purification of *Symbiodinium* sp. from *P. elisabethae*

Flash frozen or live coral were homogenized in a blender with 0.22μm filtered seawater and 10mM EDTA, and filtered through 4 layers of cheesecloth. Algal symbionts were pelleted out by centrifugation at 250 x g and subsequently washed 10 times with 40 ml clean filtered seawater and pelleted by centrifugation at 750 x g. *Symbiodinium* sp. cells were further purified on Percoll® step gradient of 20%, 40%, and 80% two or more times until <1% impurities were seen using light microscopy. Cells isolated from live coral were diluted to final concentration $2x10^6$ cells/ml using a hemocytometer and maintained in filtered seawater.

***In Vivo* incubation with radioactive substrates and radioactive measurements**

80 ml of 4 x 10^5 cells/ml were incubated with 100μCi $NaH^{14}CO_3$ at room temperature under ambient light for 24 hours. For 48 hour experiments, 9 ml of 2 x 10^6 cell/ml were incubated with $Na^{14}HCO_3$ (specific activity of 2.29 μCi/ml) for 48 hours at room temperature under constant artificial light source. Data presented consists of mean and standard error of three independent experiments.

Intact cells were extracted using HPLC grade chloroform and ethyl acetate. Crude extracts were partitioned between methanol/water (9:1) and hexanes, followed by partitioning between methanol/water (1:1) and chloroform. Pseudopterosins A through D were purified by normal phase HPLC with a hexane/ethyl acetate gradient (60:40 to 100% ethyl acetate in 40 minutes) using UV detection at 283 nm. Peak areas corresponding to the pseudopterosins were used to quantify amounts from standard curves. Elisabethatriene was extracted from lyophilized cells using hexanes

and purified on reverse phase HPLC (isocratic in methanol) at 240 nm. Radiochemical purity of all compounds was ensured by purification two times through HPLC. During the second HPLC run, fractions were collected at minute intervals and counted in a LKB Wallac 1219 Rackbeta liquid scintillation counter. Efficiency for ^{14}C and ^{3}H was checked using internal standards. Radioactivity (in dpm) includes subtraction of background and was, at a minimum, double background levels.

Base hydrolysis of Ps B, C and D was accomplished by addition of 1 ml of 5% potassium hydroxide in methanol to approximately 200 μg of pseudopterosins A through D. The reaction was stirred overnight at room temperature. To quench the reaction 3 ml of water was added and the pH adjusted to 7. PsA was extracted using chloroform and dried over anhydrous sodium sulfate and evaporated under N_2 gas.

CHAPTER FOUR

AN INDUCIBLE OXIDATIVE BURST IN SYMBIOTIC AND FREE-LIVING DINOFLAGELLATES AND INHIBITION BY THE PSEUDOPTEROSINS

4.1 INTRODUCTION

The biological production of hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS) as an immune response to pathogens has been well documented during phagocytosis in human neutrophils and mouse macrophages (Morel et al., 1991; Baldridge and Gerard, 1933; Pick et al., 1980; Dwyer et al., 1996) as well as a ubiquitous defensive response to injury and pathogenic invasion in higher plants (Bolwell, 1995,1997). In most of these cases the production of reactive oxygen species occurs as a membrane NADP(H) oxidase mediated oxidative burst. The oxidative burst is an acute induced release of ROS such as superoxide (O^{2-}), hydroxyl radicals ($^{\cdot}OH$) or hydrogen peroxide (H_2O_2), which can lead to acute cytotoxic effects to the pathogens, as signaling molecules for the induction of secondary defensive responses or as a catalyst for oxidative cross-linking processes (Bolwell, 1997).

4.1.1 Reactive oxygen species in the marine environment

Hydrogen peroxide is an ROS that exists in seawater at concentration that vary 10-fold, with measurements ranging from $10^1 - 10^2$ nM (Wong et al., 2003). It has been suggested that the main source of hydrogen peroxide in seawater is from algal photosynthesis and photochemical interactions, with secondary input from atmospheric deposition and localized release of pulses of H_2O_2 when some marine organisms experience biological stress (Wong et al., 2003; Palenik and Morel, 1988; Palenik et al., 1987). Thus far the biological production of ROS as an oxidative burst has only been described in a small number of marine species. Kupper et al., (2001) found an oxidative burst in the brown alga *Laminaria* in response to oligosaccharide elicitors and Ross et al., (2004) found an oxidative burst induced by physical wounding in the green alga *Dasycladus vermicularis*. Collen and Davidson (1999) also found that the brown alga *Fucus* spp. can produce increased amounts of ROS in response to physical stressors. There are a number of publications in the literature which measure elevated ROS levels due to high light irradiation and in response to environmental stressed conditions such as temperature variations, nutrient depletion and heavy metal contamination in phytoplankton. These include the raphidophyte flagellates *Heterosigma* (Twiner and Trick, 2000), *Olisthodiscus* (Kim et al., 1999b) and *Chattonella* (Oda et al., 1994) and the dinoflagellates *Cochlodinium* (Kim et al., 1999a) and *Symbiodinium* sp. (Lesser,1997; 1996). Table 4 summarizes ROS production in marine organisms and the proposed mechanism of ROS production and release. In most of these cases the mechanisms of ROS production were not

elucidated in the context of current signal transduction technology, or were coupled with photosynthetic processes.

Species	Elicitor	Mechanism	Reference
Mammalian Macrophages	Foreign cells	NAD(P)H oxidase	Baldridge and Gerard, 1933
Laminaria digitata	Oligoguluronates	NAD(P)H oxidase	Kupper et al., 2001
Dasycladus vermicularis	Mechanical stress	NAD(P)H oxidase	Ross et al., 2004
Fucus spp.	Freezing, dessication	Damage to thylakoid membranes	Collen and Davidson, 1999
Cochlodinium polykrikloides	Light Intensity	Photochemical reactions	Kim et al., 1999
Cyanobacteria (Anabeanasp.)	UV-B	Photo-dynamic action	He and Hader, 2002
Heterosigma akashiwo	Light irradiance Iron Stress Temperature	Photochemical reactions and enzyme dependant	Twiner and Trick, 2000
Symbiodinium sp.	High temperatures and high solar UV	Photoinhibition of photosynthesis	Lesser, 1997

Table 4: Summary of marine species studied for oxidative stress and oxidative bursts

4.1.2 Project goals

In the present study we characterize an inducible oxidative burst in symbiotic dinoflagellates of the species *Symbiodinium* (Clade B1) isolated from the gorgonian coral *P. elisabethae* (PE) and the a close sister taxa dinoflagellate *Heterocapsa pygmaea* (a.k.a *Glenodinium* sp.) (Santos et al., 2002) in response to physical injury. Induction of the burst occurs in response to physical stress caused by sonic sound, a well studied abiotic elicitor of plant defense responses. The sublethal effects of low intensity sonic sound causes increased membrane permeability, increased membrane ion fluxes, production of ROS and the increasing production and release of defensive secondary metabolites in plant cells (Lin et al., 2001; Wu and Lin, 2002). We report here the kinetics and characterization of this oxidative burst in *Heterocapsa pygmae* and PE *Symbiodinium* sp. from sonic sound exposure, ionophores and various pharmacological probes.

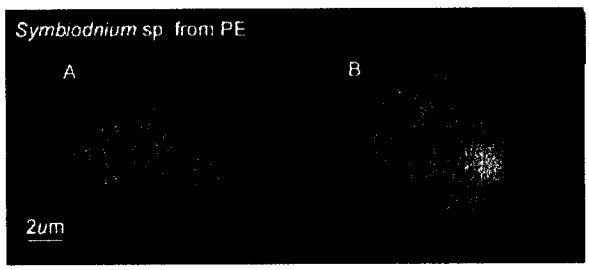
As part of this assessment of the inducible oxidative burst in *Symbiodinium sp.* isolated from PE, we test a hypothesis that this dinoflagellate cell has a resistance to injury relatable to high cellular concentrations of the diterpenoid marine natural products, the pseudopterosins present in these dinoflagellate cells (Mydlarz et al., 2003). The pseudopterosins are potent anti-inflammatory and analgesic compounds with a unique mode of action (Look et al., 1986; Ettouati and Jacobs, 1987; Mayer et al., 1998; Moya and Jacobs, 2004). Using the oxidative burst in *H. pygamea* we studied the effects of the pseudopterosins in reducing and preventing the oxidative burst.

4.2 RESULTS AND DISCUSSION

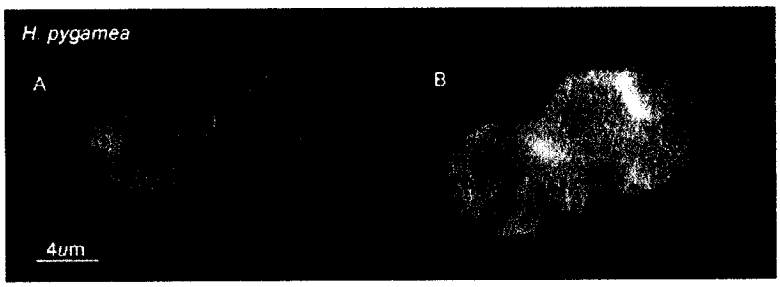
4.2.1 Induction and characterization of the oxidative burst due to physical injury in *Symbiodinium* sp. from PE and *H. pygamea*

Symbiodinium sp. from *P. elisabethae* (PE) and *H. pygamea* cultures were subjected to a non-lethal dose of low frequency sonic sound (20 kHz) in 3 (10 s) pulses. The low intensity and short pulses of sonic sound used in this experiment did not cause cell lysis. At the 20 KHz frequency it takes several minutes of sonication in order to completely disrupt microalgal membranes (Branson, 1974). These sonic bursts cavitate the cells and thereby permeabilize the cell membrane causing a physical stress (Branson, 1974). In our studies, this physical disturbance elicited an oxidative burst response that was visualized using epifluorescence microscopy. As can be seen in figure 16, (panel 1 and 2) normal control cells of *H. pygmae* and *Symbiodinium* sp. from PE fluoresce red, while sonic sound produced green fluorescence which corresponds to the ROS produced from the oxidative burst reacting with the fluorescent probe, dichlorofluoresceine diacetate (figure 16, panel 3). In order to quantify the amounts of ROS produced during an oxidative burst, a spectrofluorometric protocol using the same fluorescence probe was developed that quantified the oxidative burst pmol H_2O_2/cell.

Panel 1]



Panel 2]



Panel 3]

Figure 16: Epifluorescent micrograph of Panel 1] A) Control *Symbiodnium* sp. from PE and B) Physically injured *Symbiodnium* sp. from PE. Panel 2] A) Control *H. pygmaea* and B) Physically injured *H. pygmaea*. Excitation 488 nm, emmission 510 nm longpath. Green DCFH-DA fluorescence indicates presence of ROS. Panel 3] Reaction of DCFH-DA to cause fluorescence.

Physically injured *Symbiodinium* sp. from PE produced a small oxidative burst (0.042 ± 0.0045 pmol H_2O_2/min/cell), while unexpectedly, the free-living model dinoflagellate, *H. pygamea* produced an oxidative burst (3.37 ± 0.26 pmol H_2O_2/min/cell) that was 80 times greater than that of *Symbiodinium* sp. from PE. In kinetic studies of the oxidative burst over time (Figure 17), the upper limit of H_2O_2 released in the dinoflagellates tested took place within 1 minute of the sonic sound pulses and the levels of H_2O_2 remained high for 10 additional minutes. While *Symbiodinium* sp. from PE recovered from the burst after 20 minutes as levels of H_2O_2 approached zero, *H. pygamea* cells did not and the levels of H_2O_2 exhibited a steady rate of decrease from the maximum at 3.37 pmol/cell to approximately 2.21 pmol/cell for the remainder of the period tested (40 minutes). At this rate of detoxification, it was calculated by a best fit regression line, that it would take approximately 110 minutes for the levels of H_2O_2 to return to zero. This slow reduction of H_2O_2 levels is presumably due to a slowing production rate and enzymatic detoxification of the ROS.

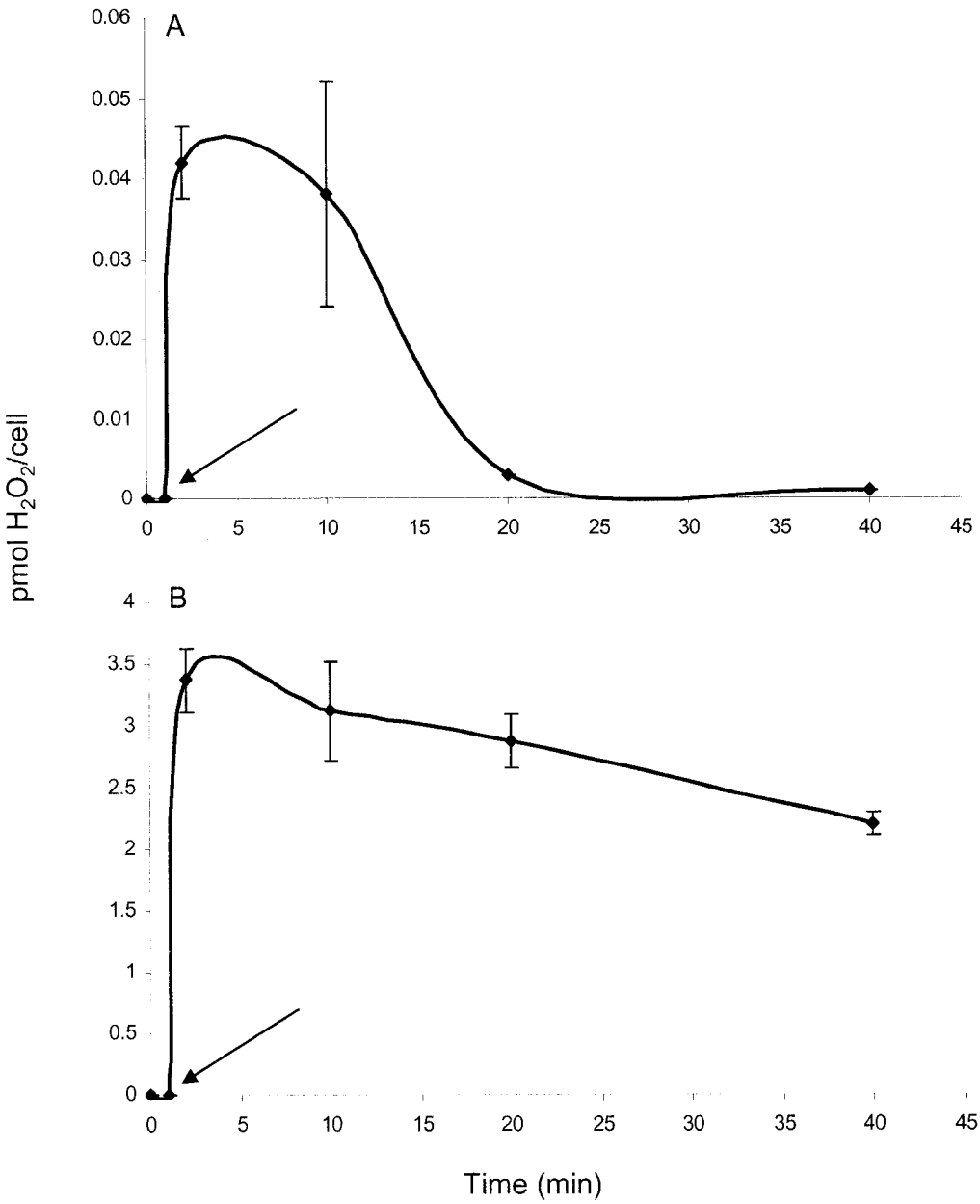


Figure 17: Kinetics of the oxidative burst caused by sonic sound in A) *Symbiodinium* from PE and B) *H. pygmaea*.(n=5) Arrows indicate point of injury.

To determine the enzymatic origin and the chemical composition of the oxidative burst in *H. pygmaea* and PE *Symbiodinium* sp. in response to physical injury, the cells were incubated with diphenylene iodonium chloride (DPI) and catalase (Table 5). DPI is a irreversible inhibitor of NAD(P)H oxidase and 50 uM of the drug inhibited the oxidative burst by 100% (n=3) in PE *Symbiodinium* sp. cells and 95 ± 2.2 % (n= 3) in *H. pygamea* cells. The near complete inhibition of the oxidative burst caused by sonic sound by DPI is consistent with presence of superoxide-generating NAD(P)H oxidases which are constituitive enzymes in the oxidative bursts of mammalian neutrophils and higher plants (Bolwell et al., 1995; Kupper et al., 2001; Dwyer et al., 1996). Catalase the enzyme which initiates the decomposition of hydrogen peroxide to water and oxygen was used to determine the chemical species present in the oxidative burst. Typically, 1 unit of enzyme decomposes 1 μmol of hydrogen peroxide. In PE *Symbiodinium* sp. catalase at 50 U/ml reduced 100% of the fluorescence emission indicating that the oxidative burst in PE *Symbiodinium* sp. was composed of nearly 100% hydrogen peroxide In *H. pygamea* a range of 50 U/ml to 500 U/ml was used to inhibit the oxidative burst. The anti-oxidant effects of catalase were dose-dependant and ranged from 79 ± 2.2% at 50 U/ml to 96% at 500 U/ml (Table 5). Even though this was a steady decrease of fluorescence emissions, the inhibition never reached 100%. This indicates that the oxidative burst in *H. pygmaea* is composed mainly of hydrogen peroxide, but may also have a small fraction of other free radicals that react with the fluorescent probe. The high levels of catalase necessary to inhibit the oxidative burst in *H. pygmaea*

also support the observation that this free-living dinoflagellate is releasing a higher concentration of ROS than PE *Symbiodinium* sp. The oxygen radical scavenger, ascorbic acid successfully scavenged all the reactive oxygen species produced and eliminated the fluorescence signal in both species (data not shown).

Treatment	PE *Symbiodinium* sp. % Inhibition	*H. pygmaea* % Inhibition
50 μM DPI	100%	95 ± 2.2 %
50 μM Catalase	100%	79 ± 2.3 %
100 μM Catalase	-	86 ± 2.5 %
500 μM Catalase	-	96 ± 0.9 %

Table 5: Summary of inhibitory effects of diphenylene iodium chloride (DPI) and catalase on PE *Symbiodinium* sp. and *H. pygmaea*

4.2.2 Oxidative Burst in related symbiotic dinoflagellates

We compared the oxidative burst due to sonic injury in *Symbiodinium* sp. freshly isolated from PE, from a cultured strain of *Symbiodinium* sp. and *Symbiodinium* sp. freshly isolated from *P. Americana* (PA) (Figure 3). The cultured *Symbiodinium* sp. produced an oxidative burst almost twice the magnitude of PE *Symbiodinium* sp. and PA *Symbiodinium* sp. produced a burst three times greater than that of PE *Symbiodinium* sp. The relatively muted oxidative burst of this select group of symbiotic dinoflagellates was on average 50 fold less than the burst in the free-living *H. pygmaea* (3.37 pmol H_2O_2/min/cell vs. a mean of 0.074 pmol H_2O_2/min/cell). This large difference in the magnitude of the oxidative bursts between symbiotic and free-living dinoflagellates represents an unexpected phemomena which may prove beneficial to the symbionts and could be related to the specific symbioses that these dinoflagellates have established with invertebrate hosts. Excess H_2O_2 can cause damage to photosystem II at the D1 dimer protein within the symbiotic dinoflagellate (Richter et al., 1990). It can also diffuse into the host tissues where similar interference with membrane functions might occur (Asada and Takahashi, 1987). This has been reported to lead to decreased photosynthesis and diminished translocation of photosynthate and may cause exocytosis of the symbionts from the host cell (Lesser, 1997). These published results and the recent measurements reported here, raise the possibility that in addition to known anti-oxidant enzymes present in these cells, there may be distinctive host signals or

natural products that are produced that down regulate or mitigate the synthesis of oxygen radicals.

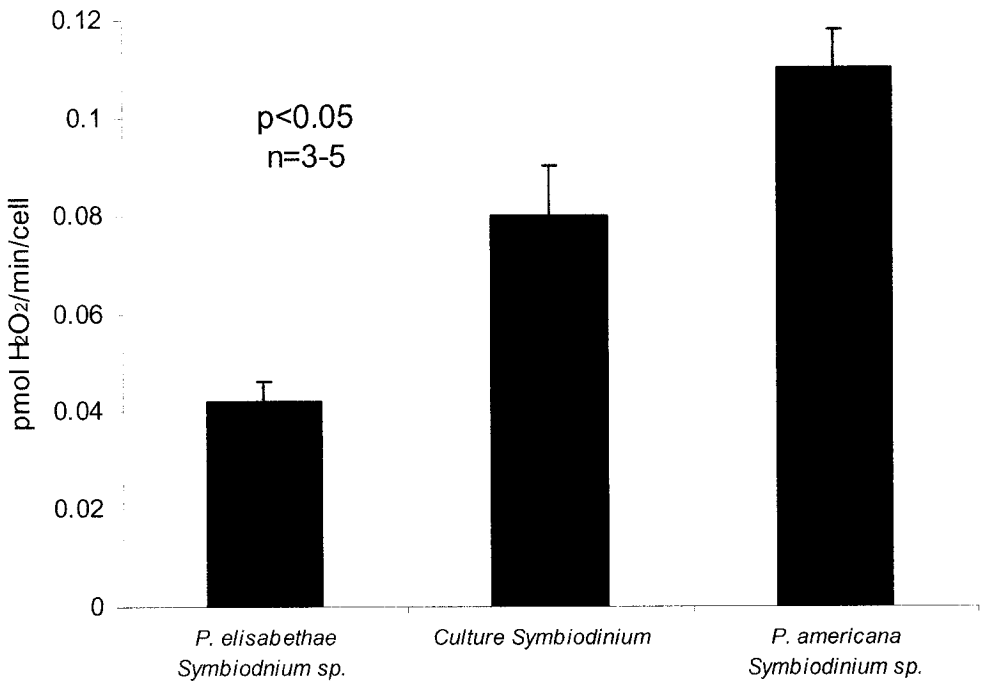


Figure 18: Comparative oxidative bursts in various *Symbiodinium* spp.

The lipid fraction of PE *Symbiodinium* contains up to 15% of the pseudopterosins which are potent anti-inflammatory and membrane stabilizing compounds, (Mydlarz et al., 2003). The PA *Symbiodinium* sp and the cultured *Symbiodinium* sp. do not contain these compounds. This fact, along with the observation in this study that PE *Symbiodinium* sp. exhibited a statistically significant increased resistance to sonic injury when compared with PA *Symbiodinium* sp. and *Symbiodinium* cultures can raised the possibility that the unique lipid signature of PE *Symbiodinium* sp. is conferring a natural role in protecting against a damaging oxidative burst. Even though there are environmental, physiological and morphological differences between these symbiont cells and coral hosts, all the *Symbiodinium* species used in this study were identified as Clade B1 sub-type by the ITS-rDNA region genotyping technique (LaJeunesse 2001; 2002). More specifically, PE and PA *Symbiodinium* sp. have the same number of base pairs of the cp 23S-rDNA domain V allele (184 bp) (Santos et al., 2002) thus the two symbionts are phylogenetically similar.

Heterocapsa pygmaea is a common free-living dinoflagellate that has been used extensively as a model for physiological comparisons with symbiotic dinoflagellates due to their similar sizes and the ease of culturing (Santos et al., 2003; Govind et al., 1990; Iglesia-Prieta et al., 1993; Boczar and Prezelin, 1986) *Heterocapsa* as a genus is representative of many dinoflagellate species as they are planktonic and pandemic (Watson and Loeblich, 1983) they are environmentally important because they are bloom-forming and they have been extensively studied

for their photophysiology (Johnsen et al., 1997; Johnsen et al., 1994; Nelson and Prezelin, 1990; Triplett et al., 1993). Since they produced a large oxidative burst in our studies, lack pseudopterosin biochemistry and are genetically related to *Symbiodinium*, *H. pygmaea* was investigated to test the effects of pseudopterosin addition on the physically induced oxidative burst.

4.2.3 Inhibition of the oxidative burst elicited by sonic sound in *H. pygmaea* by the pseudopterosins

The effects of the pseudopterosins on the oxidative burst caused by injury in *H. pygamea* were examined using a formulation of pseusopterosins A, B, C and D in their natural constitution (ratio of 15% PsA, 24% PsB, 38% PsC and 22% PsD, prepared by HPLC). Pseudopterosin A, B, C and D have the same tricyclic diterpene backbone, but differ in acetylation of the glycosidic moiety (Look et al., 1986). *H. pygamea* cells were pre-treated with various concentrations of the pseudopterosins for 1 hour prior to sonic injury. As can be seen in figure 19A, the pseudopterosins inhibited the oxidative burst in a concentration dependent manner (approximate IC_{50} of 8 μM). The dose response curve followed pseudo-first order kinetics (c.v 0.95), with the reaction rate of H_2O_2 production declining exponentially with increased pseudopterosin concentrations (figure 19A). A semi-log plot (figure 19B) shows that the rate of inhibition rises sharply between 7 and 10 μM, with near saturation at concentrations greater that 10 μM. This sharp decline in the inhibition curve may be caused by complex pseudopterosin receptor interactions, where the effect of one pseudopterosin potentiates, augments or alters the effect of the other pseudopterosins

in the mixture. This cooperative interaction would cause a large positive increase in the physiological effects of the compounds. Another reason that the percent inhibition curve has a steep incline at 7 uM may be due to the fact that all these experiments were conducted with a homogeneous population of *H. pygmaea* that were genetically identical and from the same cultured strain. The uptake of pseudopterosins into *H. pygmaea* cells was monitored using NP-HPLC to analyze the dinoflagellate cell paste, after 1 hour incubation, at least 50% of the pseudopterosins added to the media was present in the cells.

In additon, since sonic sound intensly agitates the cells and permeabilizes cell membranes, the pseudopterosins may exhibit general cell membrane stabilization properties in addition to inhibition of the oxidative burst. These stabilizing effects have been observed in fertilized sea urchin embryos (Ettouatti and Jacobs, 1987 and in calcium ionophore stimulated neutrophils (Mayer et al., 1998).

The first committed intermediate, the hydrocarbon, elisabethatriene (see scheme 3) was not active in inhibiting the oxidative burst in *H. pygmaea*. Elisabethatriene is bicyclic, has no functional groups and represents an early intermediate step in pseudopterosin biosynthesis, thus indicating the bioactivity of the pseudopterosins does not lie in the hydrocarbon backbone.

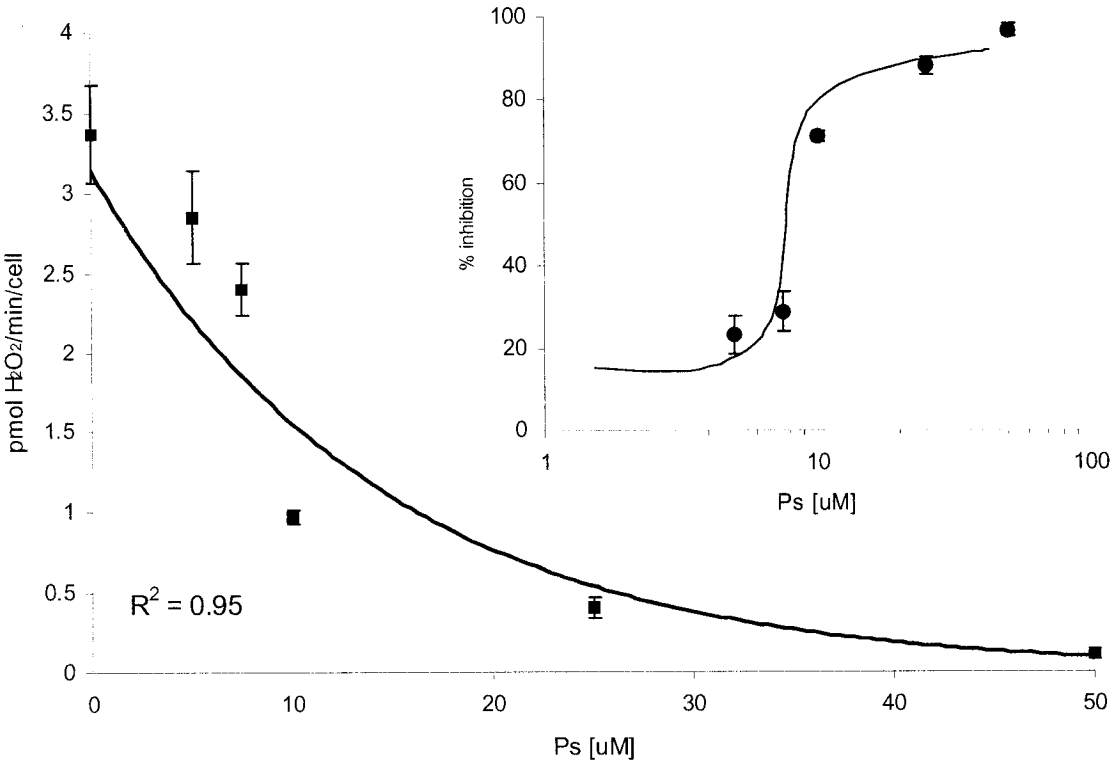


Figure 19: Inhibition of oxidative burst in *H. pygmaea* by the pseudopterosins A) Concentration dependant inhibition in H_2O_2 release B) Semi-log dose response % inhibition.

In other experiments, we compared the rate of inhibition in H_2O_2 production following treatment of PA *Symbiodinium* sp. and *H. pygmaea* with 25 μM of pseudopterosins. This concentration inhibited the oxidative burst by 80% in PA *Symbiodnium* sp (n=3, $p<0.01$) and by 86% in *H. pygmaea,* respectively (Table 6). The rate of H_2O_2 production was reduced from 0.1 pmol/min/cell to 0.02 pmol/min/cell in PA *Symbiodinium* sp. In contrast H_2O_2 production in *H. pygmaea* was reduced from 3.4 pmol/min/cell to 0.5 pmol/min/cell. Thus the absolute reduction in H_2O_2 was 36 times greater in *H. pygmaea* using the same concentration of pseudopterosins, mitigating against the view that a direct anti-oxidant/scavenging effect of the pseudoterosins was a primary mechanism.

Treatment	PA *Symbiodinium* sp.	*H. pygmaea*
Sonic sound	0.10 ± 0.008 pmol/min/cell	3.37 ± 0.26 pmol/min/cell
Sonic sound + 25 μM pseudopterosins	0.021 ± 0.008 pmol/min/cell	0.50 ± 0.008 pmol/min/cell
% Inhibition	80%	86%
Absolute change in H_2O_2 production rate	0.079 pmol/min/cell	2.87 pmol/min/cell

Table 6: Comparison of inhibitory effects of the pseudopterosins on PA *Symbiodinium* sp. and *H. pygmaea*.

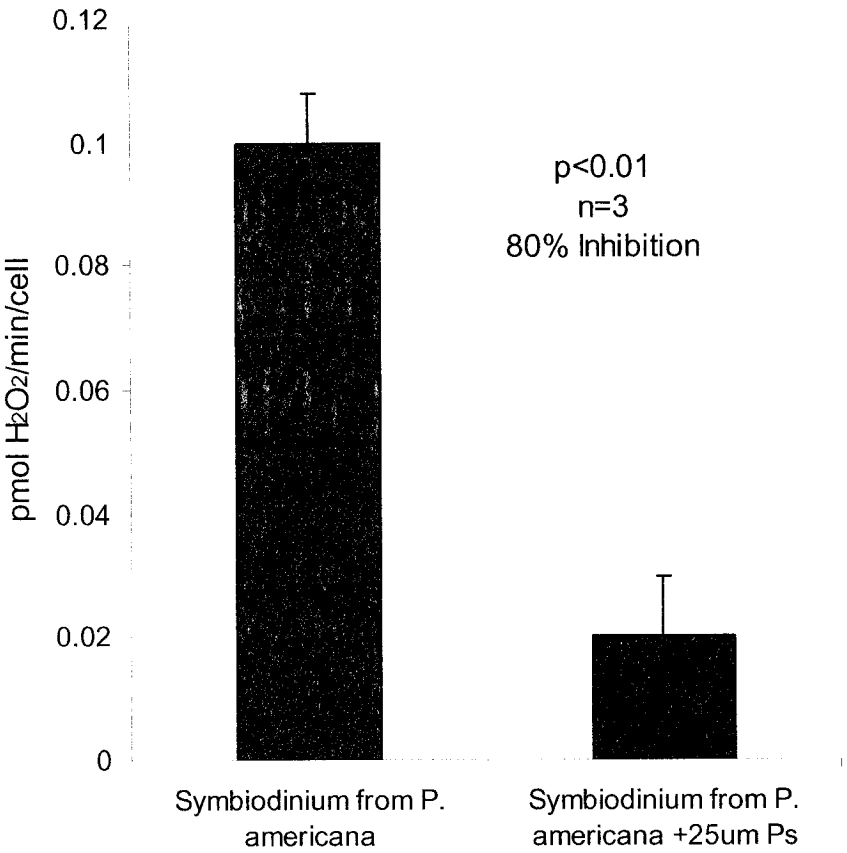


Figure 20: Inhibition of the oxidative burst *in Symbiodinium* from *P. Americana* by the pseudopterosins

To further evaluate the direct oxygen scavenging properties of the pseudopterosins an assay using a cell free system of water and H_2O_2 was developed and the direct anti-oxidant effects of the pseudopterosins were compared to the lipophilic scavenger, α-tocopherol. 5, 25 and 50uM of each compound was added to a synthetic system of hydrogen peroxide and water and the scavenging effects were measured by a decrease in fluorescence (Figure 21). At 50 uM the lipophilic scavenger α-tocopherol scavenged 70% of the hydrogen peroxide available while at 50uM the pseudopterosins scavenged a maximum of 26% of the hydrogen peroxide available. Thus the oxygen scavenging effects of the pseudopterosins appear to contribute minimally to the pharmacological effects of these compounds.

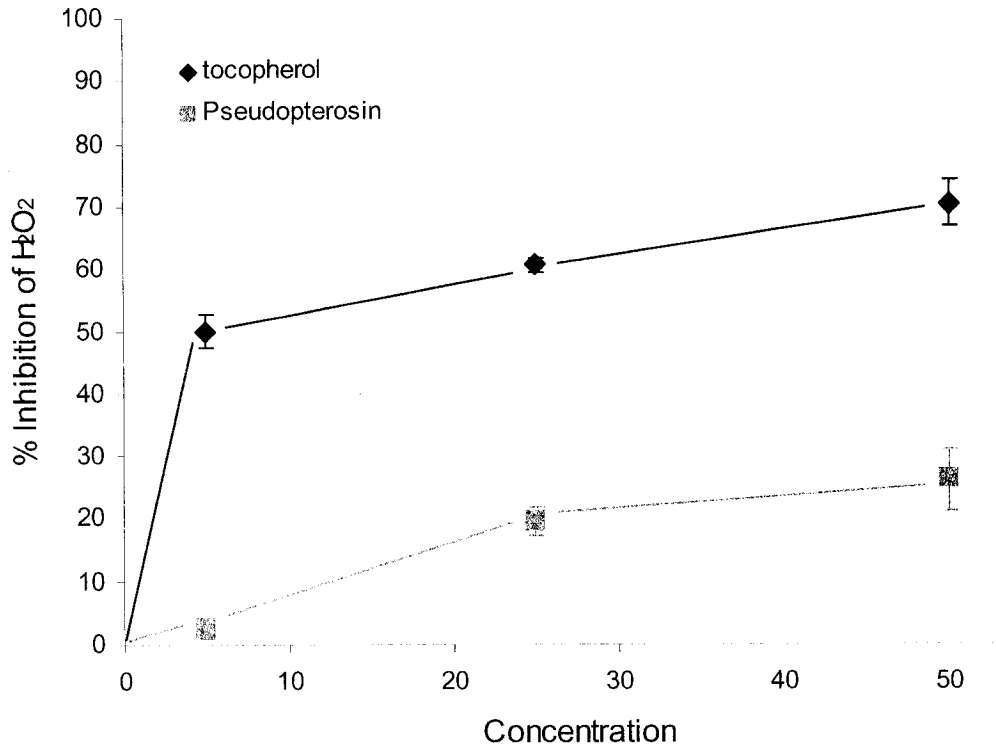


Figure 21: Anti-oxidant scavenging effects of the pseudopterosins and α-tocopherol (in DMSO)

4.2.4 Chemical irritants and ionophores cause oxidative burst in *Symbiodnium* sp. and *H. pygmaea*

In addition to the membrane permeabilization of sonic sound, ionophores were used to increase permeability of the algal membranes to specific ions. H. *pygamea* and PE *Symbiodinium* sp. were treated with Ca, K and Na ionophores to begin to identify the specific channels involved in the oxidative burst. When cells were treated with 75uM of each Valinomycin (K^+), Monensin (Na^+) and A 23187 (Ca^{2+}) for a period of 30 minutes, as well as 10uM of the G-protein activator, mastoparan, an oxidative burst was seen. Figure 22 represents a comparison of all the chemical and physical irritants in both *H. pygamea* and PE *Symbiodinium* sp.

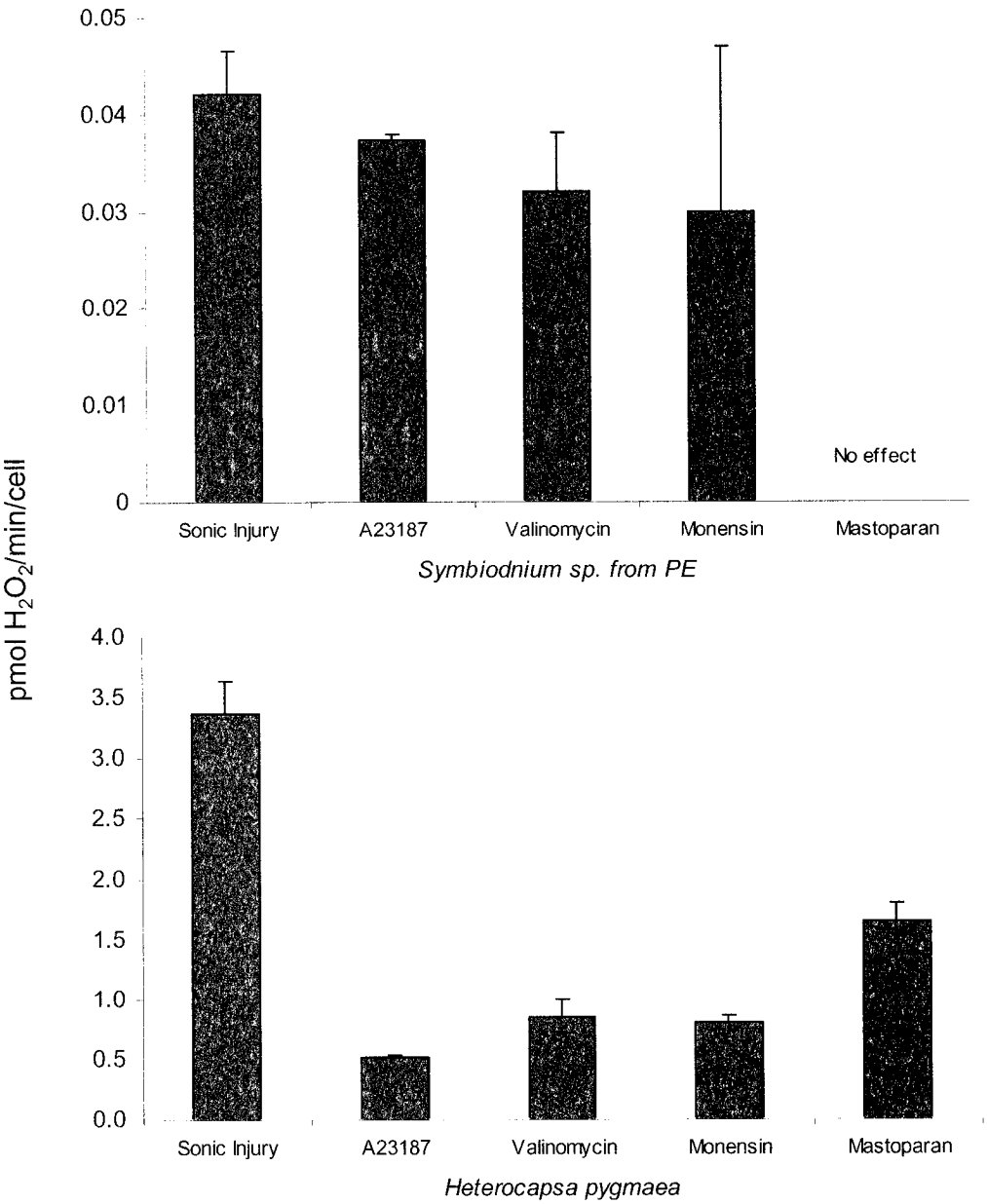


Figure 22: Oxidative burst of *Symbiodinium* sp. from PE and *H. pygmaea* due to ionophores and mastoparan.

Ionophores are pharmacological probes which enhance the flux of specific ions across membranes. A23187 increases the permeability of membranes for divalent cations, such as Mg^{2+} in addition to Ca^{2+}. In both *H. pygamea* and PE *Symbiodinium* from, the different ionophores caused oxidative bursts. The trend of a muted oxidative response in PE *Symbiodinium* sp. as seen with the physical injury also occurred with the ionophore treatment.

In PE *Symbiodnium* sp., there was no significant difference between the magnitude of the oxidative burst causes by each ionophore and the magnitude of the burst was not significantly different from the burst caused by sonic injury. This would indicate that PE *Symbiodinium* sp. are sensitive to any membrane destabilizers, whether it involves physical permeability or ionic flux. Mastoparan, the G-protein activator and wasp peptide did not cause an oxidative burst in PE *Symbiodinium* sp..

In *H. pygmaea*, the largest oxidative bursts were in response to monensin sodium and valinomycin which may indicate an important role for sodium and potassium efflux in the signal transduction cascade of the oxidative burst. In studies in the brown alga Laminaria, K^+ efflux played a key role in signal transduction of the brown algal oxidative burst (Kupper et al., 2001). Mastoparan which had no effect on PE *Symbiodinium* sp., caused the largest burst of the chemicals tested. In addition to activating G-proteins, mastoparan is also a venom which can cause membrane destabilization. The exact mechanism by which mastoparan caused the oxidative burst remains unknown.

4.2.5 Physical injury and calcium ionophore cause release of pseudopterosins from *Symbiodinium* sp. from PE

To further study the function of the pseudopterosins in the symbiont cells, the release mechanism of the compounds was examined when the cells were under stress. In section 4.2.1 and 4.2.4 of this study we showed that physical injury and calcium efflux due to calcium ionophore addition cause an oxidative burst, in this study we monitored the release of pseudopterosins that occurred coincident with the injury. Figure 23 illustrates the release of pseudopterosins from *Symbiodinium* sp. cells in response to sonic injury, calcium ionophore addition and Triton X-100 addition. All these treatments triggered the release of the compounds.

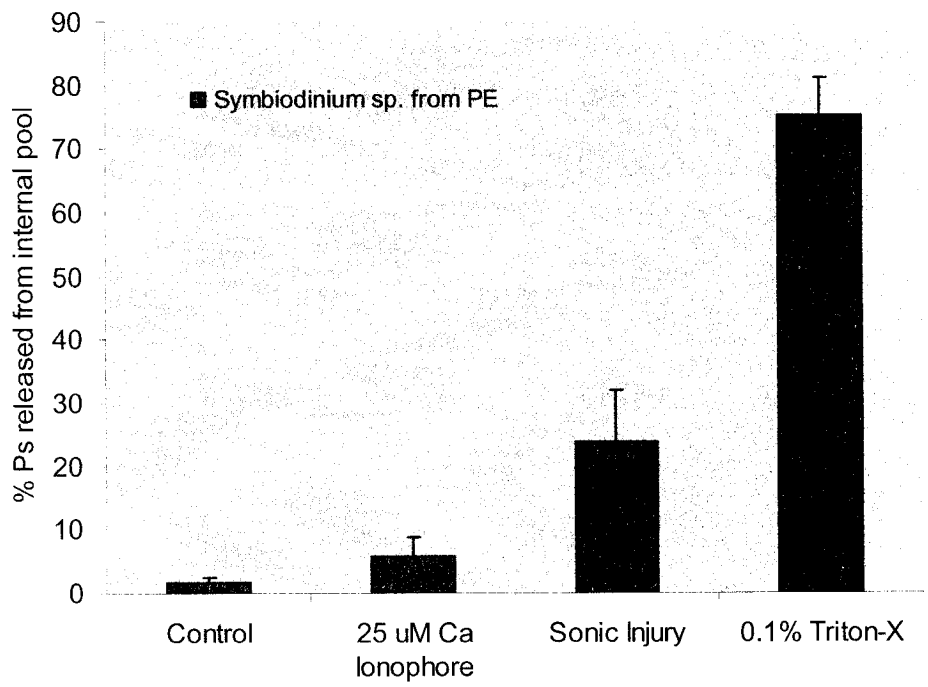


Figure 23: Release of pseudopterosins from internal pool after sonic injury, calcium ionophore and Triton X-100 treatment

The amount of pseudopterosins released from the cells was quantified by HPLC analysis. In the injured and calcium ionophore treated cells the release of pseudopterosins was a small percentage of the internal pseudopterosin pool, indicating an almost selective release. In contrast, the positive control Triton X-100 detergent extensively dissolved the cell membranes and caused a mass release of all internal cell contents, including the pseudopterosins. Using the HPLC chromatograms, the theory that there was a selective release of pseudopterosins in the injured and calcium ionophore treated cells was verified. The HPLC chromatograms of the extruded material were void of additional cellular contents and contained pure pseuodopterosins, unlike the HPLC chromatogram of the Triton X-100 treated cells, which contained many cellular metabolites.

The seemingly selective release of sizeable amounts of pseudopterosins over other intracellular metabolites in response to injury would indicate a role for the compounds in protecting the symbionts packed in the coral gastroepidermal cells or have implications to the entire coral colony, which can utilize the pseudopterosins for their own metabolism or as a defensive agent. The discriminatory release of the pseudopterosins in the calcium ionophore treatment may indicate that the pseudopterosins are stored in vesicles within the *Symbiodinium* sp. cell. Typical vesicles can be degranulated with calcium flux, and cause subsequent exocytosis of the contents.

4.2.6 Free fatty acid analysis of *Symbiodinium* sp. from PE and *H. pygmaea*

For further physiological comparisons, the free fatty acid content of PE *Symbiodinium* sp. and *H. pygmaea* cells used in these experiments was characterized. Fatty acids are important constituents of cells membranes, chloroplast membranes, they can be signaling molecules within the cell and are highly subject to enzymatic oxidation and cyclization due to environmental and physiological cues. The fatty acid distribution of PE *Symbiodinium* sp. and *H. pygmaea* cells are compared in figure 24.

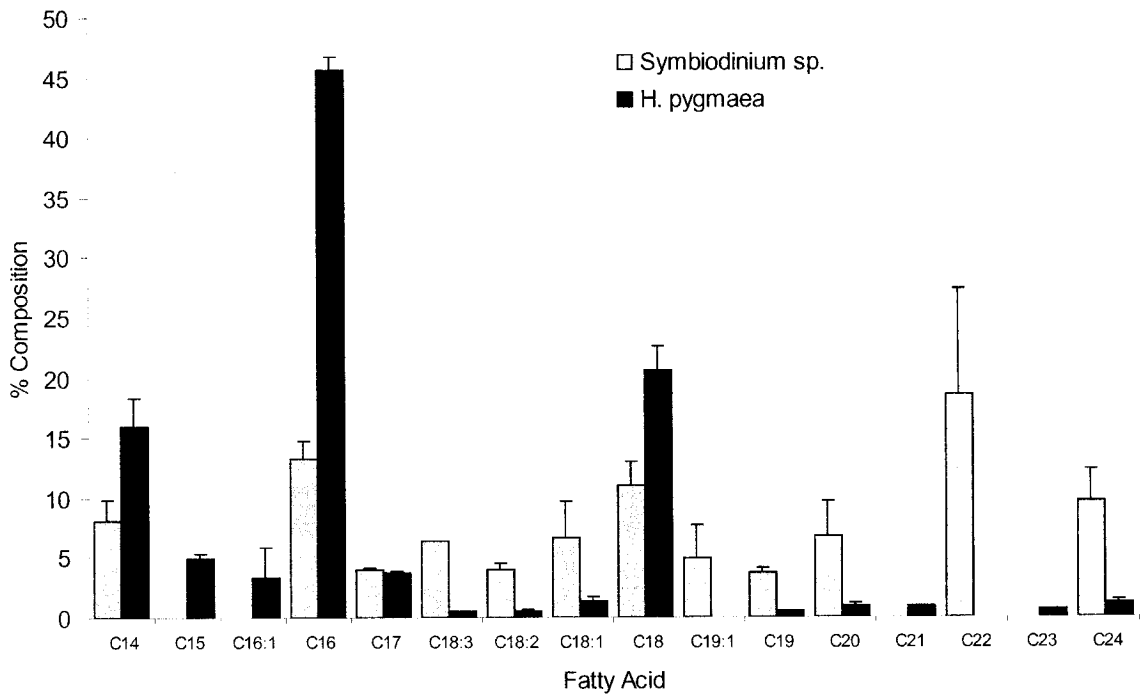


Figure 24: Percent composition of free fatty acids

One noticeable trend in the fatty acid content of both cell lines is the lack of highly unsaturated fatty acids, which are typically characteristic of dinoflagellates (Mansour et al., 1998). In free-living dinoflagellates, C16:0 and C18:5 are the most abundant fatty acids. In *Gymnodinium* sp. the abundance of C16:0 increased with culture age while the relative amount of C18:5 decreased (Mansour et al., 2003). *H. pygmaea* used in these experiments were in stationary growth phase and have been in continuous culture. Since unsaturated fatty acids are typically produced in response to environmental cues and can differ based on growth conditions, the suppression of polyunsaturated fatty acids in *H. pygmaea* can almost be expected due to the growth and culture conditions. As well, in this experiment only the free fatty acid portion of the lipids in *H. pygmaea* was examined, the phospholipids and glycolipid fatty acid content have been shown to differ in composition from each other and from free fatty acids (Leblond and Chapman, 2000), perhaps additional unsaturated fatty acids in *H. pygmaea* are present in other lipid fractions. The main saturated fatty acids of the dinoflagellates are C14:0, C16:0 and C18:0 (Zhukova and Aizdaicher, 1995) which are the abundant saturated fatty acids found in *H. pygmaea*.

The fatty acids of PE *Symbiodinium* sp. did contain substantially more mono and poly unsaturated fatty acids than *H. pygmaea* (figure 25). The PE *Symbiodinium* sp. cells used in this analysis were from wild collections and not cultures. In this study, PE *Symbiodinium* sp. contained a high percentage of C22:0 (25%). In comparative studies of fatty acids from related *Symbiodiunium* sp. there was a high concentration of unsaturated C22 fatty acids (Papina et al., 2003). This can be

possibly be explained by the fact that environmental and physiological conditions can cause the cell to adjust the amount of unsaturated fatty acids for signaling or cell membrane needs. This is supported by the fact that the free fatty acid content of *Symbiodinium* sp. does seem to differ based on the invertebrate host (Johnston, 1995) and growth within the host can cause variability in the fatty acid content and composition (Bishop et al., 1976).

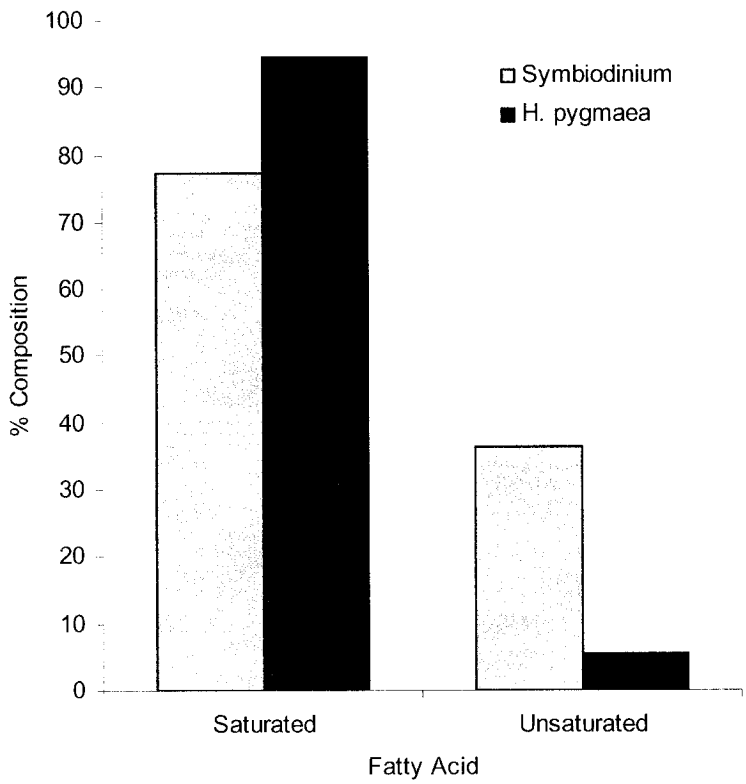


Figure 25: Comparison of fatty acid saturation levels of *Symbiodinium* sp. and *H. pygmaea* cells

4.3 GENERAL CONCLUSIONS

The present study documents the oxidative burst that occurs under physical stress and chemical stress conditions in dinoflagellates, a taxonomic group of marine phytoplankton, well known for the symbiotic relationships they form with a number of marine invertebrates and, in its free-living form, for the formation of harmful algal blooms (e.g. red tides). It is the first characterization of an enzymatic NAD(P)H mediated oxidative burst in the genera of *Symbiodinium* sp. and *Heterocapsa.* This study demonstrates that symbiotic dinoflagellates have a muted oxidative burst when compared to free-living *H. pygmaea* stressed under the same conditions. Particularly, the insensitivity to sonic membrane disruptions in PE *Symbiodinium* sp. may be in part be due to the presence of pseudopterosins that are concentrated within the cell (Mydlarz et al., 2003). *H. pygmeae* and PA *Symbiodinium* are both naturally devoid of pseudopterosins, however, exogenously added pseudopterosins to *H. pygmeae* and PA *Symbiodinium* provided the cells with similar protection and minimal response to physical injury. Therefore, we suggest a possible endogenous protective role for the pseudopterosins in the *P. elisabethae* coral – algal complex that may be ecologically significant to optimization of the symbiont lifestyle. Further experiments are aimed at elucidating the exact mechanism by which the pseudopterosins inhibit the oxidative burst.

Our results suggest that enzymatically induced (biological) production of ROS in addition to photochemical production can be a significant source of oxygen radicals in the marine environment and in coral-symbiont relationships. The

production of ROS as a result of an oxidative burst are known to have many functions in cell signaling and can alter protein and gene expression in metabolic pathways and lipid signals (Hensley et al., 2000). In dinoflagellates, several genes are expressed when the cells were under oxidative stress. In *Pyrocystis lunula* (Okamoto and Hastings, 2003) high ROS conditions caused the induction of genes involved in protein phosphorylation, signal transduction, photosynthesis and carbon metabolism, and the production of proteins for defense and detoxification of ROS molecules. More specifically, ROS have been implicated as signaling molecules in the pathways involved in cell growth, differentiation and apoptosis (Okamoto and Hastings, 2003). In the fresh water dinoflagellate, *Peridinium gatunense,* the production of H_2O_2 has been implicated in programmed cell death (Vardi et al., 1999) during dense bloom conditions that result in inorganic carbon depletion. In these studies the addition of catalase decomposed the H_2O_2 and apoptosis was prevented. These studies suggest that an oxidative burst may have an important role in algal bloom dynamics, especially when rapidly dividing cells accumulate and inorganic nutrients decline causing physiological stress on the algal population. On a physiological level, an oxidative burst can result in lipid peroxidation and formation of highly unsaturated fatty acids. These conjugated fatty acids are extremely reactive and are well known precursors to important cell signaling molecules (Hamberg, 1992). These lipid signals can act as chemotactic factors and cause cell aggregation (De Petrocellis and DiMarzo, 1994).

Lastly, from an evolutionary prospective, the presence of the oxidative burst in unicellular symbiotic and free-living dinoflagellates may indicate a conservation of these pathways as defense mechanisms in macroalgae, higher plants and even mammalian immune systems. In recent years, the paramount importance of ROS in disease processes has been recognized. For instance, redox-sensitive genes, similar to those identified in *Pyrocystis lunula* by Okamoto and Hastings (2003) have been identified in protein kinase cascades which induce inflammatory gene expression in mammalian cells (Hensley et al., 2000). Perhaps the detection and detailed analysis of an enzymatic oxidative burst in ancient, unicellular organisms such as dinoflagellates may have a widespread significance in the understanding of immune responses which contribute to chronic and degenerative diseases (Hensley et al., 2000). Thus ultimately there may prove to be a direct relationship between the effects of pseudopterosins on oxidative stress and their well known pharmacological activities.

4. 4 MATERIALS AND METHODS

Materials

2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescin diacetate; H_2DCFDA) was purchased from Molecular Probes, Eugene, OR. Diphenylene iodium chloride, Ascorbic acid, esterase (41 U/mg), catalase (3250 U/mg), mastoparan, calcium ionophore A23187, valinomyacin and monensin were purchased from Sigma.

Algal Cells

Cultures of *H. pygmaea* were generously provided by Dr. Barbara Prezelin and maintained at 17°C in L1 media without silica (Boczar and Prezelin, 1986). *P. elisabe*thae was collected in May of 2003 and January of 2004 at Sweetings Cay in the Bahamas at a depth of 10 m. *P. Americana were* collected in Long Key at 5m depth. Live coral were homogenized in a blender with 0.22μm filtered seawater and 10mM EDTA, and filtered through 4 layers of cheesecloth. Algal symbionts were pelleted out by centrifugation at 250 x g and subsequently washed 10 times with 40 ml clean filtered seawater and pelleted by centrifugation at 750 x g. *Symbiodinium* sp. cells were further purified on Percoll® step gradient of 20%, 40%, and 80% two or more times until <1% impurities were seen using light microscopy. *Symbiodinium* sp. culture 146 from *Oculina* were generously provided by Dr. Todd LaJeunesse. Cells were maintained at 25°C in filtered seawater.

Injury and drug treatment of cells

Cells diluted to 5 x10^5 cells/ml were subjected to three 10 second pulses of 20Khz sonic sound on a Fisherbrand sonioc dismembranor 200. Cell viability and shape post injury was measured using trypan blue incorporation under light microscopy. For chemically injured cells 75 uM calcium ionophore A23187, were added in DMSO. 75 uM valinomycin was added in ethanol.

Oxidative burst assay

One ml of live cells pre and post injury was added to 970 ul of sea water, 20 ul esterase (1 mg/ml, U) and 10 ul of 10mM DCFH-DA (0.5 mM final concentration) and stirred. The reaction mixture was read on a Perkin Elmer LS 50B Luminescence Spectrophotometer, excitation 488nm and emission 525 nm. Results were recorded after 3 minutes. The relative DCFH fluorescence of the oxidative burst was calibrated to a standard curve of hydrogen peroxide prepared with 1.7 ml seawater, 20 ul esterase and 0.5 mM DCFH-DA and various concentrations of 30% hydrogen peroxide prepared as a 5 mM stock in DI water. All experiments were repeated 3-10 times, data presented consists of mean and standard error of independent experiments.

Extraction and preparation of the pseudopterosins

Crude extracts *P. elisabethae* coral were partitioned between methanol/water (9:1) and hexanes, followed by partitioning between methanol/water (1:1) and chloroform. Pseudopterosins A through D were purified from the chloroform partion by normal phase HPLC with a hexane/ethyl acetate gradient (60:40 to 100% ethyl

acetate in 40 minutes) using UV detection at 283 nm using a Hitachi L-6200A Intelligent Pump connected to a L-4200 UV-Vis detector with Varian Chrompack C18 or silica columns. Peak areas corresponding to the pseudopterosins were used to quantify amounts from standard curves. The prepared formulation of PsA, B, C and D were dissolved in DMSO for biological experiments. Elisabethatriene was extracted from lyophilized coral using hexanes and purified on reverse phase HPLC (isocratic in methanol) at 240 nm.

Fatty acid analysis

Dinoflagellate cell pastes were extracted in chloroform: methanol (2:1) to extract crude lipids. The extracts were dried in vacuo and re-suspended in 100% methylene chloride and methyl esters prepared by exposure to diazomethane gas using the Lombardi method. The crude lipid extract was partioned on silica gel using hexane:ethylacetate (9:1) and the fatty acid methyl esters analyzed on a Hewlett-Packard 5790 Gas Chromatograph coupled to a VG 70SE double focusing mass spectrometer operating in EI mode. The GC column was a DB-5MS, 30 m x 0.25 mm i.d (J&W Scientific, Folsum, CA). The injector was operated on splitless mode and the carrier gas was helium. The program started at 50°C and ramped to 125°C at 18°C/min with continued heating to 255°C at 3c/min. Fatty acid methyl esters were identified by comparison of mass, retention time and fragmentation pattern to commercial standards. Hewlett-Packard Chemstation Enhancement Software was used for data analysis.

CHAPTER 5

HYDROGEN PEROXIDE RELEASE AS A PHYSIOLOGICAL STRESS SIGNAL IN GORGONIAN CORALS IN RESPONSE TO PHYSICAL INJURY AND HEAT STRESS

5.1 INTRODUCTION

5.1.1 Gorgonian physiology and stress response

The anatomy and histology of corals have been detailed by Fautin and Mariscol, 1991. The coral polyp is a cylindrical sac connected to other polyps by gastrovascular tissue and gastrovascular canals known as solenia and this assemblage forms the coral colony. In gorgonians the polyps are supported by a proteinaceous rod excreted by the axis epithelium and covered by a simple columnar epithelium that covers both the polyps and the interpolypal tissue. The internal and external epithelial layers are made up of several types of cells which function to protect the polyps, aid the polyps in capturing food and maintain the presence of symbiotic dinoflagllates within the tissue. The coral structure contains epitheliomuscular cells and a subepidermal nerve net which allows the polyps in the colony to contract for protective purposes, expand for food collection and communicate within the colony.

The mesogleal cells provide protection to the colony and represent a variety of cell types. The fibroblasts secrete matrix and collagen fibers, and the ameobocytes have demonstrated phagocytic activity as well as production of reactive oxygen species (ROS). In general the defensive and immune responses of gorgonians are not well understood and therefore considered non specific. Some basic defenses include the production of physical barriers through the action of prophenyoxidase, secretion of chemicals and bioactive molecules as discussed earlier in this dissertation (Chapter 2 and 3) and phagocytosis of microorgansimal pathogens. Olano and Bigger 2000, found that in the gorgonian *Swiftia exserta*, phagocytosis of foreign particles was associated with tissue trauma. Two hours after injury, phagocytic activity was observed in the granular amoebocytes in the immediate site of trauma. 24 hours post injury, there was extensive phagocytosis surrounding the wound. At this time, phagocytic cell types included granular amoebocytes, epidermal cells, sclerocytes, mesogleal cells, and gastrodermal cells of the solenia. These observations would suggest that trauma in *S. exserta* induces phagocytosis in cells not normally phagocytic. They were also able to detect peroxidase in the phagocytic cells.

5.1.2 Oxidative stress in corals

Recently, a few researchers have examined the use of oxidative stress biomarkers to determine coral responses to pathogens, injury and thermal stress (Downs et al., 2000; 2002; Brown et al., 2002; Hawkridge et al., 2000; Regioli et al., 1998; Lesser, 1997). The main techniques are the detection of anti-oxidant enzymes

such as superoxide dismutase and catalase, examining lipid peroxidation products and the family of peroxidases, such as glutathione peroxidase. In most examples of thermal and UV stress the production of ROS in the coral and symbionts is due to photochemical reactions. Although the exact signaling molecules for the detrimental physiological results of thermal and UV stress (coral bleaching) remains elusive, with Lesser, 1997 and Downs, 2002 supporting the hypothesis that oxidative stress drives coral bleaching.

5.1.3 Project goals

Some of the main threats to coral reefs are physical destruction due to natural storm events such as, hurricanes and wave action, in addition to destructive fishing techniques such as blasting fishing, boat anchor damage and ship groundings (Edinger et al., 1998; Riegl and Luke, 1998). Another main threat to coral reefs is thermal stress; the trend towards a warming climate has had many implications to the marine environment (Harvell, 1999). In the past, several severe coral bleaching events have corresponded with the El Nino Southern Oscillation, a time when the surface waters at tropical and sub-tropical latitudes warms significantly. Mass-bleaching events have been observed in the Cook Islands, Western and American Samoa, French Polynesia (Hoegh-Guldberg and Salvat, 1995) and the Great Barrier Reef (Marshall and Baird, 2000) Disease dynamics have also changed with increasing water temperatures (Harvell, 1999). Unfortunately the mechanisms underlying these stresses are poorly understood.

In this study the oxidative stress response in the form of an oxidative burst was examined in three species of tropical zooxanthellate gorgonian corals and one temperate azooxanthellate gorgonian. The oxidative burst was elicited using a physical stress in the form of sonic sound cavitation and heat stress. These stressors were chosen to mimic the main threats to coral health and coral diversity in the reef habitat.

5.2 RESULTS AND DISCUSSIONS

5.2.1 Physical injury induces an oxidative burst in gorgonian corals

Clippings of the whole coral association of *Pseudopterogorgia elisabethae*, *Pseudopterogorga americana, Eunicea fusca* and *Lophogorgia chilensis* were subjected to a non-lethal dose of low frequency sonic sound (20 kHz) in 3 (10 second) pulses. The low intensity and short pulses of sonic sound used in this experiment did not cause cell lysis, but some coral cells as well as symbiont cells were extruded during the physical agitation (as seen by examination under light microscopy). As seen in figure 26 all four corals showed variation in the magnitude of their oxidative burst.

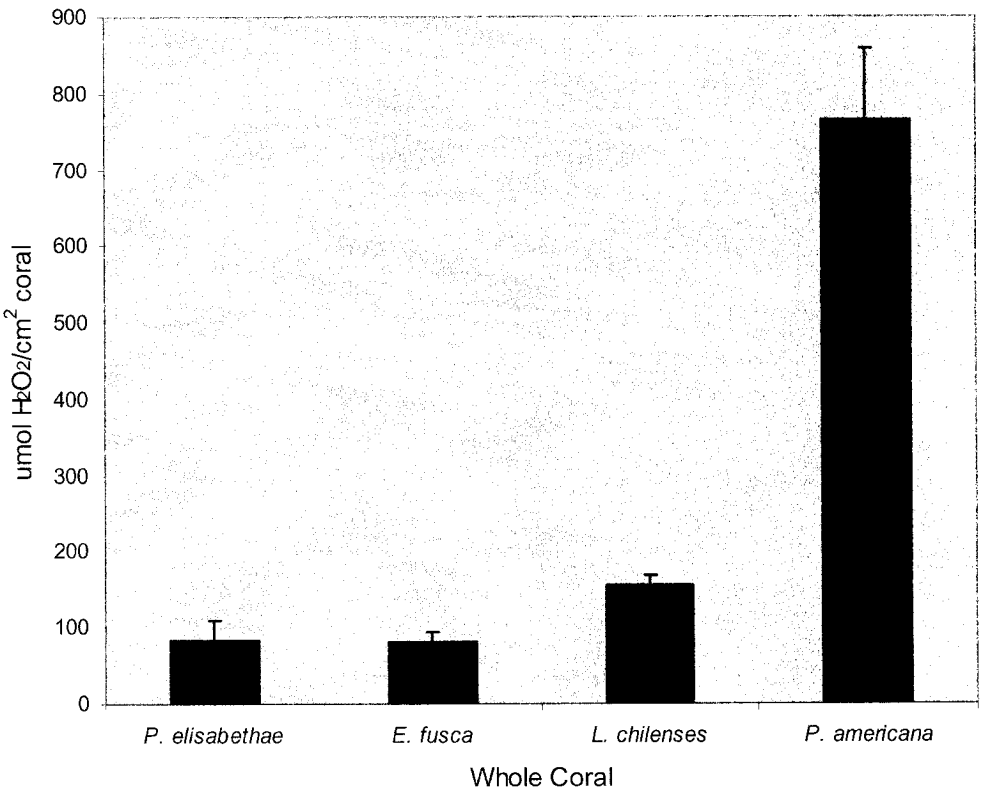


Figure 26: Oxidative burst due to sonic cavitation (injury) of clippings of four gorgonian corals (coral and symbionts intact). n=5.

P. elisabethae and *E. fusca* had responses that were 8-fold lower than the morphologically similar and closely related *P. Americana* ($n=5$, $p<0.01$) and about half of *L. chilensis*. As was demonstrated in previous experiments (Chapter 4, Mydlarz and Jacobs, 2004), the pseudopterosins are present in abundance in *P. elsiabethae* have cell stabilizing properties that may lead to increased resistance of *P. elisabethae* to injury. Interestingly, *E. fusca* contains a large amount (> 7% lipid weight) of the fuscosides (refer to figure 3 for structure), diterpenoid glycosides that exhibited very potent anti-inflammatory properties that were equal if not greater than the pseudopterosins (Jacobson and Jacobs, 1991a and 1991b). Both the pseudopterosin and fuscoside classes of compounds are structurally and pharmacologically unique and no similar compounds have since been reported from gorgonian corals.

P. americana does not contain a large concentration of diterpenoid compounds, they do, however contain some seco-steroids which demonstrated weak anti-inflammatory activity (He et al., 1995; Kerr et al., 1996). Healthy *P. americana* do have a large amount of mucosal polysaccharides and non-pathogenic bacteria that may play a role in potentially protecting the coral from disease (Johnston, 2003).

This surface protection may not be involved in cellular stabilization and therefore does not seem to impede the oxidative burst.

Lophogorgia chilensis, an azooxanthellate coral does exhibit an oxidative burst twice the magnitude of *P. elisabethae* and *E. fusca*. This is significant since this is evidence that these coral polyps are capable of an oxidative burst.

5.2.2 Characterization of the oxidative burst due to physical injury

To determine the enzymatic origin and the chemical composition of the oxidative burst in *Lophogorgia chilensis* and *P. Americana* in response to physical injury, the cells were incubated with diphenylene iodonium chloride (DPI) and catalase. Due to specimen availability and collection constraints, these two corals were chosen to further analyze the oxidative burst. *L. chilensis* represents an azooxanthellate coral and *P. Americana* demonstrated a strong oxidative burst.

DPI is a irreversible inhibitor of NAD(P)H oxidase and 50 μM of the drug did not inhibit the oxidative burst in *L. chilensis*. An increased concentration of 100 μM did have an effect of 79 ± 5 % inhibition. This inhibition of the oxidative burst caused by sonic sound by DPI implicates a similar mechanism to the superoxide-generating NAD(P)H oxidases of mammalian neutrophils. Since *L. chilensis* is azooxanthellate, the similarities in ROS production should be more analogous to ROS production in the animal kingdom than from plant models. In *P. americana* a higher dose of 150 μM DPI was chosen due to the fact that the release of H_2O_2 in *P. americana* was significantly greater than in *L. chilensis*. 150 μM inhibited the H_2O_2 release in *P. Americana* due to sonic injury by 79 ± 8% (n=3), indicating that

physical injury elicits an enzymatic oxidative burst similar to that of higher plants (Kupper et al., 2001).

Catalase, the heme-containing enzyme that catalyses the dismutation of hydrogen peroxide into water and oxygen was used to determine the chemical species present in the oxidative burst. Typically 1 unit of enzyme decomposes 1μmol of hydrogen peroxide at a pH of 7.0. In *L. chilensis*, the anti-oxidant effects of catalase were dose-dependant, and excess catalase (500-700 U/ml) actually caused an oxidative stress response possibly by irritating the coral polyps and stimulating oxygen radical signaling pathways. This effect is complex and cannot be fully understood by these experiments but provides some significant information that highlights the importance of determining dose-response curves. Concentrations of 50-100 U/ml did have an inhibitory effect and were able to degrade the H_2O_2 produced by an average of 71 ± 8 % (n=4). This could indicate that the oxidative burst in *L. chilensis* is composed mainly of H_2O_2, and suggests that the remainder represents other free radicals that will fluoresce upon reaction with DCFH-DA. Due to limited specimen availability only one dose of 200 U/ml was examined in *P. Americana*. This concentration did inhibit the fluorescence signal by 75 ± 13% (n=3) indicating that like *L. chilensis*, a majority of the ROS released was H_2O_2.

5.2.3 Peroxidase analysis of gorgonian corals

Many organisms have developed multiple mechanisms to detoxify from ROS damage following an oxidative burst. These defenses can be non-enzymatic scavengers of oxygen radicals or anti-oxidant enzymes. Peroxidases are a large

group of iso-enzymes with many cellular functions. They are involved with many processes such as lignification, wound healing, defense mechanisms, melanization, catalysis of natural product synthesis and production of glutathione and other anti-oxidants (Kawano, 2003; Hawkridge, 2000). Recently, in plants, an extracellular peroxidase has been implicated in producing free radicals from aromatic compounds and subsequently forming ROS (Kawano, 2003).

Due to the many important roles that the family of peroxidase enzymes play in the oxidative stress and defense mechanisms, the presence of peroxidase was examined in non-injured *P. elisabethae*, *P. Americana, E. fusca* and *L. chilensis* using native-PAGE gel electrophoresis. 0.3 mg of native protein of each coral was applied to the gel and subsequently activity stained with *o*-dianisine and H_2O_2 as a substrate to detect the presence of peroxidases in the protein extracts. Peroxidase activity was detected in all corals as demonstrated in Figure 27.

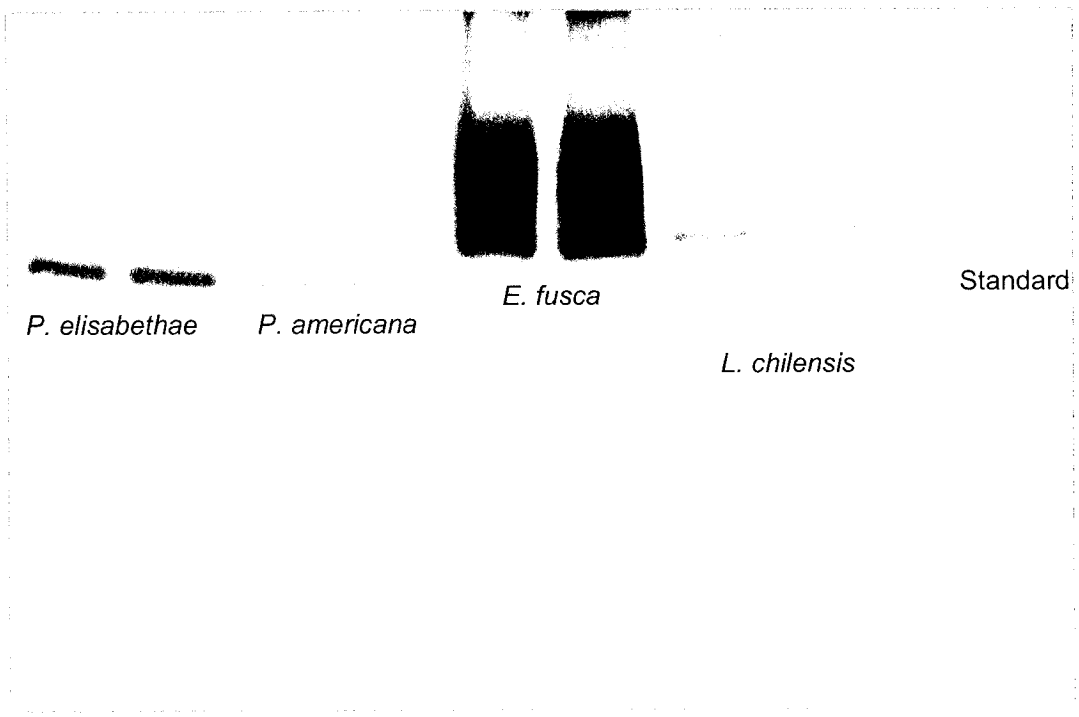


Figure 27: Native PAGE stained with *o*-dianisine for detection of peroxidase enzymes in gorgonian corals

P. elisabethae, P. Americana and *L. chilensis* all showed 2 bands of peroxidase isozymes. *E. fusca* seems to have peroxidase that is heavily glycosylated and therefore did not show proper separation on the gel. However this peroxidase detection technique is not quantifiable and we are unable to relate the amounts or

types of peroxidase enzymes to the production of ROS or the production of anti-oxidant molecules. The different peroxidase patterns of each individual coral species may play a role in the varied response of each gorgonian to physical injury.

5.2.4 Heat stress induces a release of ROS in tropical gorgonian corals

The tropical gorgonian corals, *Pseduopterogorgia elisabethae, Pseudopterogorgia Americana* and *Eunicea fusca* inhabit the waters of the Florida Keys and the Caribbean. The reefs in these areas are susceptible to warming and subsequent bleaching events. In order to determine if the oxidative burst or ROS are involved in the signal transduction pathway to heat stress, clippings of the three corals were kept in 33° C water and monitored for a period of 24 hours. Figure 28 illustrates the μmol of H_2O_2 released / cm^2 of coral.

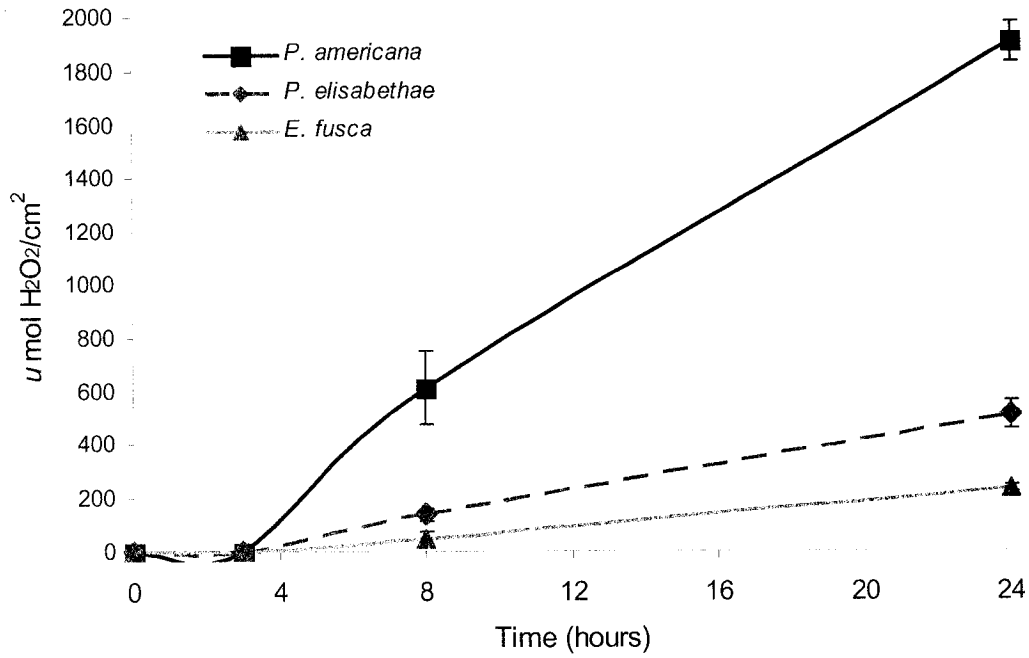


Figure 28: H_2O_2 release of tropical gorgonian corals due to heat stress.

A significant release of H_2O_2 does not occur until 4 hours post heat stress, the H_2O_2 release continues at different rates for the different corals. Similar to the injury experiments, *P. americana* has the greatest H_2O_2 release that was on average eight fold higher that that of *E. fusca* and nearly four fold greater in magnitude than the H_2O_2 release of *P. elisabethae*. The rates of H_2O_2 released for each coral are presented in table 7.

Coral	Initial Oxidative Burst (0-3 hour) umol H_2O_2/hour	Oxidative Burst (3 – 8 hour) umol H_2O_2/hour	Oxidative burst (8-24 hour) umol H_2O_2/hour	Total Rate (0-24 hour) umol H_2O_2/hour
P. americana	1	121	80	83
P. elisabethae	0	28	23	22
E. fusca	0	10	11.5	10

Table 7: Rates of H_2O_2 released in response to heat stress

P. americana had the largest magnitude of H_2O_2 released. The highest rate of H_2O_2 released for *P. americana* and *P. elisabethae* was during 3 to 8 hours of heat exposure. *E. fusca* had the same steady rate of H_2O_2 release during the entire 24 hour incubation. These data would imply that when exposed to heat, the corals need to reach a threshold of trauma before the stress responses are triggered. Once the stress responses are triggered, the corals seem to have a species dependant reaction. Coral exudates were examined under light microscopy post the 24 hour experiment, the exudates contained mostly coral cells and mucosal particles , but very little symbiont cells indicating that although the oxidative stress signal was present, bleaching had not readily occurred during this time period. True soft corals and gorgonian corals are susceptible to environmental stresses and disease, but in contrast to hard corals,

the complete release of symbionts is not as common or as obvious during environmental monitoring.

The coincidence of high temperatures and high irradiation with increased coral bleaching events is well documented (Hoegh-Guldberg, 2002). Several studies have shown that temperature stress in symbionts can lead to the detachment of the dinoflagellates from host cells (Gates et al., 1992). In addition, it has been reported that during mass-bleaching events the bleaching response and susceptibility to bleaching of scleratinoid coral taxa varies (Marshall and Baird, 2000). Furthermore different corals have different thresholds to temperature stress and variation among coral individuals of the same species has been seen during bleaching events. Cellular and molecular processes have been suggested as the basis for such variation (Downs et al., 2002).

The pharmacological basis for the release of ROS during heat stress in gorgonian corals was also examined. DPI, the NAD(P)H oxidase inhibitor was used to determine the source of ROS released during heat stress. *P. americana* was incubated with 150 μM DPI and heated to 33°C for a period of 24 hours. DPI inhibited the release of ROS by 36 ± 12 % (n=3), as evident by the standard error, there was significant variability in the inhibitory effects of DPI. For this reason the experiment was repeated using a larger dose of DPI (200 μM) and incubated at 33°C for a period of 12 hours. DPI did inhibit the release of ROS by 25 ± 6% (n=4), even with the altered experimental conditions the inhibitory effects were within the same range as the 24 hour experiment. This variability and poor inhibition of ROS release

by DPI can be possibly explained by the fact that more than one mechanism by which heat trauma can cause oxidative stress is functioning in *P. americana*. Heat stress can cause an enzymatic NAD(P)H oxidase mediated burst, or disrupt free radical flux from photosynthetic apparatus due to conformational changes in photosynthetic membranes (Lesser, 1987). Both these mechanisms have been documented in the literature. In barley seedlings, heat treatments caused an enzymatic oxidative burst and H_2O_2 accumulated after 15 minutes and 3 hours and lasted for several hours (Vallenian-Bindschendler, 1998), similar to the response seen in this present experiment. In *Symbiodinium microadriaticum* (Iglesis-Prieto et al., 1992) thermal stress inhibited photosynthesis due to an uncoupling of energy absorption and photochemistry. The authors presume this decrease in photosynthesis occurred due to heat induced lipid changes in the thylakoid membranes but the involvement of ROS was never addressed. The authors do note that the underlying mechanisms of thermal coral bleaching is due to signal transduction pathways which remain unknown.

Morphological differences between the DPI treated corals and the controls were observed in all experiments. DPI treated corals retained more of their original shape and had produced more mucosal/polysaccharide material post heat stress than the heated controls, which had substantially fallen apart, raising the possibility that the range of 25 to 36% inhibition of ROS production may have had some physiological significance. This can support the premise that heat causes various physiological stresses and membrane and protein breakdown in addition to a

complex oxidative stress response in which more than one mechanism of ROS production is at play. Further experiments are necessary to completely elucidate the effects of thermal stress on ROS production and to determine the contribution of an enzymatic oxidative burst.

5.3 SIGNIFICANCE

The present study of coral response to physical and temperature stress is the first to detect an oxidative burst in zooxanthellate and azooxanthellate gorgonian corals. The amount of H_2O_2 produced in each coral varies with coral species and may play a role in gorgonian vulnerability to injury, disease and climate-warming induced bleaching. These results support the observations of Marshall and Baird (2000), Brown et al. (2000 and 2002a) and Glynne et al. (1989) who noticed species variability and susceptibility to stressors in both scleratinian and gorgonian corals by algal invasion, herbivory, disease and bleaching.

While many of the mechanisms by which both scleratinian and gorgonian corals respond to stress remain unknown (Douglas, 2003), the significance of our study is that all corals do not physiologically react in the same manner. The plasticity observed in the responses of coral species to stress may be due to genetic factors which control the regulation of stress responses and may confer competitive advantages in the reef environment. Essential signaling molecules and signal transduction pathways which vary in different species of corals may have important ecological and physiological implications.

5. 5 MATERIALS AND METHODS

Materials

2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescin diacetate; H_2DCFDA) was purchased from Molecular Probes, Eugene, OR. Diphenylene iodium chloride, Ascorbic acid, esterase (41 U/mg) and catalase (3250 U/mg) were purchased from Sigma. 10% tris polyacrilimide gels were purchased from Biorad.

Coral specimens

P. elisabethae was collected in May of 2003 and January of 2004 at Sweetings Cay in the Bahamas at a depth of 10 m. *P. americana* and *Eunicea fusca* were collected from Alligator reef, off of the Keys Marine Laboratory in Long Key at 5-10 m depth. These tropical gorgonians were collected by Dr. Russell Kerr, Dr. Lory Santiago, Tyrone Ferns and staff of Keys Marine Lab. Corals were shipped to the culture facility at UCSB and kept in aquaria at 25°C. *Lophogorgia chilensis* was collected at Naples Reef off of Naples at a depth of 20 m by Shane Anderson.

Injury, heat and drug treatment of corals

Pieces of coral were subjected to three 10 second pulses of 20 KHz sonic sound on a Fisherbrand Sonic Dismembranor 200 or kept in a heat block at 33°C. Catalase was added in DI water and DPI was added 1 hour prior to injury in DMSO. Vehicle and heat controls also consisted of coral clippings and were kept in the same conditions as the experiments. Post-experiment, the diameter and length of the coral clippings were measured using a varnier caliper and measurements were used to

determine the surface area of the corals. All experiments were repeated 3-5 times, data presented consists of mean and standard error of independent experiments.

Oxidative burst assay

One ml of seawater supernatant pre and post coral injury was added to 970 ul of sea water, 20 ul esterase (1 mg/ml, 41 U) and 10 ul of 10mM DCFH-DA (0.5 mM final concentration) and stirred. The reaction mixture was read on a Perkin Elmer LS 50B Luminescence Spectrophotometer, excitation 488nm and emission 525 nm. Results were recorded after 3 minutes. The relative DCFH fluorescence of the oxidative burst was calibrated to a standard curve of hydrogen peroxide prepared with 1.7 ml seawater, 20 ul esterase and 0.5 mM DCFH-DA and various concentrations of 30% hydrogen peroxide prepared as a 5 mM stock in DI water. Hydrogen peroxide measurements represented the hydrogen peroxide released from coral into surrounding seawater.

CHAPTER SIX

GENERAL CONCLUSIONS AND HYPOTHETICAL MODEL OF SIGNAL TRANSDUCTION PATHWAYS LEADING TO AN OXIDATIVE BURST IN DINOFLGELLATES AND GORGONIAN CORALS

6.1 DETAILED DESCRIPTION AND VERIFICATION OF WORKING MODEL

A hypothetical model based on the experiments performed in this study (Chapter 4 and 5) and based on known plant and animal oxidative burst models was developed to suggest the causes and effects of an oxidative burst in gorgonian corals, *H. pygmaea* and symbiotic dinoflagellates (Figure 29 A and B). This model takes into account various signal transduction steps that have been studied in plant models and is based on the schematic representations of oxidative burst activation by Low and Merida (1996) and Wojtaszek (1997). The signal transduction steps of the oxidative burst elicitation are ae follows:

1) Mechanical injury and sonic sound act as an abiotic elicitor of the oxidative burst (Lin et al., 2001; Wu and Lin, 2002 and Low and Merida, 1996). This

has been verified in our model and does occur in dinoflagellates and whole gorgonian corals (Chapter 4 and 5).

2) The elicitor molecules bind to a receptor and activate a series of signal transduction steps which can include kinases and phosphatases, phospholipase A_2 and phospholipase C, G-protein activation and adenylate cyclase. These steps were not described in this present study and are based on the reviews of Wojtaszek (1997) and Low and Merida (1996).

3) Ion channels such as calcium, hydrogen, potassium and sodium when opened can signal an oxidative burst of superoxide anion which is rapidly converted to hydrogen peroxide by superoxide dismutase (Wojtaszek, 1997). This has been verified in our study to occur in symbiotic and the free-living dinofalgellate *Heterocapsa pygmaea*. When ionophores for potassium, calcium and sodium were added to cell suspensions, a release of ROS was detected.

4) The activation of the membrane bound NADPH oxidase due to steps 1) ans 2) generates ROS and an oxidative burst (Kupper, 2001; Wojtaszek, 1997). This was proven to occur in dinoflagellates and gorgonian corals by the use of diphenyiodiumchloride (DPI) which selectively inhibits NADPH oxidases. In our studies the oxidative burst due to sonic cavitations was inhibted when the cells or coral were treated with DPI prior to injury.

5) The ROS released from the activation of the NADPH oxidase can occur as superoxidee anion, hydroxyl radical and hydrogen peroxide. These oxygen radicals are labile and can be transformed from one species to the other enzymatically and

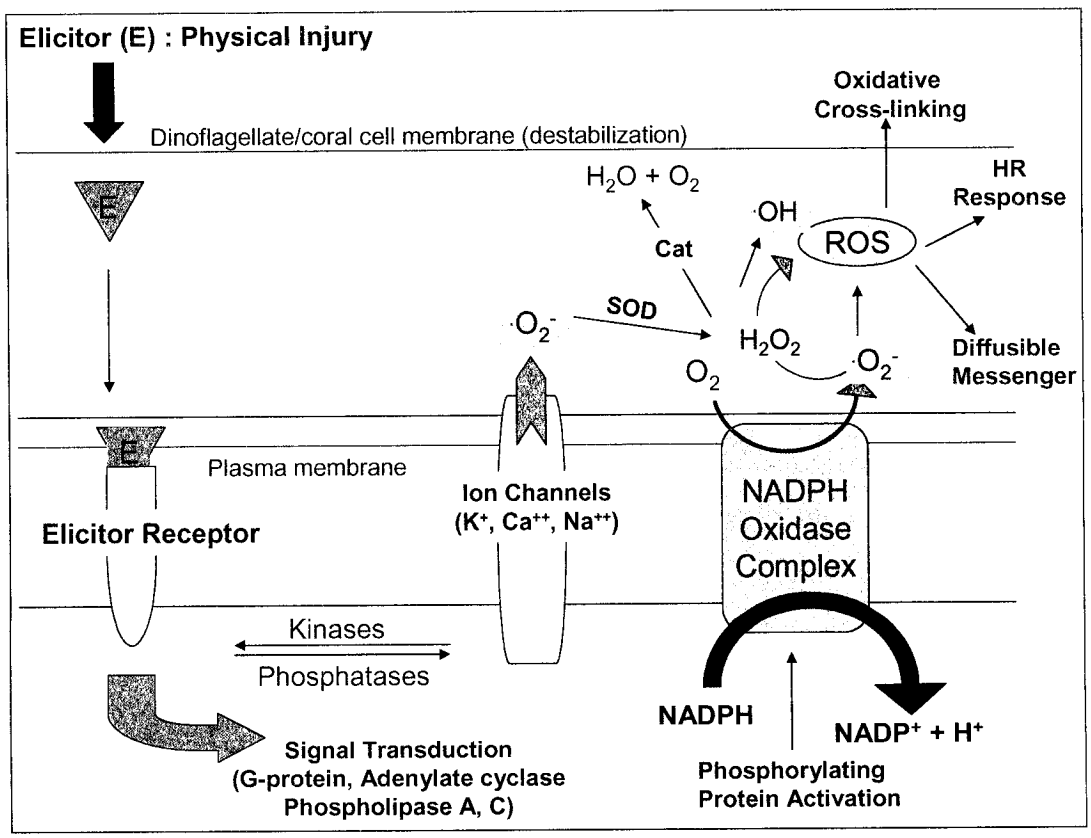
non-enzymatically. To determine the major chemical species of ROS in dinoflagellates and gorgonian corals, catalase was added to dissociate hydrogen peroxide to water and oxygen. In most cases catalase inhibited the fluorescence signal by over 70%, indicating that a majority of the ROS released in these organisms was hydrogen peroxide.

6.2 INHIBITORY EFFECTS OF THE PSEUDOPTEROSINS IN RELATION TO HYPTHETICAL MODEL

The mechanisms behind the inhibitory effects of the pseudopterosins on the oxidative burst were unfortunately not elucidated. Based on past and present pharmacological data, possible targets for the pseudopterosins in the signal transduction pathway of the oxidative burst can be postulated. Mayer et al. (1998) found that the pseudopterosins were inactive as phospholipase A inhibitors and cytokine release *in vitro*, therefore possibly eliminating these enzymes as mechanism of actions. Mayer et al., (1998) and Ettouati and Jacobs (1987) found that the pseudopterosins had cell membrane stabilizing properties in neutrophils and sea urchin embryos, respectively. These data represent a potential mechanism by which the pseudopterosins protect the dinoflagellate and coral cells against that cell membrane destabilization caused by sonic cavitations. Recent work in our lab by Moya and Jacobs (2003) has suggested that the pseudopterosins act on a G-protein receptor. This could too be a potential target for the pseudopterosins in the signal transduction of the oxidative burst.

Overall, the results of this thesis work provides a base of knowledge on which to pursue the study of biosynthetic origins of other important natural products in host-symbiont complexes, to examine the endogenous roles of marine natural products, and to begin to define the immune responses of dinoflagellates and corals to various ecological stressors.

A)



B)

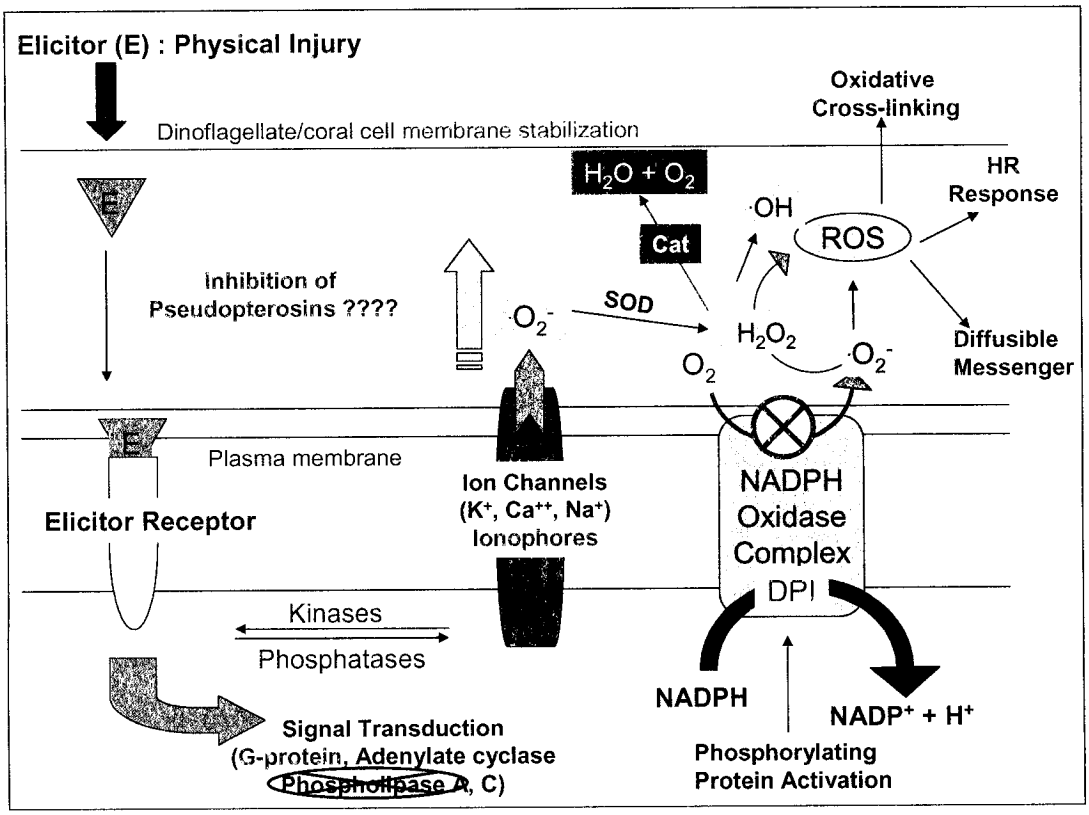


Figure 29: A) Scheme of oxidative burst of signaling pathways in corals and dinoflagellates based on plant and animal models B) Verification of the model using pharmacological probes to elucidate the signal transduction pathway of oxidative burst in corals and dinoflagellates.

REFERENCES

Asada K., Takahashi, M., 1987. Production and scavenging of active oxygen in photosynthesis. In: Kyle, D.J., Osmond, C.B., Arntzen, C.J., (Eds) Photoinhibition. Elsevier, Amsterdam, pp 228-287.

Bacurau, R.F.P., De Bittencourt, P.I., Homem, Jr., Newsholme, P., Rosa, L.F.,Costa B.P. 1999. Metabolic response of macrophages to injury promoted by the activated complement system. Cell Biochemistry and Function, 17(3), 175-182.

Baeuerle, R., Luetke-Brinkhaus, F., Ortmann, B., Berger, S. and Kleinig, H.1990. Prenyl lipid and fatty acid synthesis in isolated *Acetabularia* chloroplasts. Planta 181, 229-233.

Baldridge, C.W, Gerard, R.W., 1933 The extra respiration of phagocytosis. Am J Physiol 103, 235–236

Bandurragga, M.M, Fenical, W., Donovan, S.F., Clardy, J., 1982. Pseudopterolide, an irregular diterpenoid with unusual cytotoxic properties from the Caribbean sea whip *Pseudopterogorgia acerosa*. Journal of the American Chemical Society. 104, pp 6463-6465.

Baslow MH. 1969. Marine Pharmacology. The Williams and Wilkins Company, Baltimore, MD.

Bishop, D. G.,Bain, J. M., Downton, W.J.S., 1976. Ultrastructure and lipid composition of zooxanthellae from *Tridacna maxima*. Australian Journal of Plant Physiology, 3(1), 33-40.

Bishop, D.G.,Kenrick, J.R., 1980. Fatty acid composition of symbiotic zooxanthellae in relation to their hosts. Lipids, 15(10), 799-804.

Boczar, B.A., Prezelin, B.B. 1986. Light- and magnesium chloride-dependent characteristics of four chlorophyll-protein complexes isolated from the marine dinoflagellate, *Glenodinium* sp. Biochimica et Biophysica Acta . 850, 300-9.

Bohlmann, J., Meyer-Gauen, G., and Croteau, R.1998. Plant terpenoid synthases: Molecular biology and phylogenetic analysis. Proc. Natl. Acad. Sci. 95, 4126-4133.

Bolwell G.P., Butt, V.S.,Davies,.R.D., and Zimmerlin, A., 1995. The origin of the oxidative burst in plants. Free Rad. Res. 23, 517-532.

Bolwell G. P. and Wojtaszek P., 1997. Mechanisms for the generation of reactive oxygen species in plant defence - a broad perspective. Physiological and Molecular Plant Pathology 51, 347-366

Bonini C, Cooper C.B, Kazlauskas R, Wells RJ, Djerassi C. 1982. Minor and trace sterols in marine invertebrates.41. Structure and stereochemistry of naturally occurring 9,11-secosterols. Journal of Organic Chemistry. 48, 2108-2111.

Brash A.R., 1989. Formation of an allene oxide from (3R)-8-hydroperoxyeicosatetraenoic acid in the coral Plexaura homomalla. Journal of the American Chemical Society. 111, 1891-1892.

Branson Ultrasonics Corporation. 1974. Instruction manual for sonifier model W140. Danbury, Connecticut.

Broka, C.A, Chan, S., and Peterson, B., 1988. Total synthesis of levo-pseudopterosin A. J. Org. Chem. 53, 1584-1586.

Brown, B.E., Dunne,R.P.,Goodson,M.S.,Douglas,A.E.,2000.Bleaching patterns in reef corals. Nature 404,142–143 .

Brown, B.E., Downs,C.A., Dunne,R.P., Gibb,S.W., 2002.Exploring the basis of thermotolerance in the reef coral *Goniastrea aspera*. Marine Ecology Progress Series 242,119–129.

Brown, B.E., Dunne, R.P.,Goodson, M.S.,Douglas,A.E., 2002a.Experience shapes the susceptibility of a reef coral to bleaching. Coral Reefs 21,1191–12 26.

Buszek, K.R., and Bixby, D.L.,1995. Total synthesis of pseudopterosin A and E aglycon. Tetrahedron Letters 36, 2129-2132.

Ciereszko, L.S., 1989. Sterol and diterpenoid production by zooxanthellae in coral reefs: A review. Biological Oceanography 6, 363-374.

Coleman, A.C., Mydlarz, L.D., Kerr, R.G., 1999. *In vivo* and *in vitro* investigations into the biosynthetic relatedness of the pseudopterosins. Organic Letters 1, 2173-2175.

Coleman, A.C., Kerr, R.G., 2000. Radioactivity-guided isolation and characterization of the bicyclic pseudopterosin diterpene cyclase product from *Pseudopterogorgia elisabethae*. Tetrahedron 56, 9569-9574.

Collen, J., Davison, I.R., 1999. Reactive oxygen production and damage in intertidal *Fucus* spp. (Phaeophyceae). J. Phycol. 35, 54–61

Crick D.C., Suders J., Kluthe C.M., Andres D.A., Waechter C.J., 1995. Selective inhibition of cholesterol biosynthesis in brain cells by squalestatin I. Journal of Neurochemistry. Bol. 65, pp 1365-1373.

Culioli, G., Daoudi, M., Mesguiche, V., Valls, R., Piovetti, L., 1999. Geranylgeraniol-derived diterpenoids from the brown alga *Bifurcaria bifurcata*. Phytochemistry , 52(8)

Cvejic, J.L., and Rohmer, M. 2000. CO_2 as main carbon source for isoprenoid biosynthesis via the mevalonate-independent methylerythritol 4-phosphate route in the marine diatoms *Phaeodactylum tricornutum* and *Nitzschia ovalis*. Phytochemistry 53, 21-28.

Deutscher M.P.,1990. Methods in enzymology Volume 182: Guide to protein purification. Academic Press Inc. San Diego, CA.

De Petrocellis, L. and Di Marzo, V. 1994. Aquatic invertebrates open up new perspectives in eicosanoid research: biosynthesis and bioactivity. Prostaglandins, Leukotrienes and Essential Fatty Acids, 51, 215-229

Dezwart L.L., Meermen J.H.N, Commandeur J.N.M., and Vermuleun N.P.E., 1999. Biomarkers of free radical damage; applications in experimental animals and in humans. Free Radical Biology & Medicine, 26, 1/2, pp. 202–226

DOE .1994. Handbook of methods for the analysis of various parameters of the carbon dioxide system in sea water; version 2.1. Dickson, A.G. and Goyet, eds, ORNL/CDIAC-74

Douglas, A.E., 2003. Coral bleaching – how and why? Marine Pollution Bulletin, 46, 385-392.

Downs, C.A., Mueller,E., Phillips, S., Fauth, J., Woodley, C. M.., 2000. A molecular biomarker system for assessing the health of coral (*Montastrea faveolata*) during heat stress. Mar. Biotechnol. 2, 533–544.

Downs, C.A., Fauth, J.E., Halas, J.C., Dustan, P., Bemiss, J., Woodley, C.M., 2002. Oxidative stress and seasonal coral bleaching. Free Radical Biology & Medicine 33 (4), 533-543.

Dwyer S.C., Legendre L., Low P.S., Leto T.L., 1996. Plant and human neutrophil oxidative burst complexes contain immunologically related proteins. Biochim Biophys Acta, 1289, 231–237.

Ettouati W.S, Jacobs R.S., 1987. Effect of pseudopterosin A on cell division, cell cycle progression, DNA, and protein synthesis in cultured sea urchin embryos. Molecular Pharmacology, 31, pp 500-505.

Faulkner D.J., 1984. Marine natural products: Metabolites of marine invertebrates. Natural Products Reports, pp 575-579.

Fautin, D.G., Mariscol, R.N., 1991. Cnideria:anthozoa. In: Microscopic anatomy of invertebrates, Vol 2. Placozoa, Porofera, Cnideria and Ctenophora. Hyman, L. (ed), Wiley-Liss, New York, 267-358

Fenical W. 1987. Marine soft corals of the genus *Pseudopterogorgia*: A resource for novel anti-inflammatory diterpenoids. Journal of Natural Products, 50, 6, pp 1001-1008.

Fenical, W.,1997. New pharmaceuticals from marine organisms. Trends in Biotechnology 15, 339-341.

Fenical W., Okuda R.K., Bandurraga M.M., Culver P., Jacobs R.S.,1981. Lophotoxin: a novel neuromuscular toxin from Pacific sea whips of the genus *Lophogorgia*. Science 212(4502), 1512-4.

Gates,RD., Baghdasarian,G., Muscatine, L.,1992. Temperature stress causes host cell detachment in symbiotic cnidarians: implications for coral bleaching. Biological Bulletin 182, 324–332.

Ghisalberti, E.L.1992. A tricyclic diterpene from *Eremophila serrulata*. Phytochemistry, 31, 2168-2169.

Ghisalberti, E.L., Jefferies, P.R, Trevor, A, Twiss, E., 1994. New Cembrene diterpenes from resins of Eremophila species. J. of Natural Products, 57 (1), 100-104.

Govind, N. S.; Roman, S. J.; Iglesias-Prieto, R.; Trench, R. K.; Triplett, E. L.; Prezelin, Barbara B. 1990. An analysis of the light-harvesting peridinin-chlorophyll a-proteins from dinoflagellates by immunoblotting techniques. Proceedings of the Royal Society of London, Series B: Biological Sciences 240(1297), 187-95.

Gray, C.A., Davies-Coleman, M.T., Shleyer, M.H., 2000. Cembranoid diterpenes from the Southern African soft coral *Cladiella kashmani*. J. of Natural Products, 63 (11), 1551-1553

Grode, S.H., James, T.R, Cardellina, J.H., 1983. Brianthium Z, a new polyfunctional diterpene from the gorgonian *Briarium polyanthes*. Tetrahedron Letters. 24, pp 691-694.

Hagen, C.and Grunewald, K., 2000. Fosmidomycin as an inhibitor of the non-mevalonate terpenoid pathway depresses synthesis of secondary carotenoids in flagellates of the green alga *Haematococcus pluvialis*. Journal of Applied Botany 74, 137-140.

Hamberg, M.,1992. Metabolism of 6,9,12-octadecatrienoic acid in the red alga Lithothamnion corallioides: mechanism of formation of a conjugated tetraene fatty acid. Biochem. Biophys. Re. Comm., 188, 1220-1227

Harvell, C.D., Fenical, W., Greene, C.H. 1988. Chemical and structural defenses of carribean gorgonians (*Pseudopterorgorgia* spp.). I. Development of an in situ feeding assay. Marine Ecology Progress Series. 49, pp 287-294.

Harvell,C.D., Kim,K.,Burkholder,J.M., Colwell,R.R.,Epstein, P.R.,Grimes, D.J.,Hofmann, E.E.,Lipp, E.K.,Osterhaus, A.D.M.E.,Overstreet,R.M.,Porter,J.W., Smith,G.W., Vasta, G.R., 1999. Emerging marine diseases—climate links and anthropogenic factors. Science, 285,1505–1510.

Hawkridge, J.M., Pipe, R.K and Brown, B.E. 2000. Localization of anti-oxidant enzymesin the cniderians *Anemonia viridis* and *Goniopora stokesi*. Marine Biology, 137, 1-9.

He, H., Kulanthaivel, P., Baker, B.J., Kalter, K., Darges, J., Cofield, D., Wolff, L., Adams, L., 1995. New anti-proliferative and anti-inflammatory 9,11-secosterols from the gorgonian *Pseudopterogorgia* sp. Tetrahedron, 51, 1, pp 51-58.

He, Y.Y., Hader, D.P., 2002. Involvement of reactive oxygen species in the UV-B damage to the cyanobacterium *Anabaena* sp. Journal of Photochemistry and Photobiology B: Biology, 66 73–80

Hemmerlin, A. 2003. Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells. Journal of Biological Chemistry *278*, 26666-26676.

Hendrickson, J.B., 1973. The Molecules of Nature: A survey of the biosynthesis and chemistry of natural products. W.A Benjamin, INC. Reading, MA.

Hensley, K., Robinson, K.A., Gabbita, S.P., Salsman, S., Floyd, R.A., 2000. Reactive oxygen species, cell signaling and cell injury. Free Radical Biol. and Med. 28, 1456-1462.

Humann, P. 1993. Reef Coral Identification: Florida, Caribbean, Bahamas. New World Publications, INC. pp 51-53.

Iglesias-Prieto, R., Govind, N. S.,Trench, R. K., 1993. Isolation and characterization of three membrane-bound chlorophyll-protein complexes from four dinoflagellate species. Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences, 340(1294), 381-92.

Jabs, T., Tschöpe, M., Colling, C, Hahlbrock, K and Sheed, D., 1997. Elicitor-stimulated ion fluxes and O2- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. Proc. Natl. Acad. Sci. USA, 94, 4800-4805

Jacobson, P.B., and Jacobs, R.S., 1992. Fuscoside: an anti-inflammtory marine natural product which selectively inhibits 5-lipoxyoxygenase. Part I: Physiological and biochemical studies in murine inflammtory models. Part II: Biochemical studies in human neutrophils. The Journal of Pharmacology and Experimental Therapeutics. Vol. 262 (2), pp 866-882.

Johnston, M., Yellowlees, D.,Gilmour, I., 1995. Carbon isotopic analysis of the free fatty acids in a tridacnid-algal symbiosis: interpretation and implications for the symbiotic association. Proceedings of the Royal Society of London, Series B: Biological Sciences, 260(1359), 293-7.

Johnsen, G., Prezelin, B.B., Jovine, R.V.M., 1997. Fluorescence excitation spectra and light utilization in two red tide dinoflagellates. Limnology and Oceanography , 42(5, Pt. 2), 1166-1177.

Johnsen, G., Nelson, N.B., Jovine, R.V.M ., Prezelin, B.B., 1994. Chromoprotein- and pigment-dependent modeling of spectral light absorption in two dinoflagellates, *Prorocentrum minimum* and *Heterocapsa pygmaea*. Marine Ecology: Progress Series.

Katzenellenbogen, J.A., Crumarine, A.L., 1976. Selective γ alkylation of dienolate anions derived from α, β- unsaturated acids. Applications to the synthesis of isoprenoid olefins. Journal of the American Chemical Society. 98, 16, pp 4925-4935.

Kerr, R.G., Rodriguez, L.C., Kellman, J., 1996. A chemoenzymatic synthesis of 9(11)- secosteroids using an enzyme extract of the marine gorgonian Pseudopterogorgia americana. Tetrahedron Letters. 37(46), pp 8301-8304.

Kim, C.S., Lee, S.G., Lee, C.G., Kim, H.G., Jung J., 1999. Reactive oxygen species as causative agents in the ichthyotoxicity of the red tide dinoflagellate *Cochlodinium polykrikoides*. Journ Plank. Res. 21(11) .2105–2115.

Kim, D., Nakamura, A., Okamoto,T., Komatsu,N., Oda, T., Ishimatsu, A., and Muramatsu, T. 1999. Toxic potential of the raphidophyte *Olisthodiscus luteus*: mediation by reactive oxygen species. Journal of Plankton Research, Vol. 21 (6) 1017-1027.

Kim, D., Nakamura, A., Okamoto, T., Komatsu, N., Oda, T., Iida, T., Ishimatsu, A., Muramatsu, T. 2000. Mechanism of superoxide anion generation in the toxic red tide phytoplankton *Chattonella marina*: possible involvement of NAD(P)H oxidase. Biochimica et Biophysica Acta.1524, 220-227.

Kokke, W.C.M.C., Epstein, S., Look, S.A., Rau, G.H., Fenical, W. and Djerassi, C. (1984). On the origin of terpenes in symbiotic associations between marine invertebrates and algae (zooxanthellae). J. Biol. Chem. 259, 8168-8173.

Kumar, B., Andreatta, C., Koustas, W.T., Cole, W.C., Edwards-Prasad, J.and Prasad, K.N., 2002. Mevastatin induces degeneration and decreases viability of cAMP-induced differentiated neuroblastoma cells in culture by inhibiting proteasome activity, and mevalonic acid lactone prevents these effects. Journal of Neuroscience Research 68, 627-635

Kupper, F.C., Kloareg, B., Guern, J., Potin, P., 2001 Oligoguluronates Elicit an Oxidative Burst in the Brown Algal Kelp *Laminaria digitata*. Plant Physiology, 125, pp. 278–291

LaJeunesse, T.C., 2001. Investigating the biodiversity, ecology and phylogeny of endosymbiotic dinoflagellates in the genus *Symbiodinium* using the ITS region: In search of a "species" level marker. J. Phycol. 37, 866-880.

LaJeunesse, T.C., 2002. Diversity and community structure of symbiotic dinoflagellates from Caribbean coral reefs. Marine Biology, 141, 387-400.

Leblond, J.D., Chapman, P.J., 2000. Lipid class distribution of highly unsaturated long chain fatty acids in marine dinoflagellates. Journal of Phycology, 36, 1103-1108

Lesser, M.P., 1997. Oxidative stress causes coral bleaching during exposure to elevated temperatures. Coral Reefs, 16, 187-192.

Lesser M.P., 1996. Elevated temperatures and ultraviolet radiation cause oxidative stress and inhibit photosynthesis in symbiotic dinoflagellates. Limnolo.Oceanogr. 41(2), 271-283

Lin, L., Wu, J., Ho, K. P., Qi, S., 2001. Ultrasound-induced physiological effects and secondary metabolite (saponin) production in *Panax ginseng* cell cultures. Ultrasound in medicine & biology , 27(8), 1147-52.

Loeblich A.R. III, Schmidt R.J., Sherley J.L., 1981. Scanning electron micrography of *Heterocapsa pygmaea* sp. nov. and evidence for polyploidy as a speciation mechanism in dinoflagellates. J. Plankton Res. 3, 67-79

Look, S. A., Fenical, W., Jacobs R.S., and Clardy, J. 1986. The pseudopterosins: Anti-inflammatory and analgesic natural products from the sea whip *Pseudopterogorgia elisabethae*. Proc. Natl. Acad. Sci. USA, 83, 6238-6240.

Look, S.A., Fenical, W., Matsumoto, G.K., and Clardy, J. 1986.The pseudopterosins: A new class of anti-inflammatory and analgesic diterpene pentosides from the marine sea whip *Pseudopterogorgia elisabethae*. J. Org. Chem. 51, 5140-5145.

Look, S.A., Fenical, W., 1987. The seco-pseudopterosins, new anti-inflammatory diterpene glycosides from a caribbean gorgonian octocoral of the genus *Pseudopterogorgia* . Tetrahedron, 43 (15), pp 3363-3370.

Low, P.S., Merida J.R., 1996. The oxidative burst in plant defense: Function and signal transduction. Physiologia Plantarium, 96, pp 533-542.

Mansour, M.P., Volkman, J. K., Jackson, A.E.,Blackburn, S.I., 1999. The fatty acid and sterol composition of five marine dinoflagellates. Journal of Phycology, 35(4), 710-720.

Mansour, M.P.,Volkman, J. K., Holdsworth, D.G., Jackson, A.E., Blackburn, S.I.,1999. Very-long-chain (C28) highly unsaturated fatty acids in marine dinoflagellates. Phytochemistry, 50(4), 541-548.

Marshall, P.A., Baird, A.H., 2000. Bleaching of corals on the great barrier reef: differential susceptibilities among taxa. Coral Reefs, 19, 155-163.

Marshall, J-A., Nichols P.D., Hamilton, B., Lewis, R. J., Hallegraeff, G.M., 2003. Ichthyotoxicity of *Chattonella marina* (Raphidophyceae) to damselfish (*Acanthochromis polycanthus*): the synergistic role of reactive oxygen species and free fatty acids. Harmful Algae 2, 273–281

Mayer, A.M.S, Jacobson, P.B, Fenical W., Jacobs, R.S., and Glaser, K.B., 1998. Pharmacological characterization of the pseudopterosins: Novel anti-inflammatory natural products isolated from the Caribbean soft coral, *Pseudopterogorgia elisabethae.* Pharmacology Letters 62, 401-408.

Michalek-Wagner, K., Bourne, D.J. and Bowden, B.F. 2001. The effects of different strains of zooxanthellae on the secondary-metabolite chemistry and development of the soft-coral host *Lobophytum compactum.* Marine Biology *138*, 753-760.

Morel, F., Doussiere, J., Vignais, P.V., 1991. The superoxide generating oxidase of phagocytic cells. Eur. J. Biochem. 20, 523–546

Moya, C.E., and Jacobs, R.S., 2003. Unique effects of pseudopteroain A on calcium mobilization and phagosome formation. International Association of Inflammation Societies, Inflammation 2003. Vancouver, B.C. Canada

Muscatine L, and Porter J.W., 1977. Reef corals: mutualistic symbiosis adapted to nutrient poor environments. BioScience 27, 454-60.

Mydlarz, L.D, Jacobs R.S., Bohnlein, J and Kerr, R.G. 2003. Pseudopterosin biosynthesis in *Symbiodinium* sp., the dinoflagellate symbiont of *Pseudopterogorgia elisabethae.* Chemistry and Biology. 10, (11) 1051-1056.

Nelson, N.B., Prezelin, B.B., 1993. Calibration of an integrating sphere for determining the absorption coefficient of scattering suspensions.Applied Optics 32(33), 6710-17.

Nelson, N., Prézelin B.B. 1990. Chromatic light effects and physiological modeling of absorption properties of *Heterocapsa pygmaea* (aka. *Glenodinium* sp.). Marine Ecology Progress Series. 63, 37-46.

Oda, T., Ishimatsu, A., Takeshita, S., Muramatsu, T., 1994. Hydrogen peroxide production by the red-tide flagellate *Chattonella marina*.Bioscience, Biotechnology, and Biochemistry. 58, 957-8.

Okamoto, O.K., Hastings, J. W., 2003. Genome-wide analysis of redox-regulated genes in a dinoflagellate. Gene, 321, 73-81.

Olano, C.T., Bigger, C.H., 2000. Phagocytic Activities of the Gorgonian Coral *Swiftia exserta.* Journal of Invertebrate Pathology 76, 176–184.

Page, M.I., 1984. New comprehensive biochemistry: Volume 6. The chemistry of enzyme action. Elsevier Science Publishers B.V. New York, NY. pp 252.

Palenik, B., Zafririou, O.C. and Morel, F.M.M., 1987. Hydrogen Peroxide production by a marine phytoplankter. Limnolo.Oceanogr. 32(6), 1365-1369.

Palenik, B.P.,Morel, F.M.M., 1988. Dark production of H_2O_2 in the Sargasso Sea. Limnol. Oceanogr. 33, 1606–1611.

Papina, M, Meziane, T., and Van Woesik, R,. 2003 Symbiotic zooxanthellae provide the host-coral *Montipora digitata* with polyunsaturated fatty acids. Comparative Biochemistry and Physiology Part B, 135, 533–537

Papastephanou, C. and Anderson, D.G. 1982. Crassin acetate biosynthesis in a cell-free homogenate of zooxanthellae from *Pseudoplexaura porosa* (Houttyun): Implications to the symbiotic process. Comp. Biochem. Physiol. 73B, 617-624.

Pawlik, J.R., Burch, M.T., Fenical, W., 1987. Patterns of chemical defense among caribbean gorgonian corals: a preliminary survey. Journal of Experimental Marine Biology and Ecology. 108, pp 55-66.

Pick, E., Keisari, Y., Bromberg, Y.,Freund, M., Jakubowski, A., 1980. The oxidative burst in macrophages - generation of superoxide and hydrogen peroxide; lipid peroxidation and effect on cyclic nucleotide levels. Heterog. Mononucl. Phagocytes, [Proc. Int. Workshop] 331-8.

Pickney, J.L., Millie D.F., Howe K.E, Paerl H.W. and Hurley J.P., 1996. Flow scintillation counting of ^{14}C-labeled microalgal photosynthetic pigments. Journal of Plankton Research 18 (10), 1867-1810

Regioli, F., Winston, G.W., 1998. Applications of a new method for measuring the total oxyradical scavenging capacity in marine invertebrates. Marine Environ. Research, 46, 439-442.

Rice, J.R., Papastephanou, C., Anderson, D.G., 1970. Isolation, localization and biosynthesis of crassin acetate in *Pseudoplexaura porosa* (Houttyuyn) Biol. Bull. 138, 334-343.

Richards, J.H., Hendrickson, J.B., 1964. The Biosynthesis of Steroids, Terpenes, and Acetogenins. W.A. Benjamin, INC. pp 1-9, 240-253.

Richter, M., Ruhle, W. and Wild A. 1990. Studies on the mechanism of photosystem 2 photoinhibiton I. The involvement of toxic oxygen species. Photosynthesis Research. 24, pp 237-243.

Riegl, B., Luke, K.E., 1998. Ecological Parameters of Dynamited Reefs in the Northern Red Sea and their relevance to Reef Rehabilitation. Marine Pollution Bulletin. 37(8,12) 488-498.

Rodriguez AD, Pina IC, Soto JJ, Rojas DR, Barnes C. 1995. Isolation and structures of the uprolides.I. Thirteen new cytotoxic cembranolides from the caribbean gorgonian Eunicea mammosa. Canadian Journal of Chemistry, 73, pp 643-654.

Ross, C., Küpper, F., Vreeland, V., Waite, J.H., Jacobs R.S., 2004.Evidence of a Latent Oxidative Burst in Relation to Wound Repair in the Giant Unicellular Chlorophyte *Dasycladus vermicularis*. (J. of Phycology, Manuscript Submitted)

Rouhi M. 1995. Supply issues complicate trek of chemicals from sea to market. Science and Engineering News, Nov. 20, 1995, pp 42-44.

Roussis, V., Wu, Z and Fenical, W., Strobel, S.A., Van Duyne, G.D. and Clardy, J. (1990). New anti-inflammatory pseudopterosins from the marine octocoral *Pseudopterogorgia elisabethae.* J. Org. Chem. 55, 4922-4925.

Sanchez J.A., Lasker H.R ., 2004.Do multi-branched colonial organisms exceed normal growth after partial mortality? Proceedings of the Royal Society of London. Series B. Biological sciences, 271, Suppl 3 S117-20.

Santos, S.R., Taylor, D.J, Kinzie, R.A., Hidaka, M., Sakai, K., Coffroth, M.A., 2002. Molecular phylogeny of symbiotic dinoflagellates inferred from partial chloroplast large subunit (23S)-rDNA sequences. Molecular Phylogenetics and Evolution 23, 97–111

Santos, S.R., Gutierrez-Rodriguez, C., Coffroth, M.A., 2003. Phylogenetic identification of symbiotic dinoflagellates via length heteroplasmy in domain V of chloroplast large subunit (23S)-ribosomal DNA sequences. Marine Biotechnology, 5, 130-140.

Scopes, R.K., 1994. Protein Purification: Principles and practice. Third edition. Springer- Verlag, New York. Pp 22-43.

Scheuer, P.J., 1978. Marine Natural Products: Chemical and Biological Perspectives. Volume II. Academic Press, INC. New York, NY. Pp 76-159, 247-291.

Schmitz, K., Kremer, B.P., 1977. Carbon fixation and analysis of assimilates in a coral-dinoflagellate symbiosis. Marine Biology 42, 305-313.

Schwender, J., Germunden, C. and Lichtenthaler, H.K., 2001. Chlorophyta exclusively use the 1-deoxyxylulose 5-phosphate/2-C-methylerythritol 4-phosphate pathway for the biosynthesis of isoprenoids. Planta *212*, 416-423

Schlichter, D., Svoboda, A., and Kremer, B.P., 1983. Functional autotrophy of *Heteroxenia fuscescens*: carbon assimilation and translocation of photosynthates from symbiont to host. Marine Biology, 78, 29-38.

Selover, S.J., Crews P., Tagle, B., Clardy, J., 1981. New diterpenes from the common caribbean gorgonian *Briarium abestinium*. Journal of Organic Chemistry. 46, pp 964-970.

Shigemori, H., Sayaka, S., Mitsuhiro, S., Ayumi, O., Kobayashi, I. 2002. Echinodolides A and B, New Cembrane Diterpenoids with an Eight-Membered Lactone Ring from the Leaves of *Echinodorus macrophyllus*. J. Nat. Prod., 65, 82-84

Shin, J., Fenical, W., 1990. Fuscosides A-D: Anti-inflammatory diterpenoid glycosides of new structural classes from the Caribbean gorgonian *Eunicea fusca*. Journal of Organic Chemistry. 56, pp 3153-3158.

Soto, H.,Rovirosa, J., San-Martin, A. 2003. A new diterpene from Dictyota crenulata. Zeitschrift fuer Naturforschung, B: Chemical Sciences, 58(8), 795-798.

Soares, A.R., Teixeira, V. L., Pereira, R.C., Villaca, R., 2003. Variation on diterpene production by the Brazilian alga *Stypopodium zonale* (Dictyotales, Phaeophyta). Biochemical Systematics and Ecology, 31(11), 1347-1350.

Triplett, E.L., Govind, N.S., Roman, S. J., Jovine, R.V.M., Prezelin, B.B., 1993 Characterization of the sequence organization of DNA from the dinoflagellate *Heterocapsa pygmaea* (*Glenodinium* sp.). Molecular Marine Biology and Biotechnology, 2(4), 239-45.

Twiner, M.J. and Trick. C.G., 2000. Possible physiological mechanisms for production of hydrogen peroxide by the ichthyotoxic flagellate *Heterosigma akashiwo*. Journ. Plank. Res. 22 (10), 1961-1975.

Vallelian-Bindschendler, L., Scheweizer, P., Mosinger, E., Metreaux, J.P., 1998. Heat-induced resistance in barley to powdery mildew is associated with a burst of active oxygen species. Physiological and Molecular Plant Pathology. 52, 185-199.

Vardi, A., Berman-Frank, I., Rozenberg, T., Hadas, O., Kaplan, A., Levine, A., 1999. Programmed cell death of the dinoflagellate *Peridinium gatunense* is mediated by CO_2 limitation and oxidative stress. Current Biology, 9, 1061-1064.

Varvas, K., Jarving, I., Koljak, R., Vahemets, A., Pehk, T., Muurisepp, A-M., Lille, U., Samel, N.,1993. In vitro biosynthesis of prostaglandins in the white sea soft coral Gersemia fruticosa: Formation of optically active PGD2, PGE2, PGF2α, and 15-keto-PGF2α from arachidonic acid. Tetrahedron Letters, 34(22), 3643-3646.

Watson, D.A., Loeblich, A.R.III., 1983. The application of electrophoresis to the systematics of the marine dinoflagellate genus *Heterocapsa*. Biochemical Systematics and Ecology, 11(2), 67-71.

Wong, G.T.F., Dunstan, W. M., Kim D-B., 2003. The decomposition of hydrogen peroxide by marine phytoplankton. Oceanologica Acta, 26 ,191–198

Wylie, B.L., Ernst, N.B., Grace. K.J.S., Jacobs, R.S., 1997. Marine natural products as phopholipase A2 inhibitors. In Phospholipase A2: basic and clinical aspects in inflammtory diseases. Prog Surg. Basel, Karger, 24, pp 146-152.

Weinheimer, A.J., Spraggins, R.L., 1969. The occurrence of two new prostaglandin derivatives (15-epi-PGA2 and its acetate, methyl ester) in the gorgonian Plexaura Homomalla. Tetrahedron Letters, 59, pp 5185-5188.

Wu, J., Lin, L., 2002. Elicitor-like effects of low-energy ultrasound on plant (Panax ginseng) cells: induction of plant defense responses and secondary metabolite production. Applied Microbiology and Biotechnology, 59(1), 51-57

Wojtaszek, P., 1997. Oxidative burst: an early plant response to pathogen infection Biochem. J. 322, 681- 692

Zhukova, N.V., Aizdaicher, N.A., 1995. Fatty acid composition of 15 species of marine microalgae. Phytochemistry, 39, 351-356.