AN ABSTRACT OF THE THESIS OF

Mehran Fallah Moghaddam for the degree of Doctor of Philosophy in Pharmacy presented on October 29, 1991.

Title: Structural and Biosynthetic Studies on Marine Eicosanoids and Other Oxylipins

Abstract approved: _____

Dr. William H. Gerwick

In a survey of biomedicinal potential of Caribbean and Oregon marine algae, the crude organic extracts of *Platysiphonia miniata* and *Cotteniella filamentosa* exhibited antimicrobial activity against two pathogenic microorganisms and showed the presence of several potentially unique compounds. Purification and molecular structure elucidation utilizing 1D and 2D NMR, high and low resolution mass spectroscopy, UV, IR, and optical rotation data showed the presence of two mammalian autocoids, 12*S*-HETE and hepoxilin B_3, in the extracts of these algae. In mammals these two important compounds result from 12-lipoxygenation of arachidonic acid. This was a significant finding because 12-lipoxygenase activity was not reported in the plant kingdom prior to this work.

An Oregon marine red alga, *Gracilariopsis lemaneiformis*, shown to be a rich source of 12-lipoxygenase products, was used to investigate the biogenetic origin of mammalian eicosanoids in marine algae. Crude enzyme studies of this organism demonstrated the enzymatic production of its eicosanoids. Incubation of arachidonic acid with this enzyme preparation led to biosynthesis of 12-

lipoxygenase products (ie. 12-HETE, 12*R*,13*S*-diHETE, 12*R*,13*S*-diHEPE, and 12-oxo-dodecatrienoic acid).

12*R*,13*S*-diHETE is a novel algal metabolite of arachidonic acid. The biogenetic origin of the oxygen atom at C-13 of this compound, a unique location for oxidation in eicosanoids, has been of interest since its first isolation of this compound. In order to probe this question effectively, arachidonic acid was incubated with our crude enzyme mixture in the presence of $^{18}O_2$. Mass spectroscopic and ^{13}C NMR analysis of the ^{18}O-labeled 12*R*,13*S*-diHETE showed that molecular oxygen was the source of the two hydroxyl groups in this compound. In addition, Our crude enzyme preparation was instrumental in biosynthesis of 1-^{14}C 12*R*,13*S*-diHEPE which is not commercially available for the purpose of pharmacological evaluations.

The role of oxylipins in marine algae physiology is not clear at this point. The striking similarities between mammalian and algal 12-lipoxygenase pathways may make algae good models for studies designed to provide a basic understanding of the biological roles this enzyme and its products play.

Structural and Biosynthetic Studies on Marine Eicosanoids and Other Oxylipins

by

Mehran Fallah Moghaddam

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirement for the
degree of

Doctor of Philosophy

Completed October 29, 1991

Commencement June 1992

APPROVED:

Redacted for Privacy

Associate Professor of Medicinal Chemistry in charge of major

Redacted for Privacy

Dean of College of Pharmacy

Redacted for Privacy

Dean of Graduate School

Date Thesis is presented October 29, 1991

Typed by researcher for Mehran Fallah Moghaddam

I would like to dedicate this work to

my mother, Tahereh Karimzadeh,

my sister, Mehrnoosh Fallah Moghaddam,

and my father Ahmad Fallah Moghaddam.

It should be noted that it was my mother's appreciation of the importance of education and her never ending support and encouragement throughout my life that enabled me to reach this level of education.

ACKNOWLEDGEMENT

I would like to extend my sincere gratitude to the following:

Dr. William H. Gerwick, my major professor, for his academic and financial support and supervision,

Drs. George H. Constantine, Robert E. Larson, Stephen J. Hawkes, Stephen Kaattari, and Terry Lomax for serving on my graduate committee,

Drs. David Ballantine, Gayle Hansen, and Kathy Miller for their collaborative work and the identification of our marine algae,

Dr. Fred Stormshak for the collaborative work with the animal studies,

Dr. James Washburn for my SCUBA training,

Ms. Phola Cheun for her superb performance as my undergraduate assistant, her help in editing my writing, and her true friendship and support,

My family in Iran and the Soleman family in the U.S. for their continuous support and encouragement throughout my academic life,

NIH and Oregon and Puerto Rico Sea Grant programs for their financial support of my projects,

Oregon State University College of Pharmacy for providing a productive environment to work in throughout my Ph.D. program.

TABLE OF CONTENTS

Chapter I. INTRODUCTION 1

I.A. An Overview of Bioactive Compounds from Nature 3

I.A.1. Terrestrial Natural Products 4

I.A.2. Marine Natural Products 6

I.B. Lipoxygenase Pathway 11

I.B.1. Intracellular Sources of AA 13

I.B.2. Oxylipin Formation 18

I.B.2.a. Non-Enzymatic Formation 18

I.B.2.b. Enzymatic Formation 18

I.B.2.c. Hydroperoxide Transformations 30

I.B.2.c.1. Reduction to Hydroxy Fatty Acids 30

I.B.2.c.2. Conversion to Fatty Acid Aldehydes 36

I.B.2.c.3. Rearrangement to Epoxy Hydroxy Fatty Acids 39

I.B.2.c.4. Enzymatic Dehydration into Allene Oxides 44

I.B.2.c.5. Conversion into Divinyl Ether 45

Chapter II. DISCOVERY OF 12*S*-HYDROXY-5,8,10,14-EICOSATETRA-ENOIC ACID (12*S*-HETE) AND 10-HYDROXY- 11,12-*TRANS*-EPOXY-EICOSATRIENOIC ACID (HEPOXILIN B_3) IN THE TROPICAL RED ALGAE *PLATYSIPHONIA MINIATA* AND *COTTONIELLA FILAMENTOSA* 48

ABSTRACT 48

INTRODUCTION 49

RESULTS AND DISCUSSIONS 51

EXPERIMENTAL 60

Chapter III. 12-LIPOXYGENASE ACTIVITY IN THE RED MARINE ALGA *GRACILARIOPSIS LEMANEIFORMIS* 64

ABSTRACT 64

INTRODUCTION 65

RESULTS AND DISCUSSIONS 67

EXPERIMENTAL 72

Chapter IV. BIOSYNTHESIS OF 12*R*,13*S*-DIHETE AND 12*R*,13*S*-DIHEPE FROM THE MARINE ALGA, *GRACILARIOPSIS LEMANEIFORMIS* FOR EVALUATION OF MECHANISM OF FORMATION AND BIOLOGICAL ACTIVITY 76

ABSTRACT 76

INTRODUCTION 77

RESULTS AND DISCUSSIONS 80

EXPERIMENTAL 84

BIBLIOGRAPHY 89

Appendix. PRELIMINARY INVESTIGATIONS OF CHEMICAL CONSTITUENTS OF THE OREGON RED ALGA *GLOIOSIPHONIA VERTICILLARIS* 96

ABSTRACT 96

INTRODUCTION 97

RESULTS AND DISCUSSIONS 98

EXPERIMENTAL 101

LIST OF FIGURES

Figure		Page
I.B.1	Simplification of Oxylipin Production	11
I.B.2	Structure of AA and Some Oxylipins	12
I.B.3	Model of the Phosphoinositol Cascade in Animals and Its Possible Coupling to Oxylipin Production	15
I.B.4	Autoxidation of Fatty Acids	19
I.B.5	Autoxidation of Fatty Acids to Prostanoids	20
I.B.6	Possible Radical Mechanism of Fatty Acid Lipoxygenation through Substrate Activation	23
I.B.7	Concerted Mechanism of Lipoxygenation through Formation of an Organometallic Complex	24
I.B.8	Various Hypothetical Transition State of Iron during Lipoxygenation	25
I.B.9	Simplification of LO Mechanisms	27
I.B.10	Illustration of Antrafacial Relationship between abstraction and O_2 Addition	27
I.B.11	Formation of Leukotriene B_4	34
I.B.12	Hydroperoxide Lyase Reaction with 12-HPETE	37
I.B.13	Non-enzymatic Production of Fatty Acid Aldehydes by Lewis Acids in An Aprotic Solvent	38
I.B.14	Hematin-assisted Intramolecular Transfer of the terminal Hydroxy Group from 12-HPETE	40

I.B.15	Proposed Mechanism for the Formation of *Trans*-Epoxide by Acid Catalysis	41
I.B.16	Structure of Lipoxin A_4 and B_4	42
I.B.17	Proposed Mechanism for Formation of α-Ketol and the Macrolactones	44
I.B.18	Proposed Mechanism for Divinyl Ether Formation	46
II.1	Structures of 12*S*-HETE and Hepoxilin B_3 and their Respective Bis-ester Derivatives	52
II.2	Contour Plots of ^1H-^1H COSY and RT COSY of Hepoxilin B_3 Acetate, Methyl Ester	57
IV.1	Potential Mechanisms of 12*R*,13*S*-diHETE Formation	78

LIST OF TABLES

Table		Page
II.1	High Field NMR data for Bis-ester Derivative of 12*S*-HETE	55
II.2	High Field NMR data for Bis-ester Derivative of Hepoxilin B_3	56

LIST OF APPENDIX TABLES

Table		Page
A.1	High Field ^{1}H and ^{13}C NMR Data for the Unknown Compound	104
A.2	^{1}H-^{1}H COSY and ^{1}H-^{1}H NOESY Correlations to Each Proton Resonance in the Unkown Compound	105

STRUCTURAL AND BIOSYNTHETIC STUDIES ON MARINE EICOSANOIDS AND OXYLIPINS

CHAPTER I. INTRODUCTION

The inability to effectively treat the newly emerging diseases such as AIDS as well as some old diseases provides us with the incentive to encourage and maintain drug discovery programs. Furthermore, new drugs are needed to combat the development of drug resistance to the already existing medicinal agents. A significant proportion of existing drugs are from natural sources. Additionally, the majority of synthetic biomedicinals have natural bases. Natural Products Chemists and Pharmacognosists study substances from natural sources from a chemical and pharmacological stand point. These investigators often provide the starting material from which new drugs and biomedicinal tools can be developed.

The major objective of the work presented in this thesis was the investigation of marine algae as a source of biomedicinal substances. As a result, marine red algae were identified as a rich source of mammalian 12-lipoxygenase metabolites of arachidonic acid. These substances are currently in use as biomedicinal tools to determine the role lipoxygenases play in mammalian physiology. The market value for these compounds is extremely high and marine red algae may provide us with a less expensive source, hence, expediting the physiological research in this area. In addition, these marine organisms produce novel metabolites of arachidonic acid which show interesting biological activities *in vitro* and *in vivo*.

The remainder of this chapter consists of two sections. Section I.A. is a brief review of natural products already in biopharmaceutical use. Section I.B. provides a review of literature regarding the lipoxygenase metabolism of fatty acids in order to provide background knowledge on the research presented.

I.A. An Overview of Bioactive Compounds from Nature

Historically, humans have always sought natural substances with medicinal properties for the treatment of ailments. The Incas were familiar with the antimalarial properties of the bark of the cinchona tree, from which quinine later was isolated; and in medieval times, foxglove, the source of digitalis was used (1) for treating heart conditions. Even today, a large proportion of our pharmaceutical agents for treating various disorders come from natural sources or have been synthesized based on natural product structures (1). There is a great diversity of structural classes of bioactive compounds in nature. Not all of natural products obtained ultimately prove effective as therapeutic agents, but, they often provide medicinal chemists with insights into the design of new classes of valuable drugs or biopharmaceutical tools. The loss of natural products through species extinction or "the death of birth" (1), has raised serious concern among those who understand the extent of this biotic destruction. Biotic impoverishment may be viewed as chemical impoverishment and loss of a species may be regarded as loss of chemicals that are potentially unique in nature and not likely to be invented independently in the laboratory. These may ultimately be of great use - particularly in medicine (1). In order to illustrate the extent to which natural products play a role in our pharmacopeia, some examples of valuable terrestrial and marine natural products will be given.

I.A.1. Terrestrial Natural Products

Because of easy excess to terrestrial plants, animals, and microorganisms, people have used these sources for medicinal applications for centuries, and humans are not alone in appreciating the use of these sources for medicinal purposes. Animals such as chimpanzees in Africa have been observed to ingest young leaves of *Aspilia* plants when ill. This observation led to the isolation of a compound, thiarubine-A (2), which has subsequently shown to possess potential antitumor activity. Interestingly, at about the same time this compound was isolated from the roots of *Chaenactis douglasii*, a plant used medicinally by native indians of Canada (2).

It is difficult to estimate how much of nature's chemical treasury has so far been uncovered. Thousands of alkaloids have been characterized chemically, of which hundreds have proved active as anesthetics, analgesics, narcotics, vasoconstrictors and dilators, respiratory stimulants, muscle relaxants, neuroactive agents, insecticides, and parasiticides (1). These alkaloids have been derived from flowering plants and only 2% of these plants (some 5000 of the estimated total of 250,000 species) have been examined thus far. In addition to terrestrial plants, which comprise only a fraction of the organic world, terrestrial microorganisms, animals and marine organisms are even less well studied.

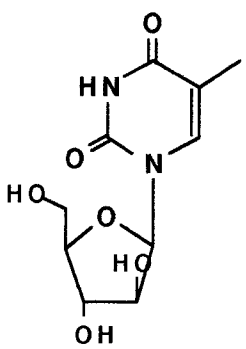
There are a number of valuable natural products currently in medical use. The vinca alkaloids, vincristine and vinblastine, which were isolated from the Madagascar rosy periwinkle, *Catharanthus roseus*, have been successfully used in treating proliferative diseases such as Hodgkin's disease, breast and testis cancer,

and acute lymphocytic leukemia (3). Some of the other chemotherapeutic agents of natural origin useful against neoplastic diseases include, doxorubicin, bleomycin, and mitomycin (3). In addition, the immunosuppressant cyclosporin and parasiticidal ivermectin were discovered from a Norwegian fungus and a Japanese mold, respectively (1). The pharmaceutical values of ivermectin and cyclosporin were realized in the course of routine screenings of microorganisms for biomedicinal activity. Ironically, the study of the fungal source of cyclosporin was almost dropped when it did not show significant antibiotic activity in the original bioassays (1). In its first five years in the U.S. market, cyclosporin annual sales reached $100 million (1). Most drug discoveries have been serendipitous and the result of continuous efforts in drug discovery programs. Hence, the efforts in natural product chemistry which result in introduction of new classes of compounds should not be hindered by a lack of immediate success in identifying their pharmacological utility. In addition to their use as chemotherapeutic agents, natural products have also provided us with useful biochemical tools. Oil from the seeds of *Croton tiglium* (Euphorbiaceae), a terrestrial plant native to Southeast Asia, had long been recognized for its irritant properties. Later, it was found that murine skin cancer brought about by carcinogenic hydrocarbons could be aggravated by application of croton oil. The active principle of this oil was characterized as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or phorbol ester (4). Phorbol ester, a naturally occurring co-carcinogen or tumor promoter, is used widely as a tool in toxicological studies.

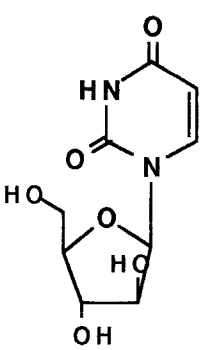
I.A.2. Marine Natural Products

Marine organisms have not been as easily accessed by humans throughout the ages, and therefore, marine natural products chemistry is a relatively new field. As a result, discovery of drugs from this group of organisms depends largely on bioassays rather than accounts of folk medicine. With the development of Self Contained Underwater Breathing Apparatus (SCUBA) techniques, and more recently, of submersible vessels, marine organisms have become very accessible and are likely to become an increasingly important natural source for drug development. Marine organisms include a large number of animal, plant, and microbial species (estimated in 1977 to be 500,000 species in 30 phyla (5)) which represent, for the most part, an untouched and potentially rich source of new biopharmaceuticals.

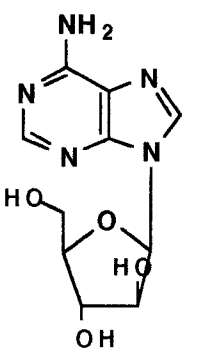
One of the hallmarks of marine natural products chemistry was the discovery of spongothymidine (1) and spongouridine (2) from the marine sponge *Cryptotethia crypta* in 1955 (6). These bioactive compounds were the progenitors to the antiviral agent Ara-A (3) and the anti-neoplastic drug Ara-C (4), respectively



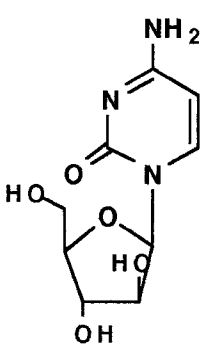
1



2



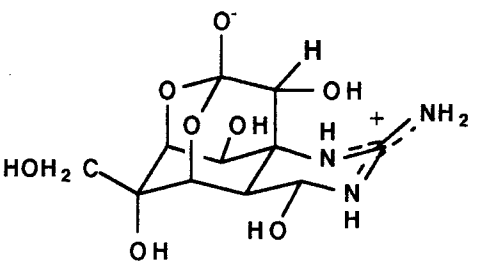
3



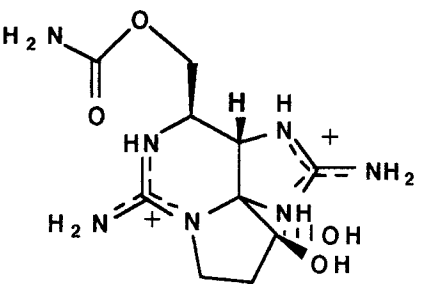
4

(4). However, chemical investigations of marine organisms seem to be concentrated within the last twenty years (4), and numerous successful projects have been completed in that time period, some of which will be presented in the ensuing discussion.

Tetrodotoxin (TTX, 5), a potent neurotoxin, is one of the most toxic non-proteinaceous substances known and was first isolated from pufferfish (*Tetraodonitidae*). Since then it has been isolated from a number of organisms (7). Tetrodotoxin was the first marine toxin to be recognized as a selective sodium channel blocker (4). Saxitoxin (STX, 6), belongs to a family of marine toxins, the



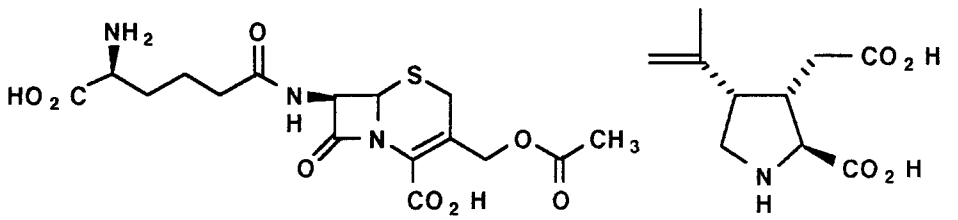
5



6

gonyautoxins, produced by dinoflagellates, *Gonyaulax* spp., which are responsible for the formation of red tides in cold and temperate oceans. These toxins cause paralytic shellfish poisoning in humans. Saxitoxin is also a selective sodium channel blocker and the two aforementioned toxins have enabled neurobiologists in identification of sodium channels and study their functions (7).

Cephalosporium acremonium was the first source of cephalosporin C (7), and was isolated in 1948 from the sea near a sewer outlet off the Sardinian coast. Cephalosporin C is a β-lactam antibiotic and is the base from which first, second,

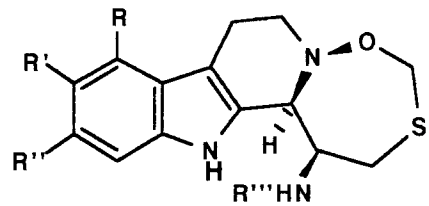


7 8

and third generation cephalosporins have been semisynthetically produced (3).

Kainic acid (**8**), an amino acid, was first isolated as the active component of the red alga *Digenea simplex*. This alga had been used for a long time in Japan for its ascaricidal properties against human intestinal parasites (4). More recently, kainic acid has been used as a neuropharmacological tool in studying the neuromechanisms in Huntington's chorea, a rare but fatal human disease (4). The annual demand of kainic acid in U.S., which was about 20 kg in 1986, is met by extraction of *D. simplex* in Taiwan (4).

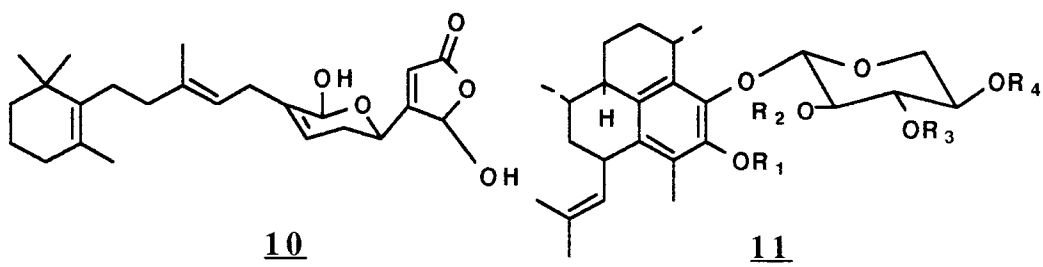
In 1981, Rinehart et al. reported isolation of a cyclic depsipeptide called didemnin B from a tunicate, *Tridemnum solidum*. It was originally isolated as an antiviral and cytotoxic agent (8). Later, this compound showed *in vivo* antitumor activity and currently is undergoing clinical trials as an antitumor drug under the auspices of the National Cancer Institute. Didemnin B is claimed to be the most promising drug prospect from recent marine natural product research (4). Another group of promising metabolites from tunicates are the eudistomins (**9**, 9), a group of β-carboline compounds with significant antiherpetic activity.



9

In 1969 the biomedical research community was alerted to the unexamined potential of marine organisms as sources of useful biochemicals when Weinheimer and Spraggins (10) reported on the isolation of prostaglandins from a Caribbean gorgonian, *Plexaura homomalla.* The discovery of a mammalian hormone in a marine coral was a novel finding. In addition, it was surprising that prostaglandins comprised up to 1.3% of the wet weight of the animal.

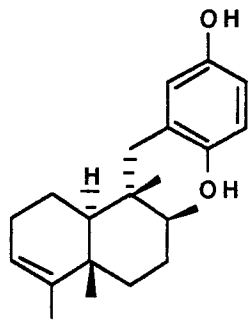
Manoalide (**10**), a C_{25} (sester) terpene which was initially isolated (11) from the Palauan sponge,*Luffariella variabilis*, was found to have strong anti-inflammatory activity. It is currently undergoing clinical trials for the treatment of psoriasis. Another group of marine anti-inflammatory compounds, the pseudopterosins (**11**), which are diterpenoid glycosides, were isolated from the gorgonian coral *Pseudopterogorgia elisabetha* (12). While these compounds are still under investigation, their degree of analgesic and anti-inflammatory activity has



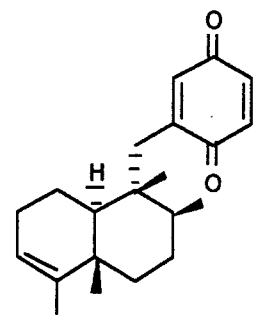
10 11

been shown to exceed that of indomethacin.

In 1974, avarol (12)was isolated from a mediterranean sponge, *Dysidea avara* (13). Later, this compound and its oxidation product avarone (13) were tested against the AIDS virus *in vitro* and both compounds showed a significant cytoprotective effect (14).



12



13

This is not by any means a comprehensive discussion of valuable natural products from marine and terrestrial sources. However, it serves to demonstrate the significance of naturally occurring compounds as biomedicinals, particularly so, from the marine environment. Marine organisms remain largely unexplored. Pending the careful preservation of this valuable resource, it could serve as a sophisticated library of naturally occurring and novel biomedicinals.

The research presented in this thesis discusses the discovery and biosynthesis of known and novel lipoxygenase metabolites of arachidonic acid in red marine algae. Therefore, it is necessary to review the relevant literature on lipoxygenase metabolism as was reported in animals and plants prior to our findings.

I.B. Lipoxygenase Pathway

In animal tissues, the reception of stimuli at cellular membranes causes release of free fatty acids from the lipid bilayer. Membrane derived fatty acids can be released via the action of phospholipases on membrane phospholipids. These fatty acids can be enzymatically metabolized by cycloxygenase, lipoxygenase, or cytochrome P-450 in animals to give rise to a class of physiologically active compounds collectively called oxylipins (figure I.B.1). Oxylipin production is also widespread in plants. The term "oxylipin" refers to all oxygenated compounds derived from fatty acids of varying chain lengths by reaction(s) involving mono- or dioxygenase enzymes (15) such as cytochrome P-450 (Cyt. P-450), cyclooxygenase (CO), or lipoxygenases (LO).

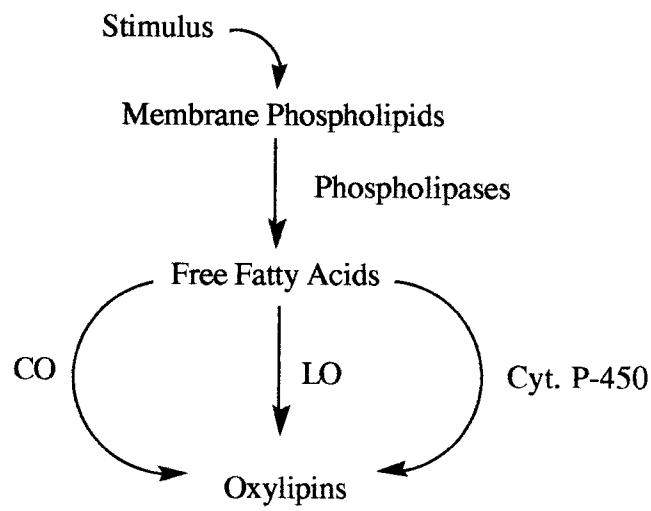


Figure I.B.1. Simplification of Oxylipin Production.

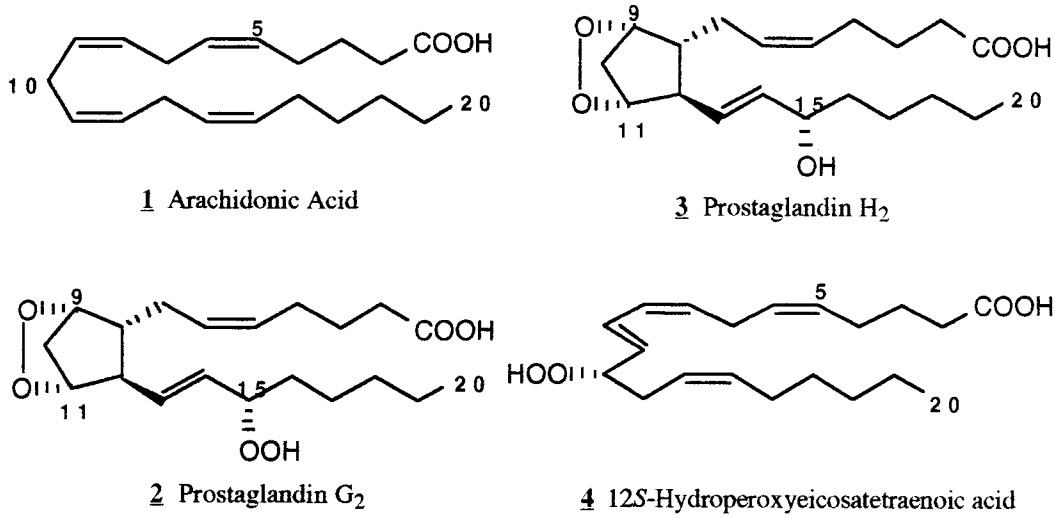


Figure I.B.2. Structures of AA and some oxylipins.

The term eicosanoid refers to those oxylipins originating from 20 carbon polyunsaturated fatty acids (PUFA) like arachidonic acid (AA, figure I.B.2, 1). AA is the precursor to many biologically important substances. Upon its release from membrane phospholipids via the action of phospholipases AA can be metabolized by the enzyme cyclooxygenase (figure I.B.1) to the endoperoxide, prostaglandin G_2 (figure I.B.2, 2) and then converted to prostaglandin H_2 (3), the precursor to many other prostaglandins (PG) and thromboxanes (TX). Alternatively, LO metabolism of AA will convert AA to its hydroperoxy derivatives, such as 12*S*-hydroperoxy-eicosatetraenoic acid (12*S*-HPETE, 4) for the case of 12-lipoxygenase (figure I.B.2). These hydroperoxy fatty acids can be manipulated subsequently via the action of several different enzymes to yield mono, di-, or trihydroxyeicosanoids, hepoxilins, leukotrienes, and various other fatty acids. Because the major emphasis of this thesis is on the lipoxygenase metabolism of arachidonic acid, this section will review the relevant literature.

I.B.1. Intracellular Sources of AA

It is now understood that the intracellular biosynthesis of eicosanoids is limited by the level of free AA inside the cells (16). Compared to other major fatty acids, AA is unusual in that the ratio of the free form of this fatty acid in the cytoplasm and blood plasma to cell membranes is extremely low (16). The main sources of AA in the lipid bilayers are phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol (17). The majority of AA in mammalian cells is esterified in the fatty acyl chains of glycerophospholipids, almost exclusively in the 2-acyl position (16). The PUFAs present in the phospholipids are believed to play an important role in maintaining the fluidity of the membrane lipid bilayers. It is generally accepted that the activity of phospholipases control free AA levels in a cell. However, one should not lose site of the fact that AA is re-esterified to the lysophospholipids continuously via the action of acyl transferase(s). Hence, the free AA levels in the tissue actually represents a balance between the liberation of the acid by hydrolysis and its reesterification into phospholipids by the activity of the acyltransferase(s) (16). Although, in resting cells re-esterification has been suggested to be the rate limiting factor which controls free intracellular AA concentrations, in stimulated cells it is the phospholipases which control levels of AA (16).

Phospholipases (PL) are a family of membrane bound, in some cases Ca^{2+} dependent enzymes which can use membrane phospholipids as their substrates upon receiving stimuli. In the case of PLA_1 and A_2 the enzymatic reaction causes the release of fatty acid chains from these phospholipids (17). These enzymes have

been found in almost every type of cell where they have been sought within the mammalian body (17).

Phospholipase A_1 can cleave the acyl chains from sn-1 positions of the glycerolipids. This type of PL sometimes occurs intracellularly in membrane-bound form and other times in soluble form (17). Furthermore, not all PLA_1s require Ca^{2+} for activation. AA is preferentially esterified to the sn-2 position of the glycerolipids and subsequently mobilized from these moieties by the hydrolytic action of PLA_2 (17). PLA_2 can be found both as membrane bound and soluble form depending on the type of cell it comes from, however, in contrast to PLA_1 it always requires Ca^{2+} for activation. This enzyme can metabolize 1-O-Alkyl-2-acyl-glycero-phosphocholine to lyso-platelet activating factor (Lyso-PAF, figure I.B.3). Lyso-PAF is then acetylated at its sn-2 position by PAF acetyltransferase to PAF, a second messenger in animal systems (18). Phosphotidylinositol is hydrolyzed by phospholipase C to 1,2-diacylglycerol (DAG) and inositol phosphate, both of which are second messengers in animal cells (18). DAG can either activate protein kinase C (PKC) which can phosphorylate proteins at serine and threonine residues and lead to physiological responses or give rise to free AA via the action of diacylglycerol lipase. Inositol phosphates can cause the release of Ca^{2+} from endoplasmic reticulum. The increased levels of cytoplasmic Ca^{2+} can activate several Ca^{2+} and calmodulin-dependent enzymes, including protein kinases and phospholipases which again cause physiological responses (18). It has been suggested that similar processes take place in plants. A difference between animal and plant systems is the lack of Ca^{2+} -dependent proteases in plants (18).

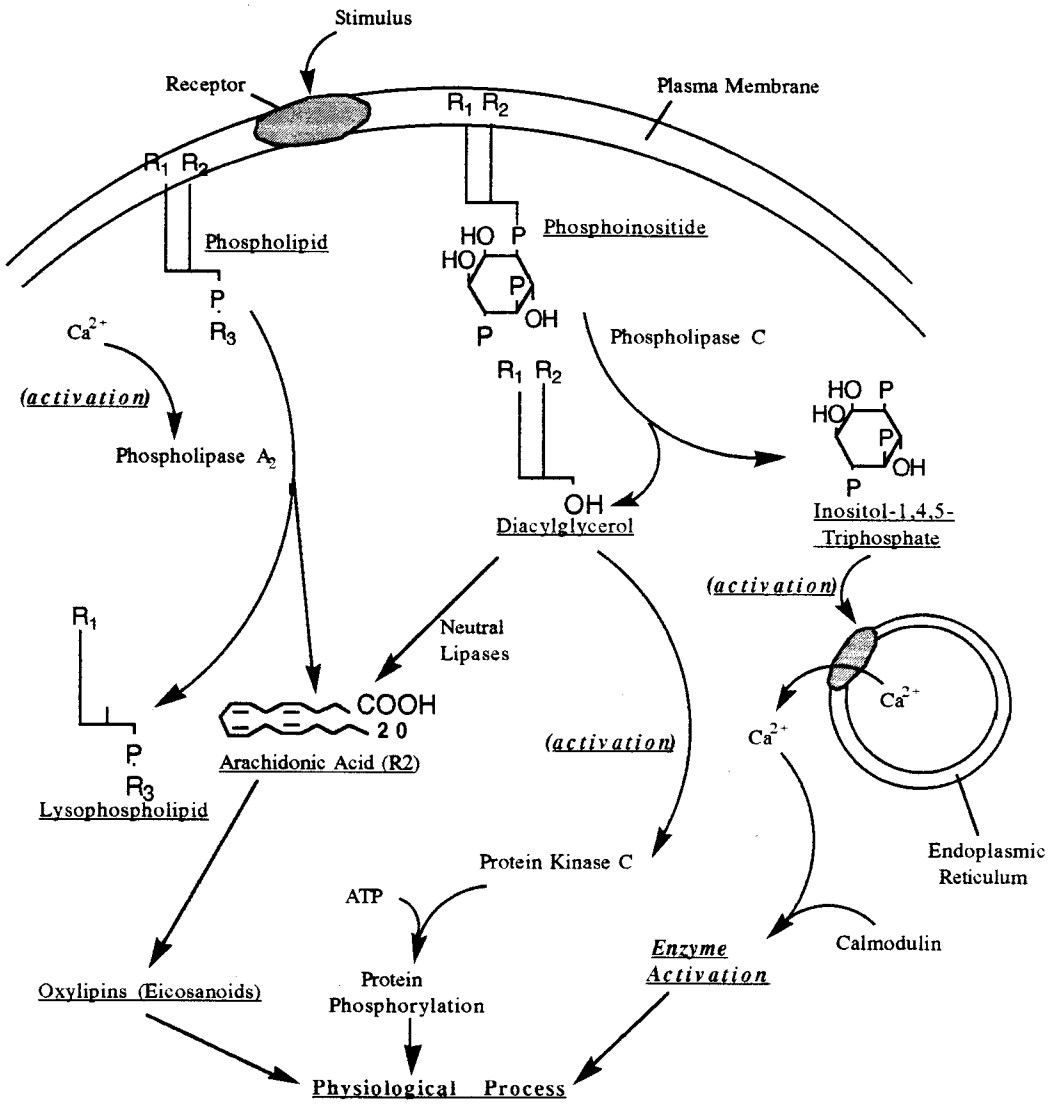


Figure I.B.3. Model of the phosphoinositol cascade in animals and its possible coupling to oxylipin production (18).

It has been suggested that PL's are involved in membrane repair processes (19). Membranes of endoplasmic reticulum and mitochondria are the primary sites of lipid peroxidation which are known to occur through a free radical propagation with formation of hydroperoxides and their degradation products. This is particularly true in pulmonary tissues (19). Rat liver mitochondria subjected to lipid peroxidation show an increase in PLA_2 concentration (19). Membrane phospholipid epoxides and phospholipid hydroperoxides are resistant to reduction by endoplasmic and cytosolic epoxide hydrolases and glutathione peroxidases. This may suggest that oxidized fatty acids associated with the membrane phospholipids are unavailable for reduction and PLA's are utilized as a protective and repair system to selectively replace the deteriorated fatty acids without replacing the whole phospholipid (19). Regulation of phospholipase activity has been suggested to be achieved by structural changes in the phospholipid bilayers. It has been hypothesized that oxidation of the fatty acid matrix creates a hydrophilic center and gives the phospholipases normally not able to approach the lipophilic areas a chance to do so and fix the affected phospholipids (19).

PL's are believed to be involved in the cytolytic action of tumor necrosis factor (TNF), a macrophage product which can cause cytolysis of certain tumor cell lines (20). Cytolysis of these cells is reduced in the precence of PLA_2 inhibitors like dexamethasone and quinacrine. In addition, TNF-susceptible cells exhibit increased release of AA and its metabolites upon TNF challenge; a process not affected by lipoxygenase or cyclooxygenase inhibitors. Furthermore, tumor cell lines selected for TNF resistance lose the capacity to mobilize AA (20). Therefore, PLA_2 seems to be involved in the cytolytic action of TNF.

It has been reported that implantation of a blastocyst into a uterine endometrium involves inflammatory type response with increased capillary permeability, release of endometrial histamine, and production of AA cascade metabolites (21). The results from this study provide evidence for the involvement of the LO enzymes and PLA_2 in the initial as well as subsequent development of early pregnancy.

Eicosanoids mediate transduction of signals for bacterial infections in insects (22). After infecting the tobacco horn worm larvae, *Manduca sexta*, with the bacterium, *Serratia marcescens*, and using inhibitors of PLA_2, LO, and CO the ability of the mentioned larvae to clear the bacterium was severely impaired. The effects of dexamethasone (a PLA_2 inhibitor) on larval survival were reversed by free AA treatment.

PLA_2 has been the target of antiinflammatory drug development. Corticosteroids such as hydrocortisone inhibit this enzyme through glucocorticoid-induced synthesis of a family of proteins called lipocortins or macrocortins (3). Indomethacin can inhibit PLA_2 in the test tube, but it is not clear whether or not this inhibition takes place *in vivo* (16). As mentioned before, manoalide and pseudopterosins have pronounced inhibitory effects towards PLA_2 which has made them excellent candidates for drug development. PLA_2 involvement in development of inflammation in animal tissue is due to its ability to mobilize AA from membrane phospholipids upon stimulation. The released AA then undergoes enzymatic oxidations which yield inflammatory compounds such as 12-HETE as well as other autocoids.

I.B.2. Oxylipin Formation

I.B.2.a. Non-Enzymatic Formation

Toxic substances, such as carbon tetrachloride, can be metabolized to produce radicals capable of peroxidation of lipid membranes and thus cell injury. Tissue irradiation may also cause production of reactive free radicals. Transition metal salts or chelates, in particular iron salts or chelates, stimulate lipid peroxidations as well (23). Fatty acids seem to become more prone to autoxidations as the number of unsaturations increases on their chains. For example, AA may be autoxidized about 2.9 X as fast as linoleic acid (23). It appears that protons closer to the ω-end of AA are abstracted by the radicals species more readily than the other protons (23). Such non-enzymatic oxidations can lead to a variety of cyclic and non-cyclic products as depicted in figures IB4 and IB5.

I.B.2.b. Enzymatic Formation

As discussed before, a group of physiologically active oxylipins such as prostaglandins originate from the enzymatic action of cyclooxygenase. However, cyclooxygenase and its products will not be discussed further; instead a less studied group of oxylipins originating from lipoxygenase (LO) pathway are the focus of this section. The following discussion starts with a general introduction of LO's and their numenclature. This will be continued by a review of radically mediated verses concerted reactions of LO's in the formation of hydroperoxide intermediates.

Finally, the biological activities of the fatty acid hydroperoxide products will be discussed.

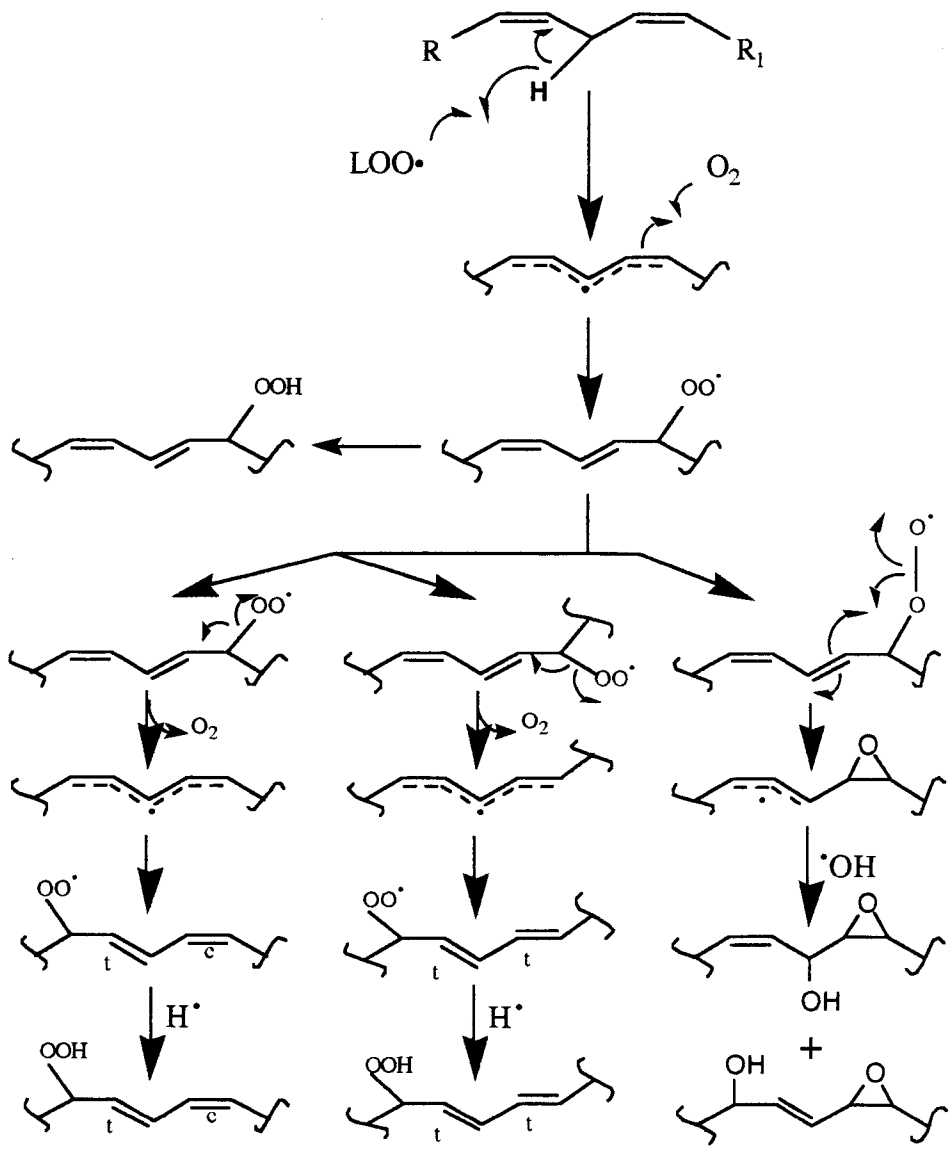


Figure I.B.4. Autoxidation of fatty acids.

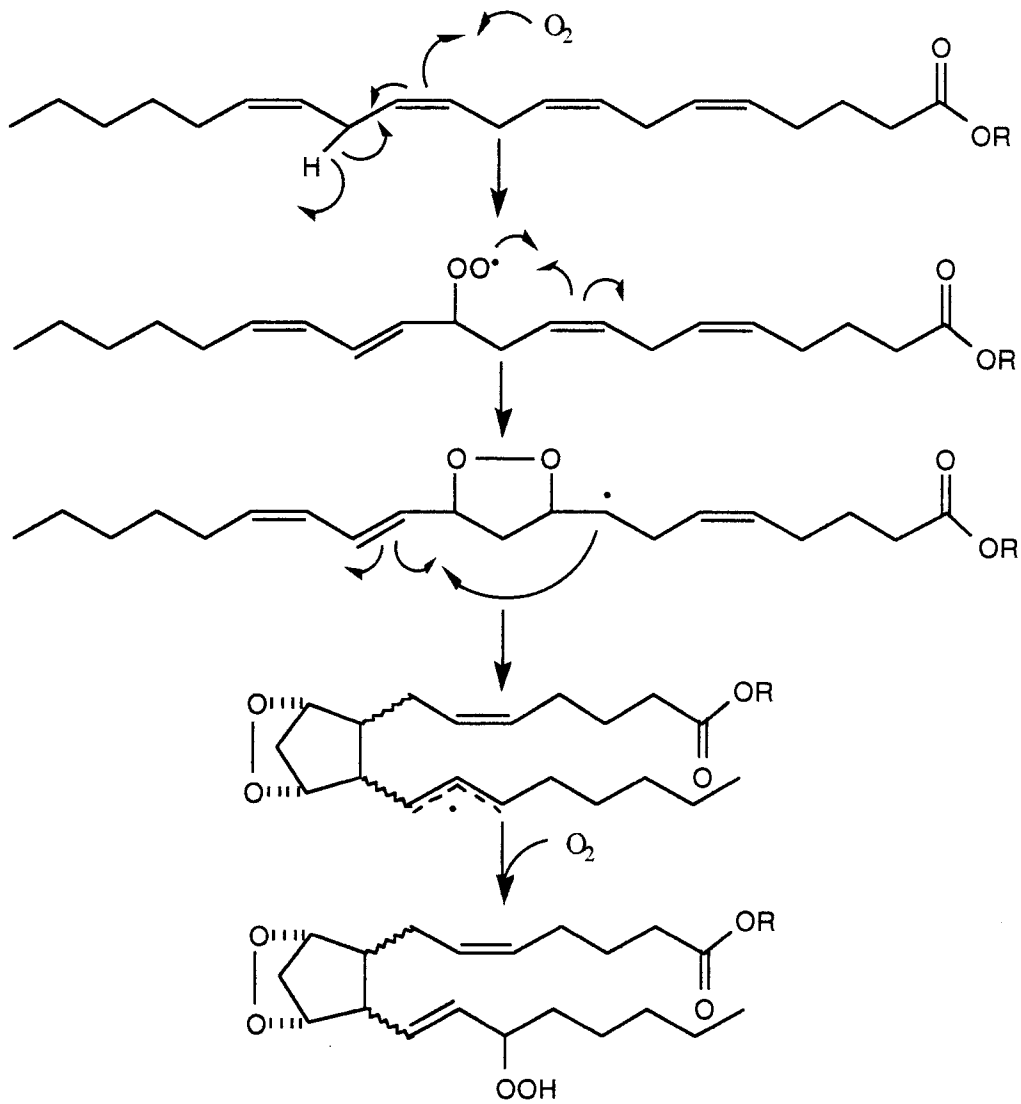


Figure I.B.5. Autoxidation of fatty acids to prostanoids.

Mammalian lipoxygenases are designated as 5-, 12-, and 15-lipoxygenases and plant lipoxygenases are generally known as 9- or 13-lipoxygenases (18). The numbers refer to the carbon atom (Δ numbering system) that is oxygenated in AA (in animals) or linoleic or linolenic acids (in plants). The best characterized lipoxygenases are from soybean seeds.

The type-1 soybean lipoxygenase (SBLO-1) has its maximum activity at pH 9 and produces mostly 13-hydroperoxy derivatives of octadecanoids. The type-2 soybean lipoxygenase (SBLO-2) has its maximum activity at pH 6.5 and produces a 50:50 mixture of 9- and 13-hydroperoxide derivatives. When supplying AA as substrate to these enzymes, both SBLO-1 and SBLO-2 behave as 15-LO although the latter one requries Ca^{2+} stimulation as does mammalian 5-LO (18). On the other hand, corn germ lipoxygenase produces 9*S* (or D)-hydroperoxy-10,12-octadecadienoic acid as the main product of linoleate metabolism at pH 6.9.

All lipoxygenases require a cis, cis-1,4-diene moiety in their fatty acid substrates and O_2 for their activity (24). It is generally agreed that LOs can exist in two forms. The native enzyme is inactive and contains one non-heme, high-spin ferrous ion (25, 26,27). In order for the enzyme to become catalytically active, the ferrous ion must be oxidized to its high spin Fe^{III} or ferric ion form. It has been suggested that the conversion of Fe^{II} to its ferric oxidation state could be accomplished by the hydroperoxide products (25). This activation reaction, referred to as Haber-Weiss reaction, is as follows (25):

$$E\text{-}Fe^{II} + R\text{-}O\text{-}OH \longrightarrow Fe^{III} + RO^{\bullet} + {}^{-}OH \longrightarrow \text{Other products}$$

Another source for these activating hydroperoxides *in vivo* is probably a small quantity of such compounds which are normally present in most tissues as a result of autoxidation (26). An alternative explanation is that perhaps a small fraction of LO enzymes have their iron in the ferric form (active form) and that these start the production of hydroperoxides which activate the inactive fraction of LO (26). Also, it has been reported that endo peroxides produced via the cyclooxygenase pathway are important for the activation of platelet lipoxygenase (28). During the catalysis, the enzyme is believed to cycle between the ferric and ferrous states and finish the catalytic cycle in the ferric form. Therefore, the enzyme does not require reactivation prior to oxidizing another molecule of substrate (26). Gibian and Galaway (24) proposed the mechanism depicted in figure I.B.6 for the active form of SBLO-1. These investigators suggested that the ferric ion can activate the substrate by abstraction of an electron from one of the double bonds in the 1,4-diene moiety which would give rise to a ferrous ion and an alkyl radical cation. This would lower the pk_a of the bisallylic methylene protons allowing for abstraction of the pro-*S* protons by a nitrogenous base in the catalytic site of the enzyme. As a result of the ensuing electron delocalization an allylic radical is generated. This radical will then react with the O_2 held in place by ferrous ion. The final result is the oxygenation of the fatty acid and reappearance of the Fe^{III} ion (24). Later, radical trapping experiments demonstrated presence of radical species associated with LO, however, this was not accepted as a proof for a radical mediated mechanism by the proponents of organoiron mediated reactions. The rejection was based on the fact that such radicals could have resulted from homolytic Fe-C bond cleavage of the proposed Fe^{III} -alkyl complex (29). Figure

I.B.7 illustrates the proposed concerted rather than radical mediated reaction for SBLO-1 (29, 30). It is believed that Fe^{III} acts as either a Lewis acid or electron acceptor because of the vacant coordination site in proximity to one of the double

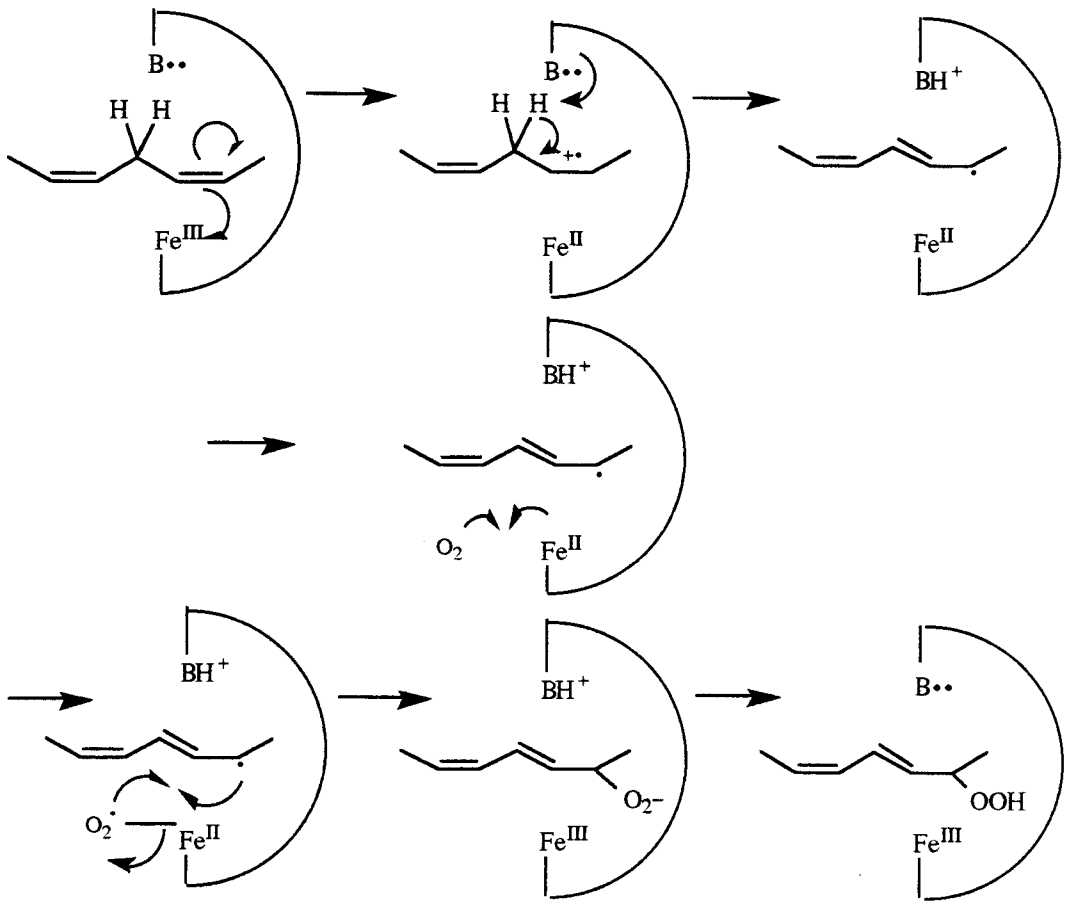


Figure I.B.6. Possible radical mechanism of fatty acid lipoxygenation through substrate activation (24).

bonds. This would promote a concerted deprotonation of the bisallylic methylene by a nitrogenase base in the enzyme catalytic site and the electrophilic addition of Fe^{III} to carbon. The resulting organoiron molecule would then be oxygenated by

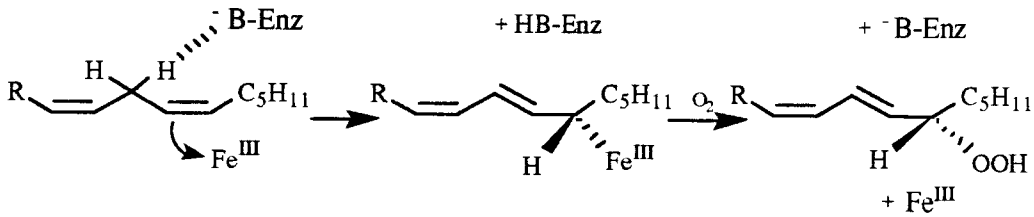


Figure I.B.7. Concerted mechanism of lipoxygenation through formation of an organometallic complex (29).

insertion of dioxygen into the Fe-C bond. However, more recent evidence seems to lend itself to the support of a radical mediated process (31). In this electron paramagnetic resonance (EPR) study linoleic acid was incubated with SBLO-1. The EPR parameters obtained for this sample agreed well with those published for oleyl peroxyl radical suggesting presence of an alkyl peroxyl radical, logically 13-peroxyl radical octadeca-9,11-dienoic acid. Furthermore, physical data obtained revealed that the peroxyl radical in the enzyme sample appeared to be more conformationally constrained than that in the frozen matrix. Therefore, it was suggested that the alkyl radical was indeed associated with the enzyme (31). This line of evidence seems to disprove the claims that the detected radicals can only be found in the reaction solution and are thus artifactual. From this review of the literature it seems that the radical mediated mechanism is more accepted and it may be summarized as depicted in figure I.B.8 (26).

One aspect of lipoxygenation which is illustrated in figure I.B.8 and has not been discussed yet is the enzyme inactivation processes. The inactivation of LO,

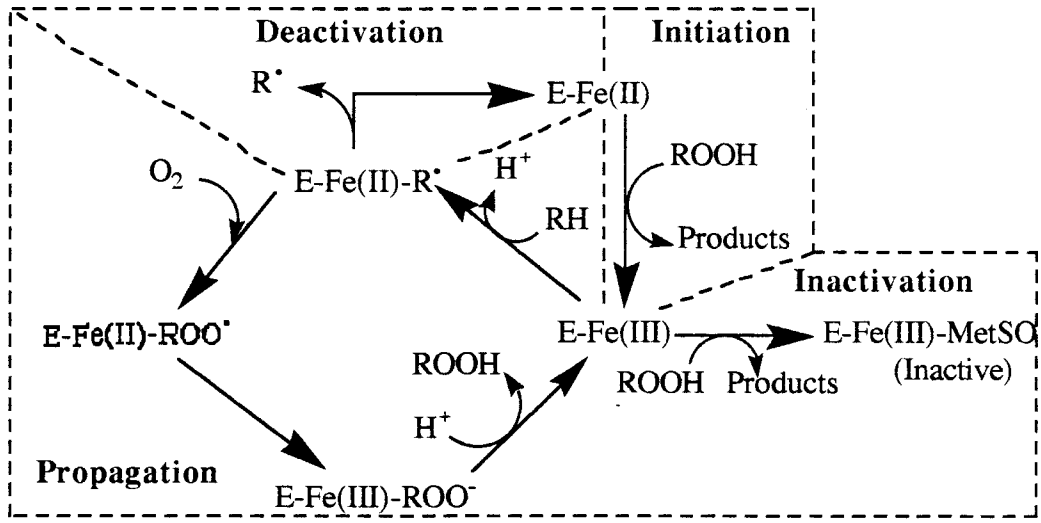


Figure I.B.8. Various hypothetical transition state of iron during lipoxygenation (26).

which to a large extent is due to the effects of lipid hydroperoxides on the enzyme in its ferric form, is non-reversible and takes place at the level of ferric enzyme-hydroperoxide complex (26). In reticulocyte LO, it has been demonstrated that the inactivation process coincides with oxidation of 1 mol of methionine in the LO to methionine sulfoxide per mol of enzyme (32). The modified methionine is presumed to be located at or near the active site. Although oxidation of amino acid residues has not been demonstrated in the inactivation of 5-LO, direct involvement of lipid hydroperoxides has been established unequivocally (26). It has been shown that 5-, 12-, and 15-HPETE can inactivate 5-LO with 5-HPETE being the most potent inactivator. This inactivation can be partially blocked in the presence of reduced glutathione (GSH) which reduces hydroperoxides to hydroxy fatty acids (26). Another inactivating factor that has not been studied very well is calcium (26). It is ironic that both calcium and lipid hydroperoxides are required for

activation of 5-LO, yet both factors are able to cause 5-LO inactivation. The mechanisms by which calcium stimulates and inactivates LOs are not understood at this time (26). The opposing effects of lipid hydroperoxides on LOs may be explained in terms of the transition state of iron present in the enzyme.

Clearly, a controversy exists about the mode of LO action. However, regardless of whether these enzymes act via concerted or radical mediated mechanisms, the final products are the same. While it is useful to be aware of both proposed modes of action (a and b in figure I.B.9), it may be necessary to remember only the net result (c in figure I.B.9) for the purposes of understanding the following discussions. As depicted in figure I.B.9 (c), LO abstracts one of the two bisallylic protons and inserts a molecular oxygen to one of the terminal olefininc carbons in the 1,4-pentadienyl moiety of a PUFA. There is an antrafacial relation between hydrogen abstraction and oxygen insertion (28, 33). Therefore, if we consider a planar 1,4 diene system, abstraction of the bisallylic H located above the plane accompanies oxidation from the bottom of the plane and vice versa (figure I.B.10, 33).

It has been suggested that the fatty acid hydroperoxides produced as a result of LO action can be harmful to cellular metabolism. Upon challenging human leukocytes by phorbol ester, release of low MW clastogenic substances were observed which induced chromosomal aberrations in cell cultures (34). This clastogenic action can be suppressed by inhibitors of AA cascade enzymes. Further investigation revealed that HPETEs are able to cause DNA breakage in the mouse embryo fibroblasts and contribute to the clastogenic factor (CF) activity of human leukocytes. The clastogenic acitivity of HPETEs are Ca^{2+} and Fe dependent. It has

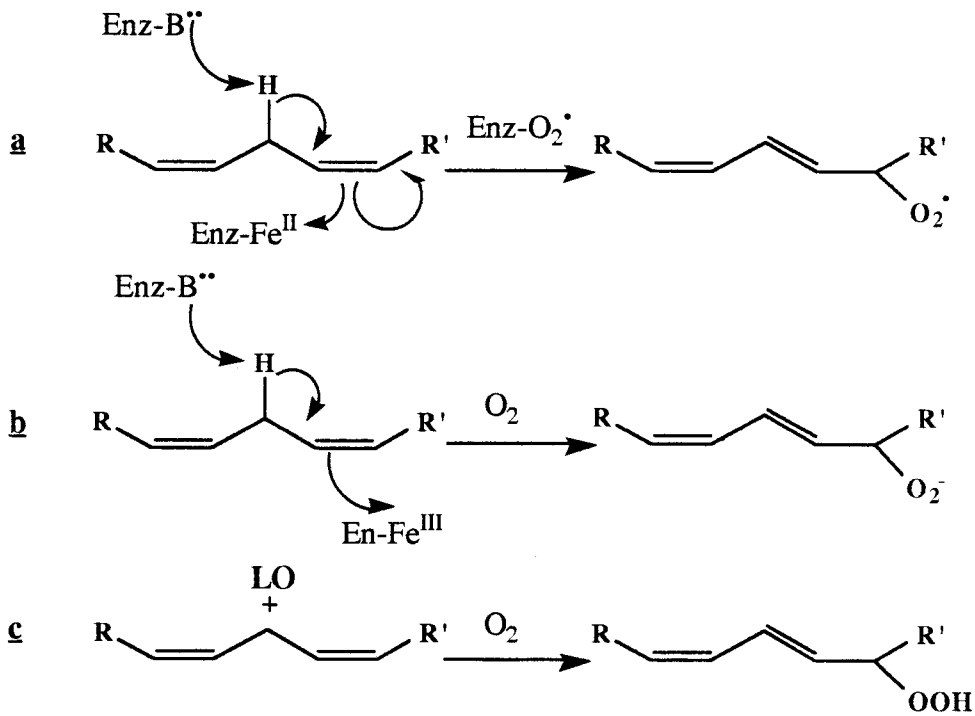


Figure I.B.9. Simplification of LO mechanisms. a. Proposed radical mediated lipoxygenation, b. Proposed concerted lipoxygenation, c. Net result of lipoxygenase reaction.

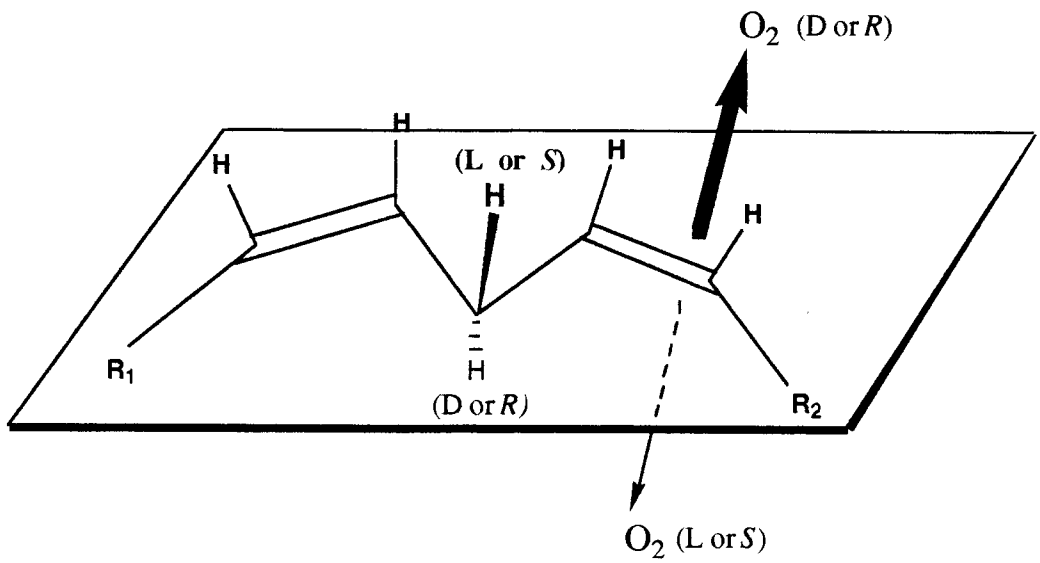


Figure I.B.10. Illustration of antrafacial relationship between abstraction and O_2 addition (32).

been proposed that alkoxy radicals (hydroperoxy radicals) formed in Fe^{2+}-catalyzed reactions may be intermediates in DNA strand breakage. HPETEs released from human leukocytes are probably of sufficient concentration to cause damage in neigboring cells. In addition, these hydroperoxides can stimulate cyclooxygenase and lipoxygenase enzymes and mobilize more Ca^{2+} from mitochondria which would amplify their biosynthesis. Glutathione peroxidase (GSH) can suppress the DNA damage because it reduces hydroperoxy fatty acids to hydroxy fatty acids which are less reactive (35). LO activity is also involved in the maturation of reticulocytes to red blood cells (36). The transition of reticulocytes to erythrocytes involves a degradation of mitochondria and ribosomes as well as partial or complete loss of certain enzymes and active transport systems. A 20 fold increase in the products of reticulocyte LO was observed during the development of a bleeding induced reticulocytosis (36), and the appearance of LO products coincided with the appearance of reticulocyte LO. *In vitro* studies using isolated rat liver mitochondria revealed that incubation of this organelle with purified LO caused an oxidation of mitochondrial membrane. This led to severe structural damage, a loss of respiratory enzyme activity, and drastic changes in the electrical properties of the membranes (36). Also, it was demonstrated in this study that lipoxygenation of polyenoic fatty acids takes place within the lipid bilayer of reticulocyte membranes. It has been hypothesized that this type of membrane lipid oxidation destabilizes the membranes and makes them susceptible to further degradation by proteases. An alternative suggestion offered by the authors was that perhaps production of LO products in the membrane was not, *per se*, a disruptive event. However, it is possible that the lipoxygenation process may provoke radical-mediated secondary

reactions which could lead to an oxidation of membrane proteins. This could then lead to proteolytic breakdown of oxidized membrane proteins; a well established process in reticulocytes (36). A similar role for LO has been suggested in the process of acrosome reaction in mammalian spermatozoa (37). The acrosome reaction in mature mammalian sperm is necessary for fertilization to occur. During this process, a Ca^{2+}-dependent breakdown and fusion of the outer acrosomal membrane and the overlying plasma membrane takes place. As a result, the soluble hydrolytic enzymes present in the acrosome are released which help in the penetration of the zona pellucida and the fusion with the oocyte plasma membrane by sperm. Inhibitors of LO, but not CO, were demonstrated to inhibit acrosome reaction in bull sperm both in presence and absence of Ca^{2+} (37). This demonstrated that 15-LO plays an important role in mammalian acrosome reaction (37). It is not clear whether or not the membrane degradative activity of LO is due to its hydroperoxide intermediates at this time.

Normally, cells are protected against the activities of LO hydroperoxide intermediates by a variety of enzymes which transform these compounds. Such enzymatic transformations include reduction into alcohols, conversion into epoxy alcohols, divinyl ethers, leukotrienes and related compounds.

I.B.2.c. Hydroperoxide Transformations

I.B.2.c.1. Reduction to Hydroxy Fatty Acids

In blood platelets 12-hydroperoxyeicosatetraenoic acid (12-HPETE) can be enzymatically reduced to 12-hydroxyeicosatetraenoic acid (12-HETE). It had been suggested that this reduction is due to the action of glutathione peroxidase (GSH-Px). The rationale behind this idea comes from experiments with rats on a selenium deficient diet (which cause a reduction in glutathione peroxidase activity). In these animals, there is a reduction in 12-HETE and an increase in 12-HPETE levels in their blood platelets (35). However, several lines of evidence challenge this claim. After selective removal of intracellular GSH and inhibition of the GSH-dependent peroxidase (GSH-Px) by 1-chloro-2,4-dinitrobenzene (CDNB), whole platelets were found to convert greater than 75% of 12-HPETE to 12-HETE (35). This finding was demonstrated in platelet membranes and cytosol, also. In addition, antiinflammatory drugs such as aspirin and indomethacin inhibit 12-HPETE peroxidase activity, but not the human GSH-Px (35). Therefore, GSH-Px does not seem to be necessary for the peroxidase activity, but it appears to serve a beneficial role in maximizing lipoxygenase activity perhaps by removal of the toxic hydroperoxides (35). In fact, the peroxidase activity may be associated with the lipoxygenase itself (38). Two different lipoxygenase activities have been detected in platelet cytosol. On a Sephadex G-150 column these enzymes eluted with an apparent M_r of 100,000 and 160,000. The smaller molecule exhibited only lipoxygenase activity, while the larger one contained both lipoxygenase and

peroxidase activities converting [1-^{14}C]HPETE to [1-^{14}C]HETE. These two enzymes were also separable on DEAE-Sephadex (38). It was hypothesized that the smaller enzyme molecule may have been an artifactual fragment of the larger one produced before or during enzyme preparation process. The reported peroxidase activity was inhibited by aspirin, indomethacin, sodium salicylate, ibuprofen, and naproxen, but not acetaminophen or phenacetin (38). Inhibition of the peroxidase activity gave rise to increased levels of HPETE. In certain sensitive individuals acute allergic responses to aspirin and indomethacin are observed. These individuals may be extremely sensitive to HPETE peroxidase inhibition which would create a sharp and intense rise in HPETE levels (39). The allergic response results from high levels of HPETE which has been shown to enhance the release of anaphylactic mediators from perfused guinea pig lung (39) and/or may serve as a precursor to other autocoids such as leukotrienes.

12-HETE was discovered in 1974 as a platelet metabolite of AA (40). Since its discovery both stereoisomers of 12-HETE have been found in many different animals as well as different tissues within the same animal. 12-HETE is a major proinflamatory metabolite of AA in human skin and was assumed to be formed by the action of epidermal 12-LO (40). The lesional skin of psoriatic patients exhibits an enhanced ability to synthesize 12-HETE. Topical application of 12-HETE obtained from psoriatic lesions onto healthy human skin produces erythema and leukocyte accumulation. Further, 12-HETE synthesized in psoriatic lesions cause chemokinesis of polymorphonuclear (PMN) leukocytes *in vitro* (41). Investigation of the stereochemical nature of 12-HETE in these lesions revealed a 12-*R* stereochemistry (41). Following this finding, an *in vitro* study proved 12*R*-HETE

to be more potent than 12*S*-HETE in chemotaxis of human neutrophils; a process partially responsible for development of inflammation (42). Furthermore, while the *R* enantiomer was chemotactic for human lymphocytes *in vitro*, the *S* enantiomer lacked such activity. After comparing the two enantiomers *in vivo*, it was demonstrated that 12*R*-HETE was indeed more potent in chemotaxis of neutrophils in human skin and therefore more proinflammatory (42). It has been speculated that 12*R*-HETE is chemotactically more potent than its enantiomer due to its interaction with the leukotriene B_4 receptor whose natural ligand has the 5*S*, 12*R* stereochemistry (43). The possibility that 12*R*-HETE present in psoriatic lesions was produced by 12-LO has been dismissed based on the following facts. As opposed to the highly enantioselective 12-LO which has always been reported to give rise to the *S* enantiomer of 12-HETE, cytochrome P-450 (isolated from rat liver microsomes) has been shown to catalyze production of a non-regioselective mixture of 5-, 8-, 9-, 11-, 12-, and 15-HETEs (44). While, cytochrome P-450 production of all of these mono-HETEs was shown to be non-enantioselective, about 80% of the 12-HETE produced by cytochrome P-450 was of *R* stereochemistry. In fact, this was the first report of a well characterized mammalian enzyme producing 12*R*-HETE (44). The identity of this enzyme was confirmed further when the production of 12*R*-HETE was inhibited by using cytochrome P-450 inhibitors such as carbon monoxide and SKF 525A (45). Therefore, existing evidence seems to support that the enzyme responsible for the development of psoriasis is cytochrome P-450 rather than epidermal 12-LO. This, however, does not detract from the importance of 12*S*-HETE.

The maturation of rabbit reticulocytes to red blood cells, earlier addressed as a role of LOs, has more recently been attributed to 12*S*-HETE (46). In addition, this compound has been implicated in mediating angiotensin II inhibition of renin release from kidney cells (47), stimulation of LHRH-induced ovarian progesterone and prostaglandin E_2 production (48), inhibition of platelet aggregation, and arterial vasodilation (49). 12*S*-HETE is produced in inflamed and anoxic tissues of the heart, and has been thought to exacerbate myocardial infarctions (50). It has been demonstrated that 12*S*-HETE attracts a large number of PMN leukocytes to the infarcted area in order to phagocytize cellular debris. However, these invading PMNs also produce high quantities of 12*S*-HETE which in turn causes vasoconstriction that is even more damaging to the already anoxic tissue (50). In addition, PMNs produce toxic oxygen metabolites (free radicals) and proteases during phagocytosis. This makes it additionally dangerous because the invading leukocytes continue to infiltrate the damaged tissue for hours after the onset of infarction, leading to continued myocardial destruction (50). It would be beneficial to inhibit 12-LO early on and limit the extent of heart damage. In addition, 12-HETE has been implicated in glomerular capillary wall injury in glomerulonephritis (51).

A variety of dihydroxy fatty acids also can be formed by LOs. Some of the leukotrienes (LT) fall into this catagory of oxylipins. The trivial name "leukotriene" was introduced because leukocytes constitute an important source of these conjugated triene unit containing fatty acids. As illustrated in figure I.B.11, cytosolic leukotriene A_4 hydrolase can hydrolyze LTA_4, itself a 5-LO product, and result in formation of leukotriene B_4 (LTB_4) or 5*S*, 12*R*-dihydroxyeicosa-6,14-*cis*-

8,10-*trans*-tetra-enoic acid (52). LTB_4 is at least 500-times more potent in its chemokinetic properties than 12*R*-HETE, which is in turn 10-20 times more potent than 12*S*-HETE (43). Borgeat *et al.* discovered an oxylipin closely related to

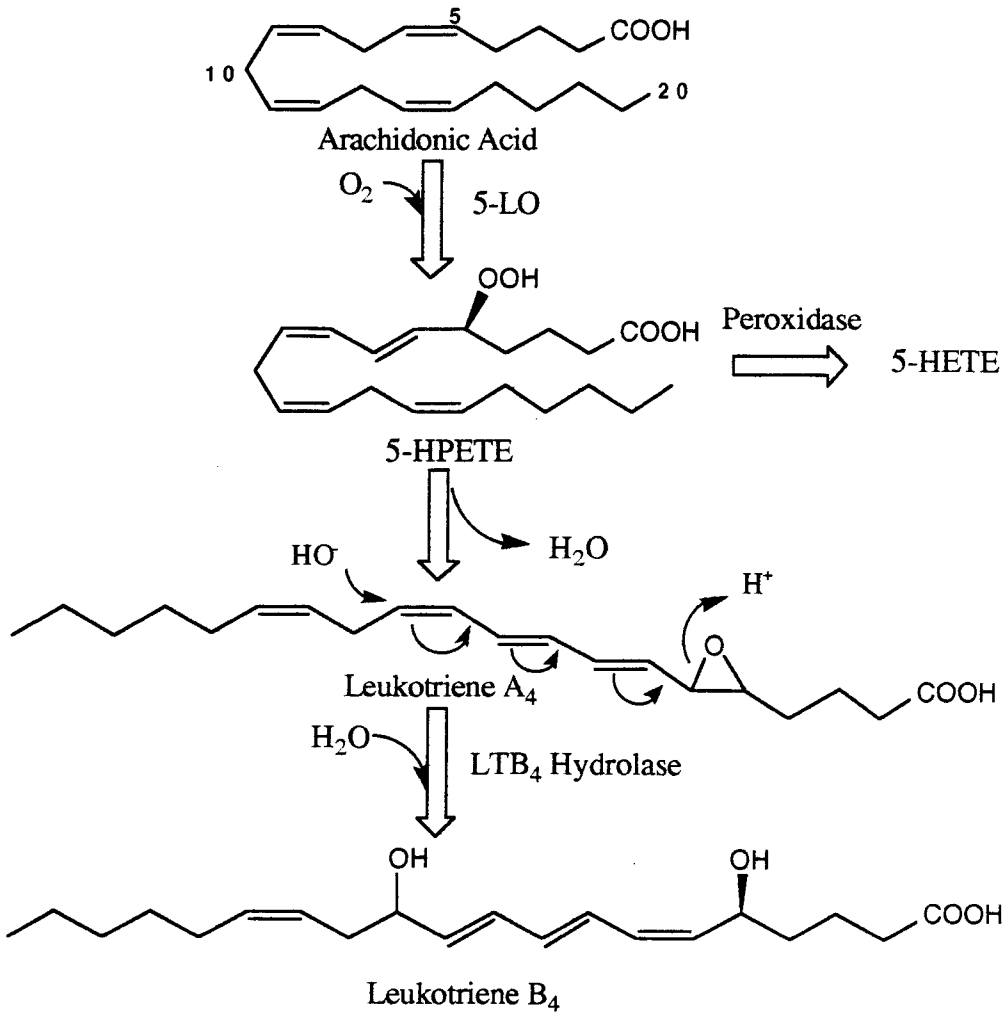


Figure I.B.11. Formation of leukotriene B_4

leukotriene B_4 (53). This compound was found to be 5*S*, 12*S*-dihydroxyeicosa-HETE, a diastereomer of leukotriene B_4. A biosynthetic study of this compound using porcine leukocytes and platelets revealed that it could be formed by 12-lipoxygenation followed by a 5-lipoxygenation or vice versa (53). Leukocytes were believed to contain mostly 5-LO and platelets mostly 12-LO. Therefore, incubation of 12-HETE with the former and 5-HETE with the latter type of cells produced 5,12-diHETE (53). However, it should by noted that 12- and 15-LO have been isolated from leukocytes and that these cells do not necessarily require 12-HETE produced by platelets to form 5,12-diHETE (54, 55). As opposed to the production of leukotriene B_4, neither route for the enzymatic production of 5,12-diHETE required an epoxy fatty acid intermediate. Furthermore, 5,12-diHETE possessed an 6*E*,8*Z*,10*E*,14*Z* double bond geometry whereas that of leukotriene B_4 was 6*Z*,8*E*,10*E*,14*Z* (53). Enzymatic formation of 5*S*,12*S*-diHETE was found to lead to a progressive inhibition of leukotriene B_4 biosynthesis which may imply that this compound has a modulatory role in LTB_4 formation (53). Non-enzymatic hydrolysis of leukotriene A_4 can lead to production of yet another group of leukotriene isomers (56, 57). These isomers include two diastereomers of leukotriene B_4, 5*S*, 12*R*-dihydroxy-6*E*,8*E*,10*E*,14*Z*-eicosatetraenoic acid and its 12*S* epimer as well as two diastereoisomers of 5,6-dihydroxy-7,9,11,14-eicosatetraenoic acid. It should be noted that both froms of 5,12-diHETE produced by non-enzymatic hydrolysis of leukotriene A_4 bear different double geometry than their enzymatically formed analogues (56, 57). It has been demonstrated that LTB_4 isomers bearing conjugated triene with one *cis* and two *trans* double bonds are more potent than the ones with all *trans* conjugations (57).

As mentioned before, linoleic and linolenic acids are the common substrates for plant LOs. The results of lipoxygenation of these fatty acids in plants are their 9 or 13-hydroperoxy derivatives. More recently, isolation of 12*S*-HETE, an arachidonic acid 12-LO product, has been reported from the plant kingdom (58). This unprecedented finding will be discussed in chapter II. *In vitro* production of 12-HETE has been demonstrated by algal (59) and corn (60) enzyme prepartations when AA was used as a substrate. Isolation of additional 12-LO products from marine algae has been reviewed by Gerwick *et .al.* (61)

I.B.2.c.2. Conversion to Fatty acid Aldehydes

Porcine leukocytes have been shown to produce 12-oxo-dodeca-5,8,10-(*Z,Z,E*)-trienoic acid when challenged with high concentrations of AA under anaerobic conditions (55). This fatty acid aldehyde was able to activate leukocytes and cause their chemotaxis. It was proposed that formation of the short chain aldehyde and the cleavage of C12-C13 bond was catalyzed by leukocyte 12-LO (55). However, a substantial body of evidence (18, 62, 63) points to the fact that the action of hydroperoxide lyase on hydroperoxy fatty acids like 12-HPETE is responsible for production of such metabolites (figure I.B.12). More recently, 15-oxo-dodecatrienoic acid was reported from rabbit leukocytes, presumably formed by the action of hydroperoxide lyase on 15-HPETE (63). As illustrated in figure I.B.13, short chain fatty acid aldehydes are formed non-enzymatically from methyl linoleate by heterolytic cleavage of hydroperoxides into aldehydes by Lewis acids in aprotic solvents (64).

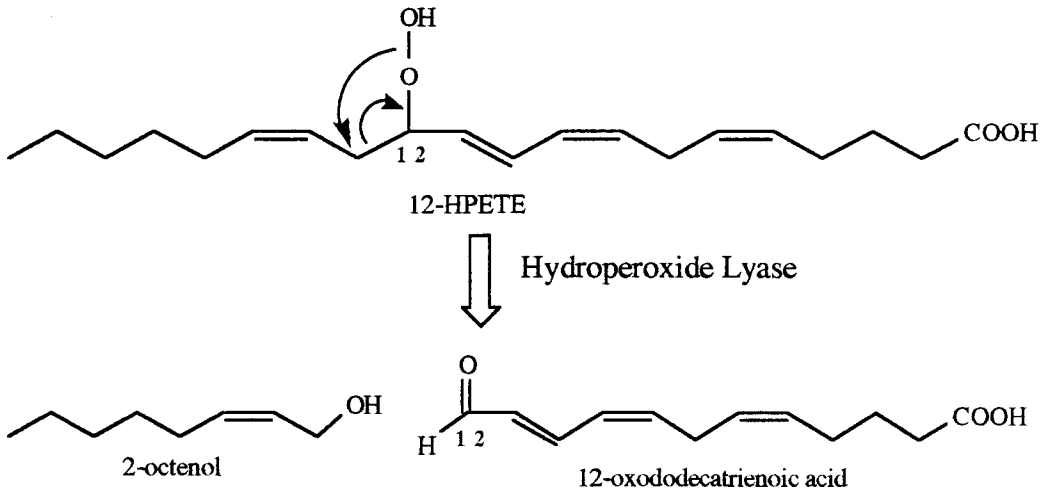


Figure I.B.12. Hydroperoxide lyase reaction with 12-HPETE.

Hydroperoxide lyase is a widely prevalent enzyme in the plant kingdom. However, because plant LO products tend to originate from linoleic and linolenic acids, the fatty acid aldehydes which result from the action of hydroperoxide lyase are of shorter chains than those found in animals (18). The hydroperoxide lyase isolated from most plant species prefers 13-hydroperoxy to 9-hydroperoxy derivatives of linoleic and linolenic acids (18). The aldehyde products are reduced to alcohols in some plants (18). Some of these products such as hexanal, nonenal, and hexenol are components of the flavor and aroma of plants. Moreover, these compounds have been identified as antifungal components of some fruits and leaves (18). One interesting difference between terrestrial plant and animal hydroperoxide lyases is that in plants the enzyme seems to cleave the bond α to the hydroperoxy bearing carbon on the carboxy end, and in the animals it cleaves the bond on the opposite side (18, 48, 49). As discussed in chapter III, marine algae appear to contain hydroperoxide lyase activity and produce products resembling those found

in animals (also 59).

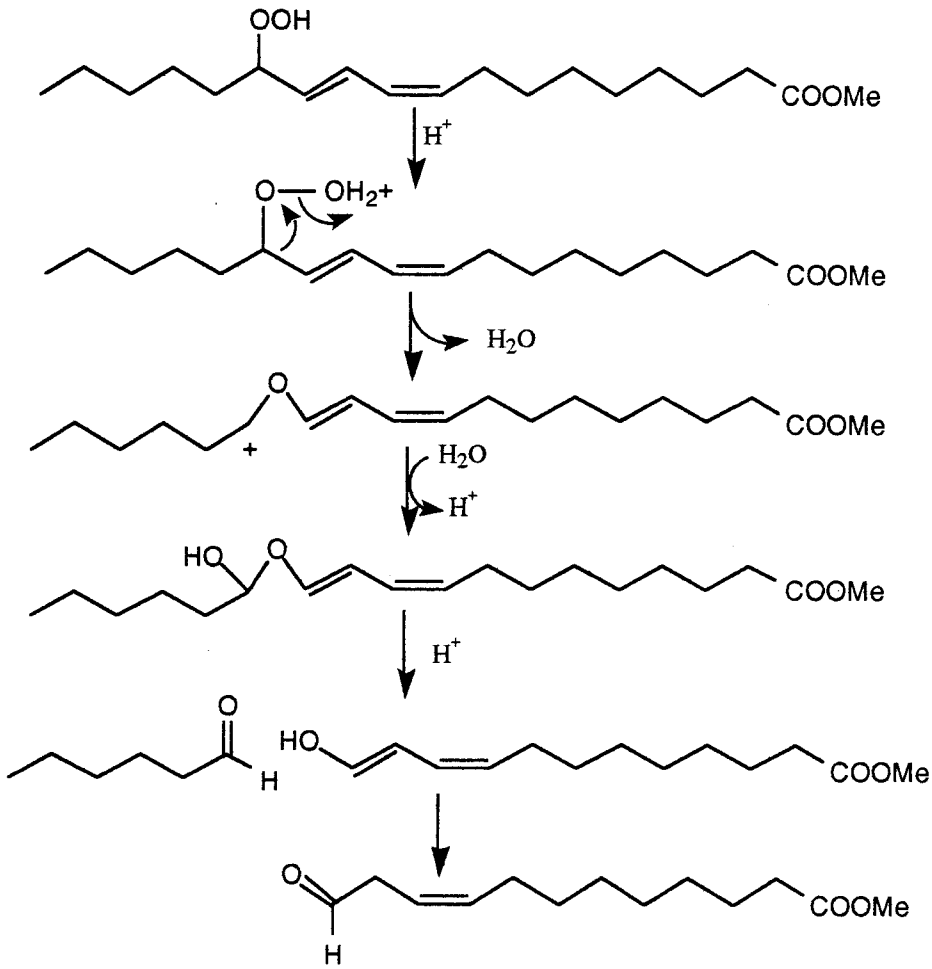


Figure I.B.13. Non-enzymatic production of fatty acid aldehydes by Lewis acids in an aprotic solvent (64).

I.B.2.c.3. Rearrangement to Epoxy Hydroxy Fatty Acids

In 1979, Walker *et al.* reported a multifunctional epoxy, hydroxy fatty acid, hepoxilin B_3 or 10-hydroxy-11,12-*trans*-epoxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid (figure I.B.14), from mammalian platelets (65). Subsequently, this compound was isolated from pancreatic islets (66), lungs (67), and *Aplysia* neurons (68). Hepoxilin A_3 or 8-hydroxy-11,12-*trans*-epoxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid, a positional isomer of hepoxilin B_3, has been isolated from the same types of tissues (67). Both of these compounds are biologically active and show insulin secretagogue activity (hepoxilin B_3 being the more stable and active isomer, 66), presynaptic inhibition activity in *Aplysia* sensory cells (68), and enhanced transport of calcium across the visceral yolk sac membrane of the guinea pig (69). As depicted in figure I.B.14, biogenesis of these compounds involves a hematin-assisted intramolecular rearrangement of 12*S*-HPETE in blood platelets (70). Hepoxilins are formed when the hydroxyl group of the hydroperoxy moiety is intramolecularly transferred to either C-10 (hepoxilin B_3) or C-8 (hepoxilin A_3). It was demonstrated that transfer of the hydroxyl group from 12-HPETE to the C-8 position, but not to C-10 position, required presence of the porphyrin moiety (70). Therefore, while hepoxilin A_3 was only formed with hematin present as a catalyst, Fe salts were sufficient to form hepoxilin B_3 (70). Molecular complexes between porphyrins and aromatic compounds have been described previously (71). Based on these findings, the authors proposed a cage system (figure I.B.14) in which the porphyryl moiety of hematin was associated with $\Delta^{8,10}$ conjugated diene of 12-HPETE, possibly aligning the through some form of Van der Waals forces for

intramolecular transfer of the OH (70). In addition, it was shown that the protein portion of hemoglobin did not play a part in the intramolecular rearrangement of 12-

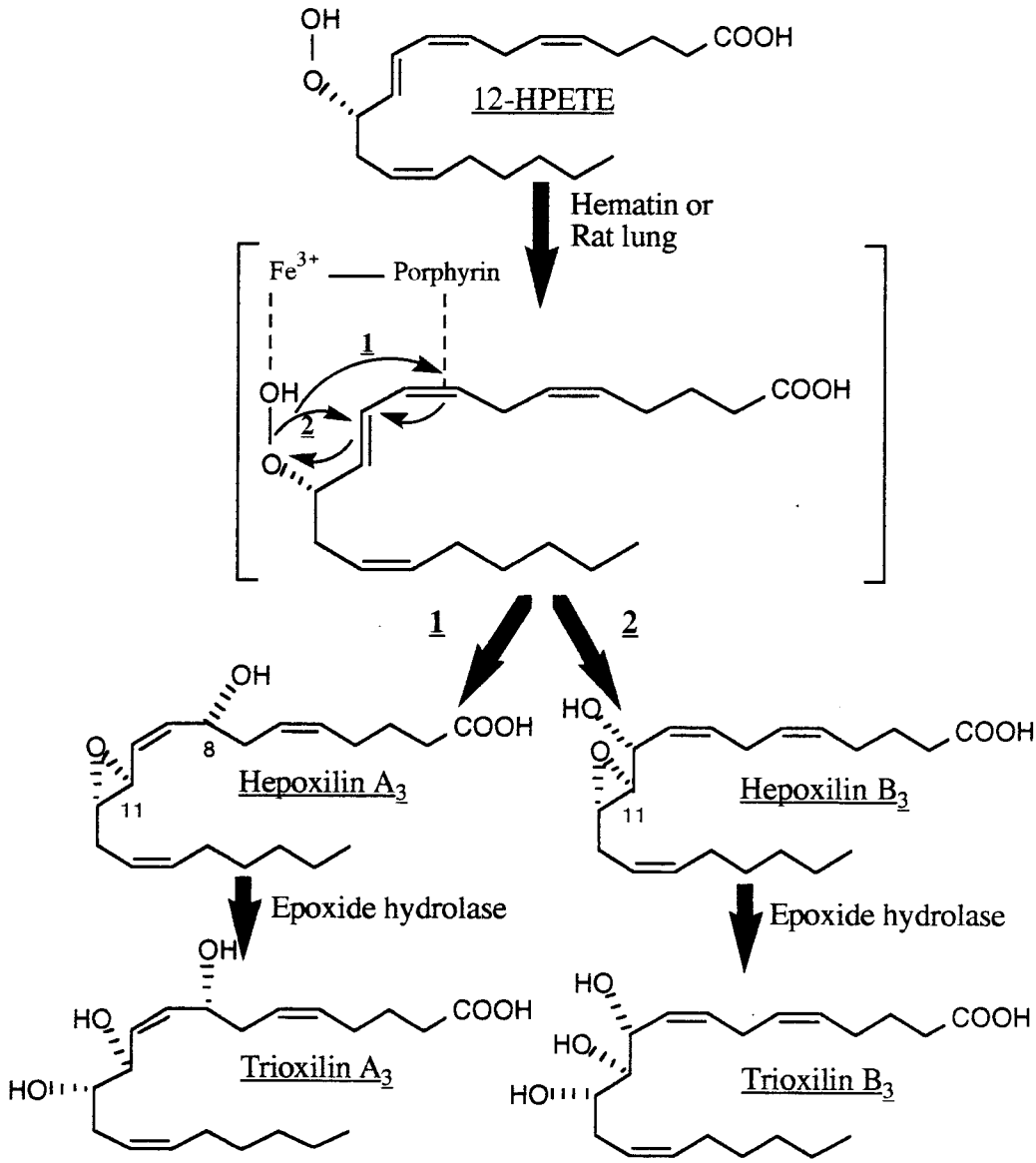


Figure I.B.14. Hematin-assisted intramolecular transfer of the terminal hydroxy group from 12-HPETE (70).

HPETE (70). It should be noted that hepoxilins can be produced non-enzymatically and an acid catalyzed mechanism has been proposed for their formation (figure I.B.15, 72). Once again, a free radical pathway may be envisioned for the non-enzymatic production of these compounds.

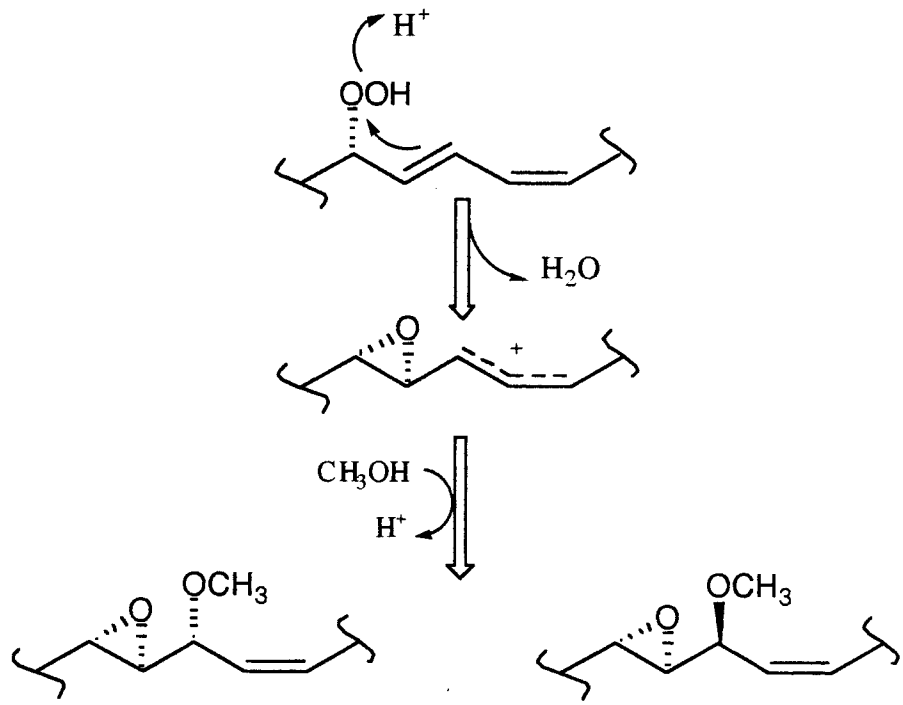


Figure I.B.15. Proposed mechanism for the formation of *trans*-epoxide by acid catalysis (72).

Hepoxilins are metabolized further to trioxilins via the action of epoxide hydrolase (EH). Pace-Asciak *et. al.*, reported that a 0-30% ammonium sulfate fraction of the high speed supernatant of rat lung converted 12-HPETE into hepoxilins A_3 and B_3 (73). Furthermore, the 30-50% ammonium sulfate fraction of the high speed supernatant contained epoxide hydrolase activity and converted the two hepoxilins to 8, 11, 12- and 10, 11, 12-trihydroxyeicosatrienoic acids,

respectively (figure I.B.14, 73). These trihydroxy compounds did not possess insulin secretagogue activity as did their precursors (66). More recently, hepoxilin epoxide hydrolase was isolated from rat liver cytosol (74). Based on its molecular mass value, pI, and substrate specificity, hepoxilin epoxide hydrolase was determined to be different than leukotriene A_4 epoxide hydrolase (74).

Other examples of trihydroxyeicosanoids include lipoxins A_4 and B_4 (figure I.B.16). Lipoxins (LX) are trihydroxytetraene fatty acids and their formation takes place through very complicated interactions between LOs (26). For example, metabolism of 15-HPETE and 5,15-diHPETE by porcine 5- or 12-LO can lead to

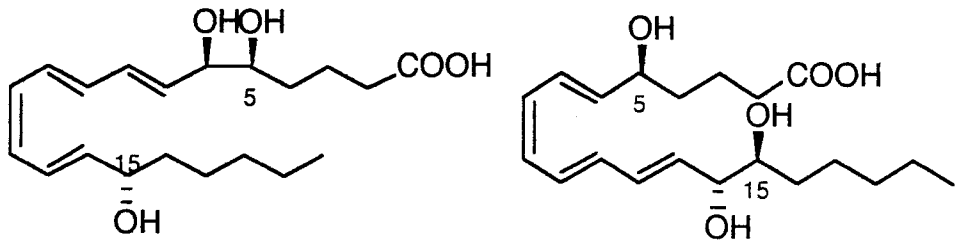


Figure I.B.16. Structures of Lipoxins A_4 and B_4.

lipoxin B_4 formation (26). 12- and 15-LOs from rabbit reticulocyte and soybean 15-LO can convert 5,15-diHETE to trihydroxytetraenes. In addition, 12- and 15-LOs from leukocytes can convert 15-HETE and AA to LXB_4 (26). Also, LX biosynthesis can involve epoxide intermediates. 12-LO from human platelets and soybean 15-LO can form LXA_4 from LTA_4 (26). Lipoxins can be also formed by non-enzymatic hydrolysis of epoxytetraene intermediates (26). LXs and trioxilins differ in that the former are the results of multiple lipoxygenations or metabolism of epoxytetraenes and bear conjugated tetraenes. Trioxilins, on the other hand, are

only formed via the action of epoxide hydrolases on their epoxide precursors and do not bear conjugated olefins. Both lipoxins A_4 and B_4 can cause a relaxation of isolated arteries, contraction of smooth muscles, activation of leukocytes and protein kinase C, as well as inhibition of natural killer cell activity (26).

Similar types of hydroperoxide degradations have been observed in plants. SBLO-1 was reported to isomerize 13*L*-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid to *threo*-11-hydroxy-*trans*-12,13-epoxy-9-*cis*-octadecenoic acid (76). In addition, the same enzyme was found to convert 15*S*-HPETE into 13*R*-hydroxy-14,15-*trans*-epoxyeicosatrienoic acid (77). In both studies, labeling experiments revealed that both oxygens of the hydroperoxide moiety were retained in the product due to an intramolecular rearrangement of the hydroperoxide. Also, it has been reported that the hydroperoxide isomerase from *Saprolegnia parasitica* can convert both 9- and 13-hydroperoxyoctadecadienoic acids to both their α,β- and γ,δ-epoxy alcohols (78). Further, this organism showed the capability to hydrolyze these epoxy alcohols to their corresponding trihydroxy fatty acids. Incubation of arachidonic acid with *S. parasitica* led to the production of 11,12,15*L*-trihydroxy-5,8,13-eicosatrienoic acid, 11,14,15-trihydroxy-5,8,12-eicosatrienoic acid, and 13,14,15-trihydroxy-5,8,11-eicosatrienoic acid (79).

I.B.2.c.4. Enzymatic Dehydration into Allene Oxides

Allene oxide formation from fatty acid hydroperoxides has been reported in the plant tissues (80) and corals (81). Corn hydroperoxide dehydrase has been shown to convert 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid into 12,13*S*-

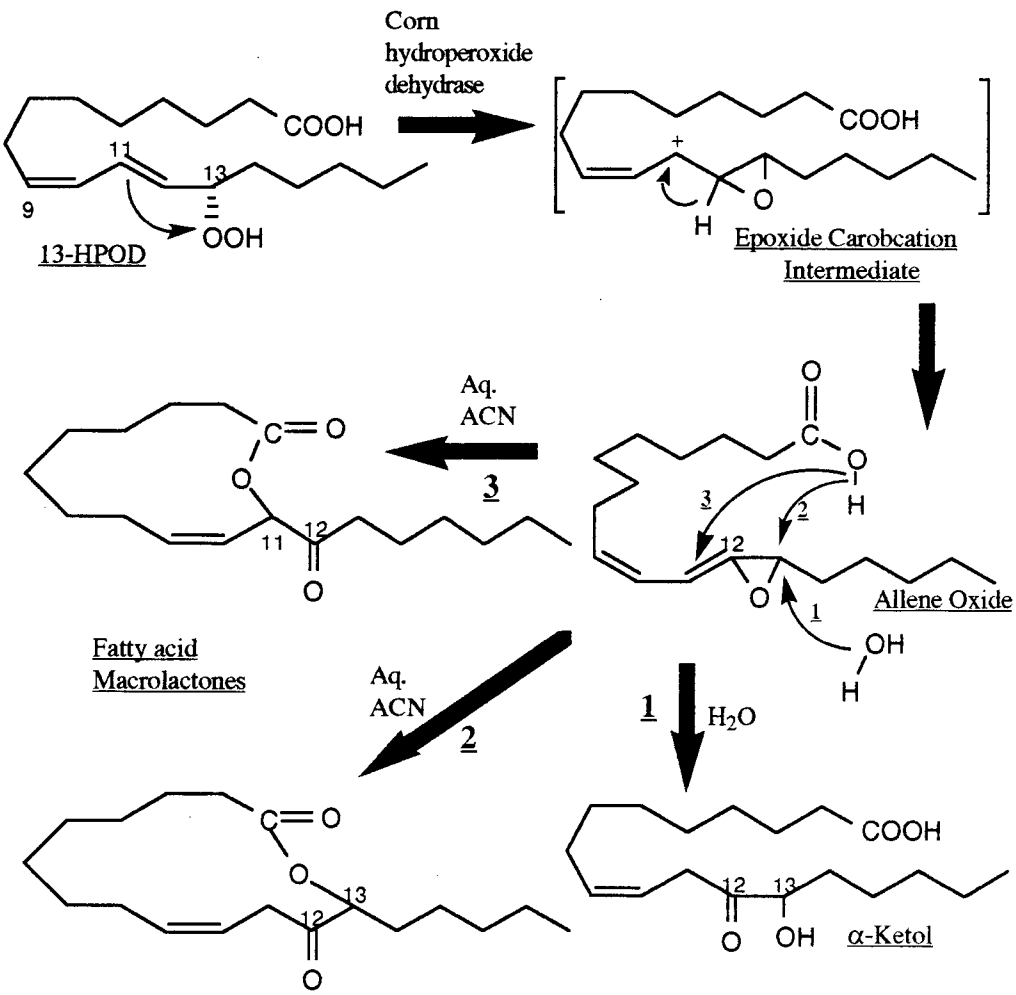


Figure I.B.17. Proposed mechanism for formation of α-ketol and the macrolactones (80).

epoxy-9Z,11-octadecadienoic acid, an unstable allene oxide (figure I.B.17). An epoxide carbocation has been proposed as an intermediate between the hydroperoxide and allene oxide (82). The allene oxide spontaneously hydrolyzes to yield an α-ketol (80). Stability of the allene oxide is considerably higher in acetonitrile, a non-nucleophilic solvent, and in this solvent slowly decomposes to two isomeric lactones (figure I.B.17). Recently, a hydroperoxide dehydrase or allene oxide synthase activity was isolated from flaxseed (83). This dehydrase enzyme was determined to be a 55-kilodalton hemoprotein. Furthermore, spectral characteristics of this enzyme revealed it to be a cytochrome P-450 (83). This finding establishes a new catalytic activity for a cytochrome P-450.

I.B.2.c.5. Hydroperoxide Conversion into Divinyl Ether

This type of metabolism is unique to the plants. In 1973, a novel divinyl ether fatty acid was isolated from potato tubers (*Solanum tuberosum*) (82). This novel fatty acid, colnelenic acid or 9-[nona-(1'*E*,3'*Z*)-dienyloxy]-non-(8*E*)-enoic acid, was shown to be enzymatically produced from 9*S*-hydroperoxy linoleic acid in potato tuber homogenates (figure I.B.18). Deuterium labeling studies showed that removal of the *pro-R* proton from the allylic methylene to the carboxy side of the 1,4-pentadiene moiety leads to the formation of an epoxide carbocation (84). Colneleic acid was degraded, enzymatically or non-enzymatically, to yield 9oxonanoic acid and was therefore suggested to be a precusor to the short chain aldehyde and aldehyde acid fragments (84). Interestingly, the products of this pathway are the same as those hydroperoxide lyase in plants.

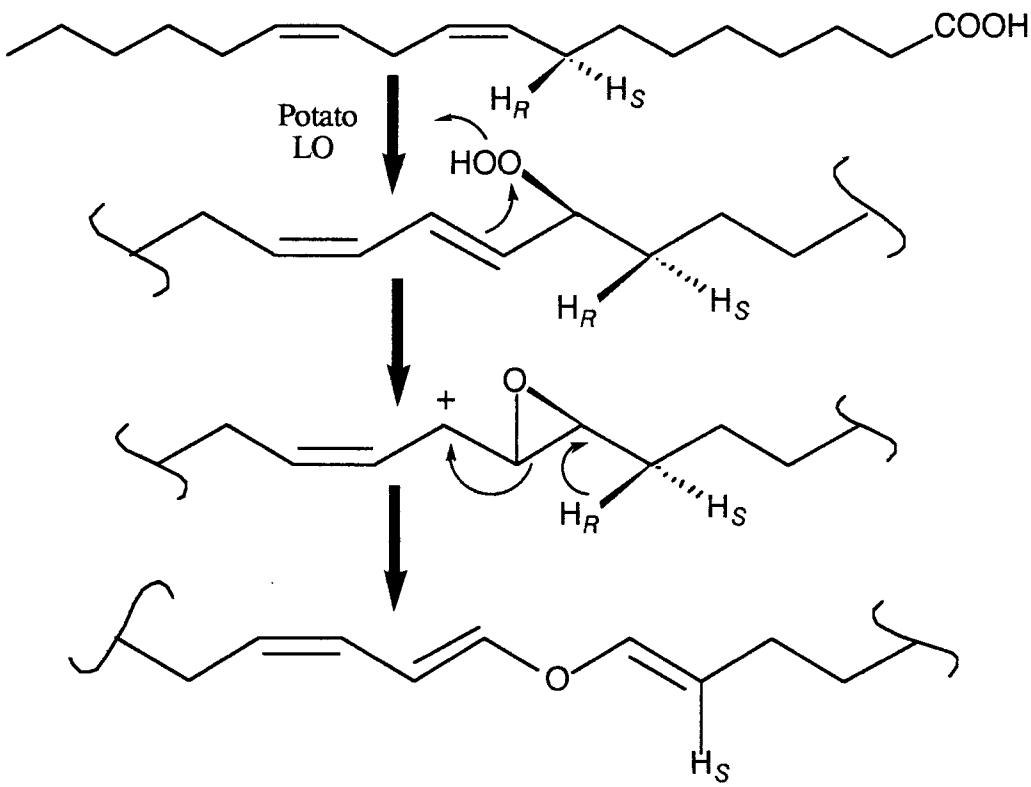


Figure I.B.18. Proposed mechanism for divinyl ether formation (84).

With the exception of the findings presented in the appendix section, all other material discussed hereafter has been either published or accepted for publications in scientific journals. Due to the multiplicity of authorship in the mentioned publications, it is necessary to indicate that this author was the primary investigator and therefore the first author on those articles.

Chapter II. DISCOVERY OF 12*S*-HYDROXY-5,8,10,14-EICOSATETRAENOIC ACID (12*S*-HETE) AND 10-HYDROXY-11,12-*TRANS*-EPOXYEICOSATRIENOIC ACID (HEPOXILIN B_3) IN THE TROPICAL RED ALGAE *PLATYSIPHONIA MINIATA* AND *COTTONIELLA FILAMENTOSA*

ABSTRACT

The potent mammalian immunohormone, 12*S*-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12*S* -HETE) and the insulin release enhancer, 10-hydroxy-11,12-*trans*-epoxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid (hepoxilin B_3) are 12-lipoxygenase metabolites of arachidonic acid and are found in various mammalian tissues. In humans, 12*S*-HETE is produced and secreted by platelet cells and elicits both chemotactic and degranulatory responses in target neutrophils. Hepoxilin B_3 has been found in pancreatic and nervous tissues. Although lipoxygenase pathways are well documented in terrestrial plants, these lipoxygenase products have never been isolated from the plant kingdom. Herein, we report the first isolation of 12*S* -HETE (1) and hepoxilin B_3 (2) as their stabilized perester derivatives (3 and 4) from two plants, the tropical red marine algae *Platysiphonia miniata* (C. Agardh) Børgesen and *Cottoniella filamentosa* Børgesen.

INTRODUCTION

Since its discovery in 1974 (40), both stereoisomers of 12-HETE have been found widely in many different animals [sea urchins (85), guinea pigs (86, 87), and humans (88, 89)] as well as different tissues within a single animal [human neutrophils (88, 89), and psoriatic lesions (90)]. This valuable lipoxygenase product (75), derived from arachidonic acid, is chemotactic to human PMN leukocytes (91), pro-inflammatory (92) and inhibitory to cell growth (93). Interestingly, 12-HETE is found at elevated levels in lesional skin of psoriatic patients and seems to play an important role in the etiology of this disease (90). However, the 12-HETE involved in psoriasis is of *R* and not the *S* configuration which is produced by platelet cells (41).

Hepoxilin B_3, a multifunctional epoxy, hydroxy fatty acid has been isolated from mammalian platelets (65), pancreatic islets (66), lungs (67) and *Aplysia* neurons (94). Analogous multifunctional fatty acids have also been isolated from higher plants (76) and fungi (78). Hepoxilin B_3 is a 12-lipoxygenase (12-LO) product of arachidonic acid and arises from hematin-assisted intramolecular rearrangement of 12*S*-hydroperoxyeicosatetraenoic acid (12*S*-HPETE) in blood platelets (95). This intramolecular oxidation mechanism results in the production of both stereoisomers of hepoxilin B_3 at carbon 10 (96) which can undergo further metabolism to trioxilin B_3 (73). An analogous metabolism, mediated by hepoxilin epoxide hydrolase (HEH), is involved in the conversion of hepoxilin A_3 to trioxilin A_3 in rat liver and other tissues (74). The literature available on the physiological role of hepoxilin B_3 is limited; however, this compound has recently been shown to

possess insulin secretagogue activity (66) and to play a possible role as a second messenger for presynaptic inhibition in *Aplysia* sensory cells (94)

As part of an extensive survey of the biomedicinal potential of Caribbean marine algae, the crude lipid extracts of more than 100 species of seaweeds were examined for a combination of antimicrobial properties and unique compounds by TLC (97). The crude organic extract of one of the algae, *Platysiphonia miniata* (C. Agardh) Børgesen (Delesseriaceae, Rhodophyta), exhibited antimicrobial activity against two gram positive microorganisms, *Staphylococcus aureus* and *Bacillus subtilis*, and showed the presence of several potentially unique secondary metabolites by TLC. Although, bioactivity was used as a way to screen our organic extracts for interesting properties origionally, it was not pursued throughout our fractionations because of the need to derivatize our extracts. Subsequently, the well characterized hydroxyeicosanoid 12*S*-HETE (**1**, figure II.1) was isolated as a synthetically stabilized derivative (**3**). Continued investigation of the complex lipid extract of this alga led to our isolation of a biogenetically related eicosanoid, the insulin-release modulator hepoxilin B_3 (**2**), again obtained following synthetic derivatization (**4**). A second red alga of the same family, *Cottoniella filamentosa* Børgesen, was chromatographed and derivatized in a similar manner to yield the same eicosanoids as their stabilized per-esters (**3**, and **4**).

Isolation of 12-HETE and hepoxilin B_3 from marine algae resulted in two separate publications (58, 98).

RESULTS AND DISCUSSION

Platysiphonia miniata and *Cottoniella filamentosa* are both small red algae (to 10 cm) which are found in 10-25 m water off the southwest Coast of Puerto Rico (99). The crude lipid extract of *P. miniata* was found to be antimicrobial to *Staphylococcus aureus* (8 mm, inhibition diameter) and *Bacillus subtilis* (8 mm, inhibition diameter, 97) and toxic to brine shrimp (100% mortality at 1 mg/ml, 9% at 200 μg/ml, 0% at 50 μg/ml). The crude extract of *C. filamentosa* was found to be antimicrobial only to *S. aureus* (ca. 8 mm, inhibition diameter). Further, both extracts showed the presence of numerous unique appearing secondary metabolites which appeared as streaks on TLC plates. Initially, this was attributed to partial degradation of these compounds on silica gel; however, a 2D TLC indicated these metabolites to be chemically stable under these conditions. Nevertheless, the chromatographic characteristics of these natural products could be enhanced by acetylation and methylation of the crude extracts. Following a combination of vacuum, flash and high performance liquid chromatography, we isolated the well recognized hydroxy-eicosanoid 12*S*-HETE (**1**, figure II.1) from *P. miniata* as a synthetic derivative (**3**, 58). Our continued investigation of the natural products of these two tropical algae led to the isolation of the mammalian insulin-release modulator, hepoxilin B_3 (**2**, figure II.1), again as a synthetic derivative (**4**, 98) from both organism.

Derivative **3** showed a UV absorption maxima at 238 nm indicative of a conjugated diene and IR absorptions consistent with one or more esters. By a combination of LR EIMS (obs. M^+ - HOAc, 100%) and ^{13}C NMR (obs. 19 ^{13}C

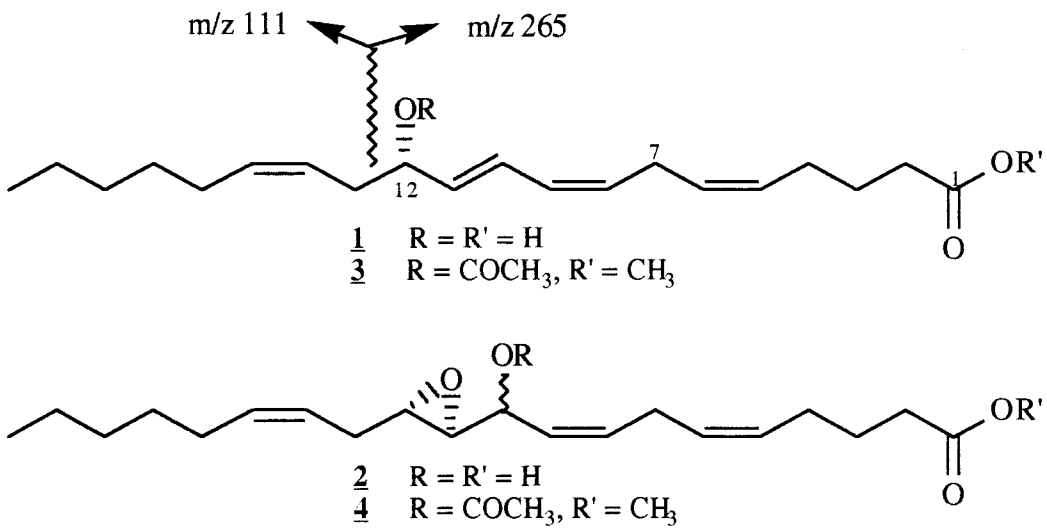


Figure II.1. Structures of 12*S*-HETE (1) and hepoxilin B_3 (2) and their respective bis-ester derivatives (3 and 4).

signals) a molecular formula of $C_{23}H_{36}O_4$ (6° of unsaturation) was deduced. As we have shown previously for related compounds (100,101,102,103), the linear spin system present in fatty acid derived compounds are very effectively probed by ^1H-^1H COSY (table 1). In this case, the overall structure of derivative 3 could be deduced from this technique alone. The position of oxidation in derivative 3 was confirmed by analysis of the LR EIMS fragmentation pattern. The two key fragments at m/z 111 (8%) and 265 (15%), which corresponded to $C_8H_{15}^+$ and M^+ - C_8H_{15} respectively (figure II.1), represented cleavage alpha to the acetoxy functionality and thus placed the site of oxidation at C-12 (85).

The stereochemistries of the C8-C9 and C10-C11 olefins were determined as *cis* and *trans*, respectively, by measurement of their diagnostic coupling constants (table II.1). The stereochemistries of the C5-C6 and C14-C15 olefins were deduced by comparison of our assigned ^{13}C NMR spectrum to model

compounds and calculated values. Shifts of δ26.72 at C-4 and δ26.39 at C-7 defined the C5-C6 olefin as *Z* and confirmed the C8-C9 olefin as *Z* as well. The only remaining allylic carbons in the ^{13}C NMR spectrum for **3** were at δ 32.95 (C-13) and δ27.70 (C-16), and thus, defined a *Z* stereochemistry for the C14-C15 as well (100, 104). The optical rotation of derivative **3** was -2.9°, which by comparison with literature values for the same derivative of a closely related compound, 12*S*-HEPE (100), defined the configuration at C-12 to be *S*. Therefore, the structure of the natural product **1** was defined as 12*S*-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid.

Derivative **4**, as isolated from the derivatized organic extract of *P. miniata*, was an optically active and colorless oil which showed IR absorptions consistent with one or more esters. A molecular formula of $C_{23}H_{36}O_5$ (6 ° unsaturation) was deduced for derivative **4** from observation of a small M^+ ion (0.06%) in the LR EIMS (Low Resolution Electron Impact Mass Spectrum) and a prominent M^+-OAc ion (34.3%) in the HR EIMS. The LR EIMS further showed fragmentations consistent with the presence of one acetate and one carbomethoxy functionality, and this was confirmed by integration of its ^1H NMR spectrum. In addition to the two ester carbonyls expected in the above functionalities, three more degrees of unsaturation were revealed by the ^{13}C DEPT (Distortionless Enhancement by Polarization Transfer) spectrum of **4** by observation of six olefinic methine carbon atoms, a structural feature also consistent with ^1H NMR integration data. Thus, derivative **4** contained a single ring.

As we have shown previously for related compounds (58, 100, 101, 103, 105), ^1H-^1H COSY (COrrelated SpectroscopY) can be a very effective probe of the

linear spin system present in fatty acid derived compounds (table II.2 and figure II.2). However, several regions of the spectrum obtained for **4** in a variety of solvents were severely overlapped and precluded a simple analysis. These difficulties in interpretation arising from this accidental degeneracy were overcome by application of ^1H-^1H Relay Transfer Correlation Spectroscopy (RTCOSY, 105).

Routine interpretation of COSY data allowed assignment of a normal $\Delta 5$, $\omega 6$ structure to derivative **4** (table II.2). Further, the linear spin system could be followed through the C5-C6 olefin to a bisallylic methylene at C7. This, in turn, was coupled to the third and final olefin in **4** located between C8 and C9. Consideration of the molecular formula with those atoms left unassigned from COSY data (excluding the acetate ester) indicated that a fragment of molecular constitution $C_4H_5O_2$ spanned these positions. Once again, COSY data, supported by assignments from the DEPT spectrum and molecular formula, allowed assignment of this third partial structure. A methine proton alpha to the acetate ester was adjacent to a disubstituted epoxide which was in turn next to an allylic methylene (table II.2). However, as the olefinic protons at C9 and C14 had the same proton chemical shifts (table II.2), it was impossible on the basis of ^1H-^1H COSY data alone to determine the orientation of this central fragment. This problem was effectively solved by use of RTCOSY (figure II.2) in which an additional relayed correlation was observed between the methine proton alpha to the acetoxy ($\delta 5.67$) and the methine proton at position 8 ($\delta 5.51$), proving that the oxidized end of this central fragment was closer to the -$COOCH_3$ end. As this relationship of olefin, epoxide and alcohol functional groups is identical to those found in hepoxilin B_3 (65), the LR CIMS characteristics of our derivatized

C#	C_6D_6 ^{13}C[a] δ	C_6D_6 ^1H δ	m	J (Hz)	$CDCl_3$ ^1H δ	m	J (Hz)
1	[b]	-					
2	33.30	2.10	t	7.4	-	t	7.5
3	24.99	1.58	tt	7.4	2.33	tt	7.5
4	26.72	1.94	dt	7.1,7.1	1.71	dt	6.6,6.6
5	129.74	5.27	m		2.02	m	
6	[c]	5.34	m		5.30-5.49	m	
7	26.39	2.83	bdd	7.4,7.4	5.30-5.49	bdd	6.1,6.1
8	131.46	5.39	m		2.91	m	
9	[c]	5.97	dd	11.0,11.0	5.30-5.49	dd	11.0,11.0
10	[c]	6.74	dd	14.4,11.0	5.96	dd	15.2,11.0
11	131.85	5.65	m		6.53	dd	15.2,7.3
12	74.19	5.60	m		5.62	m	
13	32.95	2.39	ddd	14.3,7.1,7.1	5.30-5.49	ddd	14.6,7.3,7.3
		2.49	ddd	14.3,7.1,7.1	2.37	ddd	14.6.7.3,7.3
14	124.18	5.46	m		2.42	m	
15	133.17	5.53	m		5.30-5.49	m	
16	27.70	2.01	dt	6.9,6.9	5.30-5.49	dt	6.4,6.4
17	29.58	1.25	m		2.11	m	
18	31.77	1.25	m		1.32	m	
19	22.90	1.25	m		1.32	m	
20	14.22	0.88	t	6.9	1.32	t	6.8
1'	50.92	3.37	s		0.89	s	
12'	168.80	-			3.67		
12''	20.80	1.73	s		- 2.05	s	

[a] Assigned on the basis of comparisons with model compounds.
[b] Not observed.
[c] Signals obscured by C_6D_6.

Table II.1. High field NMR data for bis-ester derivative 3 of 12*S*-HETE.

		C_6D_6			$CDCl_3$		
C#	$^{13}C^a$	^1H	m	J (Hz)	^1H	m	J (Hz)
	δ	δ			δ		
1	b						
2	33.30	2.12	t	7.4	2.33	t	7.4
3	25.00	1.60	tt	7.4,7.4	1.71	tt	7.4,7.4
4	26.73	1.98	dt	7.4,7.4	2.11	dt	7.4,7.4
5	130.17c	5.32	bdt	10.4,7.4	5.39-5.42	m	
6	127.74c	5.35	m		5.39-5.42	m	
7	27.59	2.95	m		2.94	m	
8	123.37c	5.52	m		5.62	bdt	9.8,7.4
9	134.22c	5.42	m		5.39-5.42	m	
10	70.86	5.67	dd	9.2,6.4	5.39-5.42	m	
11	55.71	2.92	m		2.96	m	
12	58.30	2.86	ddd	7.4,7.4,2.1	2.90	ddd	6.3,6.3,2.0
13	29.66	2.18	ddd	14.8,7.4,7.4	2.26	ddd	14.3,7.0,6.3
		2.30	ddd	14.8,7.4,7.4	2.42	ddd	14.3,7.0,6.3
14	124.31c	5.42	m		5.39-5.42	m	
15	133.35c	5.46	m		5.54	bdt	10.9,7.3
16	26.73	1.92	dt	8.8,8.8	2.02	dt	7.3
17	29.52	1.25	m		1.32	m	
18	31.71	1.25	m		1.32	m	
19	22.88	1.25	m		1.32	m	
20	14.23	0.88	t	7.0	0.87	t	5.7
OMe	50.94	3.36	s		3.67	s	
OAc	20.53	1.65	s		1.59	s	

a Assigned on the basis of model compounds (58, 100, 101, 104).
b Quaternary carbons not observed in distortionless enhancement by polarization transfer.
c Based on model compounds (58, 101), however, maybe interchanged.

Table II.2. High field NMR data for bis-ester derivative 4 of hepoxilin B_3 .

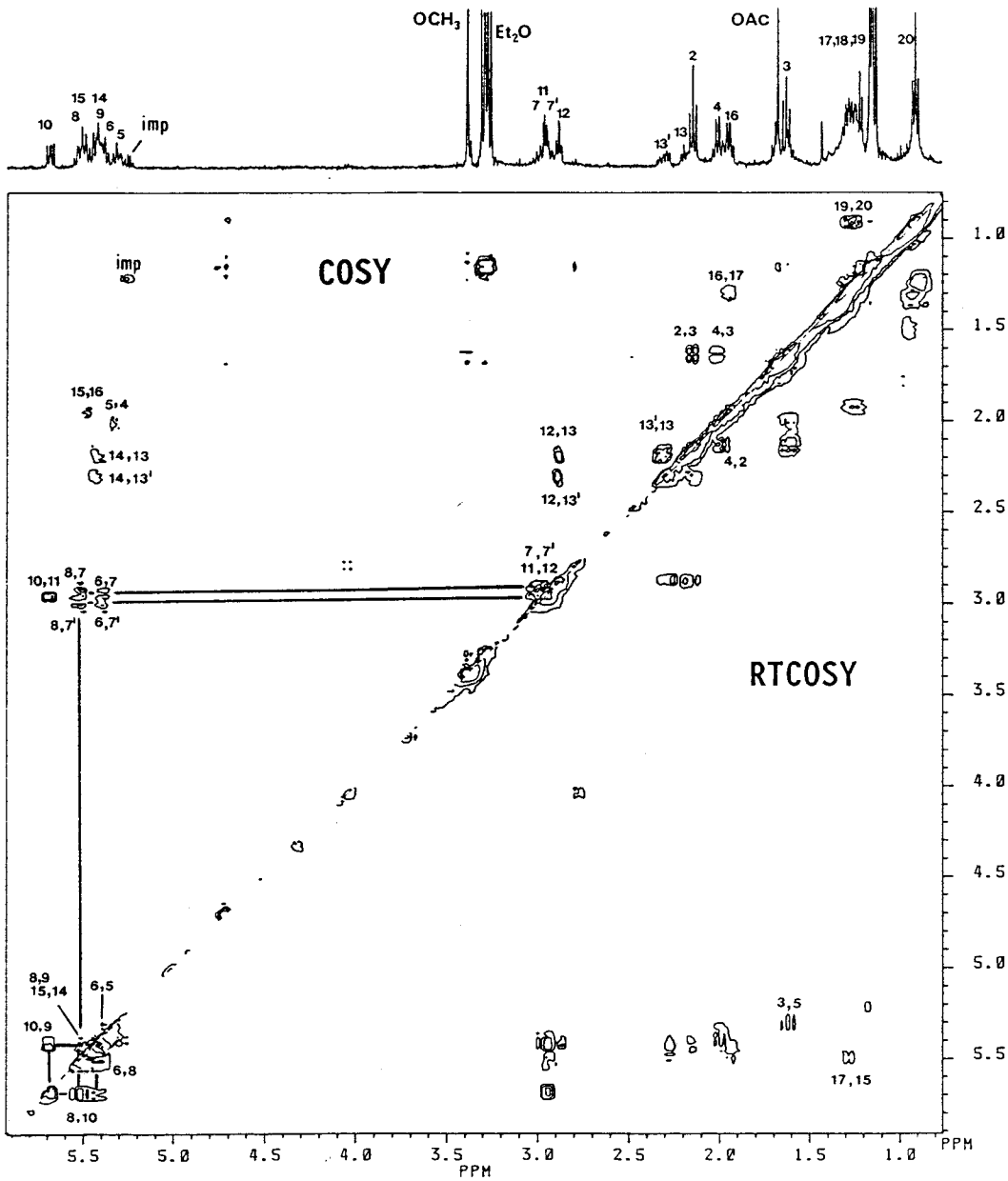


Figure II.2. Contour plots of ^{1}H-^{1}H COSY (upper) and relay transfer (RT) COSY (lower) of hepoxilin B_3 acetate, methyl ester (4).

compound 4 were compared with those obtained from the same derivative of an authentic standard of hepoxilin B_3. Hepoxilin B_3 was recently obtained from another red alga (M. Bernart and W.H. Gerwick, work in progress) and authenticated by comparing the mass spectrum of its methyl ester, trimethylsilyl ether with published data (65). The spectra obtained for the two materials were identical. Further, in this ionization mode the acetate-methyl ester derivatives give fragmentations which are more diagnostic for the sites of oxidation in the chain (see experimental).

The C11-C12 of epoxide in 4 had a *trans* stereochemistry as shown by a coupling constant value of 2.1 Hz between its protons (table II.2). The C5-C6 olefin was deduced to be of *Z* stereochemistry based on a 10.4 Hz coupling constant seen in the d-6-bz spectrum of 4 (table II.2). In $CDCl_3$, derivative 4 showed coupling constants of 9.8 Hz and 10.9 Hz for H8-H9 and H14-H15 respectively, allowing a *Z* assignment to the geometry of these two double bonds as well (table II.2). Therefore, the structure of the natural product 2 was defined as 10-hydroxy-11,12-*trans*-epoxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid. Carbon assignments, based largely on comparisons with several model compounds (58, 101, 103, 104), were fully consistent with and supportive of these stereochemical assignments. Although a total synthesis of both C(10)-isomers of hepoxilin B_3 has been published (96), lack of reported optical rotation data makes it impossible to determine the configuration of our isolate. Further, isolation of hepoxilin B_3 as stabilized derivative (4) precluded its evaluation for the antimicrobial activities associated with the crude lipid extract of these algae.

The crude lipid extract of *C. filamentosa* was derivatized and chromatographed in a similar fashion as detailed for *P. miniata* to yield both derivatives 3 and 4 following HPLC. A combination of GCMS and ^{1}H NMR analyses served to confirm the overall structural identity of these two materials as isolated from this second alga.

This was the first discovery of 12*S*-HETE and hepoxilin B_3 from a plant source and represents a major source of these valuable compounds (75) In addition, its isolation from these aforementioned plants further supports the discovery that lipoxygenase-type products are widely present in the red algae (Rhodophyta). Isolation of 12*S*-HETE as its stabilized perester derivative (2) precluded its evaluation for the antimicrobial activity associated with the crude extracts of these algae. Their function in the algae, while under exploration in our laboratory, is wholly unknown at this point.

EXPERIMENTAL

Instruments. Ultraviolet and infrared spectra were obtained on Beckman DB-GT and Perkin-Elmer 727 spectrophotometers, respectively. Nuclear magnetic resonance (NMR) spectra were obtained on Varian EM-360 and Bruker AM 400 spectrometers with TMS as an internal standard while low resolution mass spectra (LRMS) were obtained on a Varian MAT CH7 spectrometer. High performance liquid chromatography (HPLC) was performed using a Waters M-6000 pump, U6K injector and R 401 differential refractometer. Thin layer chromatograms were run using Merck aluminum-backed TLC sheets (silica gel 60 F254). Vacuum and flash chromatography was performed using Woelm 30-40 μ Silica gel. All solvents were distilled from glass prior to use.

Bioassays. Brine shrimp toxicity assays were conducted following published methodology (106). Antimicrobial assays used standard antimicrobial sensitivity methodology in which compound-impregnated paper discs (6.5 mm diameter) were incubated on the surface of seeded agar. Crude extract was tested at 2 mg/disc and applied in 20 ml of diethyl ether. After evaporation of the ether (20-30 min RT) the discs were placed on Mueller-Hinton agar (Difco) seeded with one of the following: *Staphylococcus aureus* (ATCC 12600), *Candida albicans* (ATCC 14053), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 9721). The plates were incubated at 37° C for 24 hours and the zones of inhibition measured in mm.

Collection, extraction and isolation. The marine red alga, *Platysiphonia miniata*, was collected using SCUBA from a location 1.5 km seaward of Media

Luna Reef, Puerto Rico at a depth of 17 m. Voucher specimens of the alga were deposited at the herbarium of the Department of Marine Sciences, University of Puerto Rico, Mayaguez. The fresh frozen algal material (ca. 200 gm dry weight) was extracted 3x using warm $CHCl_3$/MeOH (2:1, v/v) and reduced *in vacuo* to an aqueous slurry. This extract was partitioned between water and chloroform and the lipid layer reduced *in vacuo* to yield 1.6 g of a dark green and viscous oil. A smaller collection of *C. filamentosa* (ca. 100 g dry weight) was made 1.6 km seaward of Margarita Reef, P.R. at 24 m and was similarly extracted to yield 0.7 g of green oil.

Approximately 800 mg of *P. miniata* crude extract was treated with excess acetic anhydride in pyridine (1:1) for 16 h at which time the reaction was quenched by the addition of ice water. The product was extracted with diethyl ether (3 x 30 ml) which was then sequentially washed with 5% HCl (2 x 25 ml), saturated $NaHCO_3$ (2 x 25 ml) and water (1 x 25 ml) and then dried over anhydrous $MgSO_4$. Following filtration and *in vacuo* concentration, the acetylated products were methylated (ethereal CH_2N_2) to yield 255 mg of product. This mixture was applied to a normal phase silica gel column in the vacuum mode and chromatographed in a gradient of EtOAc/isooctane. Of the resulting eight fractions, the first contained the major quantity of the fractionated material (108.5 mg) and showed potentially interesting chemical compounds by TLC analysis (discrete compounds, some with UV absorbance at 254 nm, and giving blue-grey colors upon acidification [50% H_2SO_4] and heating). This fraction was re-chromatographed using normal phase flash chromatography (25% EtOAc/isooctane) to yield a fraction (78.5 mg) enriched in non-pigmented acid charring material. High performance liquid chromatography

(HPLC, 5% EtOAC/isooctane, 8 mm x 10 cm radially compressed μ-Porasil) of this enriched mixture yielded nine fractions the third of which was derivative 3 in pure form (2.8 mg, 0.35% of crude extract). The fractions seven and eight contained compound (2) in derivatized form (4). Further HPLC of the latter two fractions (3.5% EtOAC/isooctane, 8 mm x 10 cm radially compressed μ-Porasil) yielded pure hepoxilin B_3, acetate, methyl ester (4, 1.3 mg, 0.16% of crude extract).

The crude lipid extract of *C. filamentosa* was derivatized and chromatographed in a fashion similar to that described above to yield ca. 1.0 mg of the bis-ester derivative of 12*S*-HETE (3) and 0.4 mg of the bis-ester derivative of hepoxilin B_3 (4). These derivatives showed identical GCMS and high field ^1H NMR spectral features to the same derivatives as obtained from *P. miniata.*

12S-acetoxyeicosatetraenoic acid, methyl ester (2): The acetate, methyl ester derivative of 12*S*-HETE was a colorless oil and showed the following: $[\alpha]_D^{23°} = -2.9°$ (c = 0.28, acetone); UV (MeOH) $_{max}$ = 238 nm (ε = 11500); IR ($CHCl_3$) 2956, 2928, 1742, 1575, 1550, 1250, 1245, 1219, 1006, 979 cm^{-1}; Low resolution electron impact mass spectrometry (LR EIMS) m/z (fragment, rel. intensity) 317 (M^+ - OAc, 100%), 291 (9%), 285 (M^+ -OAc, MeOH, 22%), 265 (M^+ -C_8H_{15}, 15%), 111 ($C_8H_{15}^+$, 8%), 61 (25%); ^1H and ^{13}C NMR in table II.1.

10-Acetoxy-11,12-trans-epoxy-5(Z),8(Z),14(Z)-eicosatrienoic acid methyl ester (4): The acetate methyl ester derivative of hepoxilin B_3 was a colorless, fruity smelling oil which showed the following: $[\alpha]_D^{23°} = -10.9°$ (c = 0.11, $CHCl_3$); IR ($CHCL_3$) 2956, 1743, 1550, 1372, 1234, 1033, 999 cm^{-1}; GC EIMS m/z (fragment, rel. intensity) obs. M^+ at m/z 392 (0.06%), 372 (0.03%),

361 (M^+-OMe, 0.1%), 350 (0.2%), 332 (M^+-HOAc, 0.4%), 319 (0.4%), 314 (0.5%), 301 (1.2%), 261 (1.7%), 231 (2.2%), 221 (10.4%), 147 (13.5%), 119 (15.2%), 81 (46.4%), 43 (100%); HR EIMS (High Resolution Electron Impact Mass Spectroscopy) (positive ion, fragment, rel. intensity, deviation) obs. M^+-OMe at m/z 361.2442 (10.3%, 6.3 mamu dev.), 333.2407 (M^+-OAc, 34.3%, 2.3 mamu dev.), 332.2485 (M^+-HOAc, 34.3%, 13.4 mamu dev.); LR CIMS (Low Resolution Chemical Ionization Mass Spectroscopy) (CH_4, positive ion, fragment, rel. intensity) obs. $(M+H)^+$ at m/z 393 (8.4%), 361 ($(M-CH_3O)^+$, 12.8%), 333 ($(M-OAc)^+$, 100%), 315 (70.0%), 301 ($(M-OAc-MeOH)^+$, 56.9%), 281 ($(M-C_8H_{15})^+$, 5.7%), 283 (26.8%), 221 ($(M-HOAc-C_8H_{15})^+$, 12.6%), 211 (20.6%), 161 (35.6%), 153 ($(M-C_{13}H_{19}O_4)^+$, 5.9%), 149 (8.1%), 141 (13.5%), 139 (20.3%), 123 ($(M-C_{14}H_{19}O_5)^+$, 17.2%), 111 ($(M-C_{15}H_{21}O_5)^+$, 17.8%), 81 (17.7%), 69 (12.8%), 67 (11.6%), 61 (67.3%), 55 (11.6%); ^1H and ^{13}C NMR in table II.2.

Chapter III. 12-LIPOXYGENASE ACTIVITY IN THE RED MARINE ALGA *GRACILARIOPSIS LEMANEIFORMIS*

ABSTRACT

The temperate red alga *Gracilariopsis lemaneiformis* is a rich source of polyunsaturated fatty acids that are regio-specifically oxidized at carbon 12. In this *in vitro* study, we demonstrate the existence of a highly active lipoxygenase-type enzyme system in *Gracilariopsis*. Gas chromatography-mass spectrometry and ^{1}H NMR work on the crude enzyme reaction mixture identified production of 12-HETE, 12*R*,13*S*-diHETE, 12*R*,13*S*-diHEPE, and 12-oxo-dodecatrienoic acid. These were all 12-lipoxygenase products previously isolated from this alga. This was the first report of 12-lipoxygenase-type activity from the plant kingdom.

INTRODUCTION

In the previous chapters we discussed a widespread occurrence of a variety of pharmacologically active hydroxy-eicosanoids in red algae (58, 98, 100, 101, 102, 103, 107). This includes 12-hydroxyeicosatetraenoic acid or 12-HETE (**1**, 58), 12-hydroxyeicosapentaenoic acid or 12-HEPE (**2**, 100), 6-trans-leukotriene B_4 ethyl ester or 6-E-LTB_4, ethyl ester (**3**, Bernart, M. and Gerwick, W.H., unpublished results), hepoxilin B_3 (**4**, 98), and 12*R*,13*S*-dihydroxyeicosatetraenoic acid or 12*R*,13*S*-diHETE (**5**, 101), all of which are oxidized at C-12. In mammals, eicosanoids of this general type arise most commonly by metabolic action of 12-lipoxygenase on arachidonic acid (**6**, AA) or its congeners, although their formation also occurs to a lesser extent via a cytochrome P-450 pathway (108). Metabolites of the 12-lipoxygenase pathway are not only involved in many normal metabolic functions in humans but are also central to the etiology of numerous disease states, including psoriasis, asthma, and disorders involving the immune system. Hence, description of these enzyme systems in other life forms may enhance our understanding of fine elements of the enzymology and biosynthesis of these mammalian immunohormones, as well as give insight into evolutionary trends and molecular recognition phenomena in these classes of enzymes.

Further biosynthetic manipulation of the initial product of 12-lipoxygenase, 12-hydroperoxyeicosatetraenoic acid (**7**, 12-HPETE), ultimately gives rise to several physiologically important mammalian autocoids, including the HETE's, hepoxilins and leukotrienes (40, 73, 109). Interestingly, while higher plants (110),

algae (111), and animals all contain lipoxygenase enzymes, only animals have been shown to contain lipoxygenase with a regiospecificity for C-12 of the fatty acid chain.

Although the red algae are now a recognized source of 12-hydroxy-eicosanoids, the lipoxygenase enzyme system presumably responsible for their formation has not yet been reported. Our recent chemical work with a red alga from the Oregon coast showed *Gracilariopsis lemaneiformis* (Bory) Dawson, Acleto et Foldvik to be a rich source of AA and EPA derivatives oxidized at C-12, including 12-HETE (**1**), 12-HEPE (**2**), and 12*R*,13*S*-dihydroxyeicosapentaenoic acid or 12*R*,13*S*-diHEPE (**8**, 107). This organism was thus a logical choice for our initial efforts in the evaluation of red algae for 12-lipoxygenase-type activity.

Two separate publications (59 and 112) resulted from our findings reported in this chapter.

RESULTS AND DISCUSSION

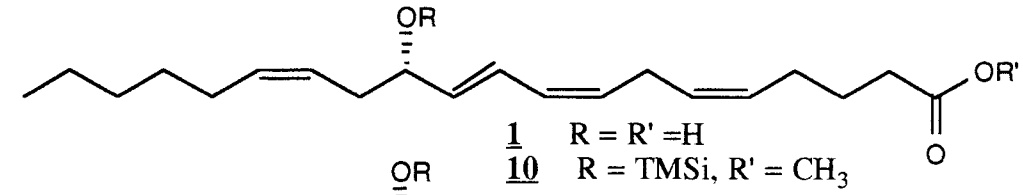
An acetone powder extract (AP) of fresh-frozen *G. lemaneiformis* , collected from the Central Oregon Coast, was incubated with AA in an air-saturated buffer solution under three experimental conditions: 1) AA + AP, 2) AA + boiled AP, and 3) AP alone. An additional control consisted of AA alone in air-saturated buffer. Following the 80 minute incubation, all samples were similarly extracted for their lipids and these were then analyzed by thin layer chromatography. On the same chromatogram, aliquots were applied from all of the above experimental conditions as well as AA standard and the acetone extracted lipids used in preparing the enzyme extract.

Although the $CHCl_3$/MeOH (2:1) extract of this collection of *G. lemaneiformis* showed a rich assortment of eicosanoid-type natural products, the acetone extract did not show major amounts of these compounds (UV-active spots charring blue with cupric acetate). This may have been due to the 'swamping effect' of other compounds and pigments in this acetone extract which overlapped the eicosanoid R_f region. Analysis of the TLC of the extract from the "AP alone" treatment (without added AA) demonstrated the absence of detectable amounts of endogenous eicosanoids or substrates in the "AP" preparations. The materials extracted from the "AA + buffer" treatment showed mainly the presence of AA (R_f = 0.5) along with minute quantities of more polar materials which represented non-enzymatic oxidation products of AA. These faint spots also appeared on the TLC of the AA standard and represented minor impurities. The "AA + AP" treatment showed only a small quantity of unmetabolized AA along with major quantities of a

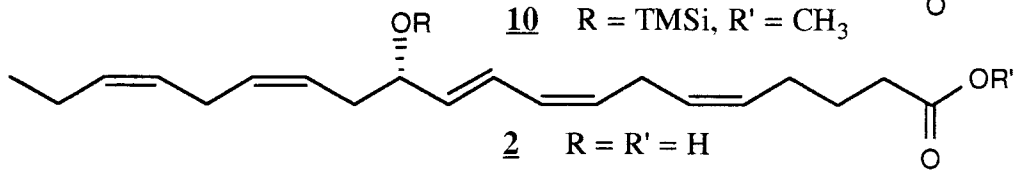
number of UV-active, blue-charring compounds with R_f's at 0 - 0.3. In contrast, the "AA + boiled AP" preparation once again yielded major quantities of AA and none of the metabolites obtained with the active enzyme preparation, suggesting that these products are formed as a result of heat labile, enzyme mediated reactions.

The lipids obtained from the "AA + AP" treatment were checked for the presence of hydroperoxides (see experimental) since these are the expected intermediates between AA and its hydroxylated metabolites, however none were found. This is probably due to a high conversion rate of hydroperoxides to other products by hydroperoxidase and perhaps other enzymes in the crude AP preparation. Alternatively, the mono-oxygenase enzyme, cytochrome P-450, could be responsible for the production of these hydroxy metabolites since this route does not normally involve hydroperoxide intermediates (108). However, our recent isolation of hepoxilin-type natural products from red algae (98) suggests a hydroperoxide intermediate (95) which is consistent with the lipoxygenase pathway.

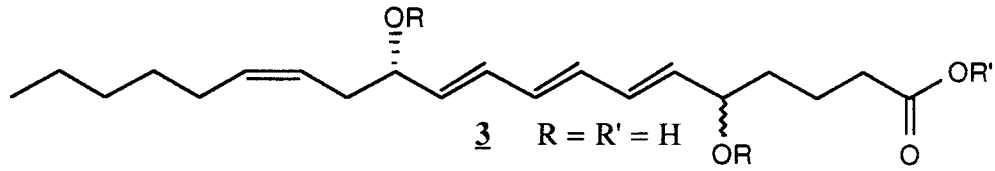
The crude mixture of the AA metabolites obtained from the "AA + AP" treatment was subjected to analysis by LR GC-MS following appropriate derivatization (see experimental). Two compounds from this mixture were identified, one as derivatized starting material (**9**, AA methyl ester) and the other as TMSi ether of 12-HETE methyl ester (**10**). A key fragment ion in the CIMS of derivative **10** was observed at m/z 295 (9%) which is diagnostic for the cleavage alpha to the carbon bearing the silylated alcohol (C12-C13) (40), and thus, identifies C-12 as the point of oxidation. The ion at m/z 193 reinforces this structural assignment as it arises from the alternate cleavage alpha to the carbon



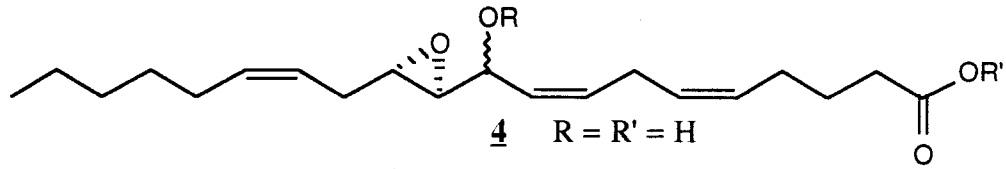
1 R = R' =H
10 R = TMSi, R' = CH_3



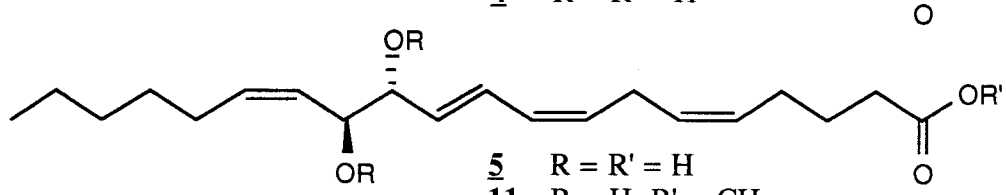
2 R = R' = H



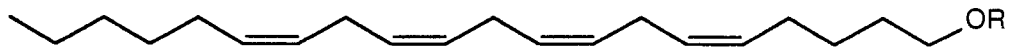
3 R = R' = H



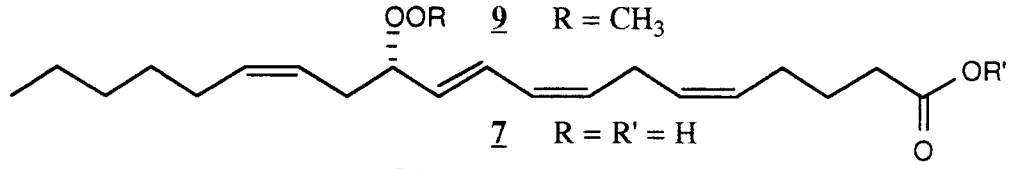
4 R = R' = H



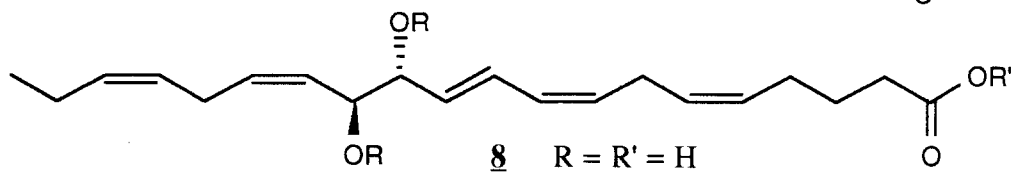
5 R = R' = H
11 R = H, R' = CH_3



6 R = H
9 R = CH_3



7 R = R' = H



8 R = R' = H
12 R = H, R' = CH_3

bearing the silylated alcohol (C11-C12). Although this latter cleavage has not been previously reported for this derivative of 12-HETE (40), it has precedence by analogy to this same derivative in other hydroxy-eicosanoids (73). This compound, present at <1% of the crude mixtrue, was then proven to be a derivatized form of 12-HETE indicating presence of highly active lipoxygenase and hydroperoxide reductase type enzymes.

In addition, from a subsequent incubation, four other compounds were isolated by repetitive NP-HPLC using various solvent mixtures. These methyl ester derivatives were each characterized by high field NMR, LR GC EIMS (of the corresponding TMS ethers for compounds **11** and **12**) as well. Derivatives **11** and **12** were deduced by ^{1}H NMR and ^{1}H-^{1}H COSY as methyl 12*R*,13*S*-dihydroxy-5*Z*,8*Z*,10*Z*,14*Z*-eicosatetraenoate (methyl 12,13-diHETE, 8% of recovered lipids) and methyl 12*R*,13*S*-dihydroxy-5*Z*,8*Z*,10*Z*,14*Z*,17*Z*-eicosapentaenoate (methyl 12,13-diHEPE, 8% of recovered lipids). In both cases, the structural assignment was confirmed by LR EIMS in which major ions for the expected fragments from C12-C13 cleavage were observed at *m/z* 295 and 199 for the TMS ether of **11** and *m/z* 295 and 197 for the same derivative of **12**. Isolation of compouds **11** and **12** reemphasized the presence of 12-lipoxygenase type activity. It is interesting to note the production of 12,13-diHEPE, an ω-3 fatty acid, despite a lack of EPA as a substrate. This suggests the presence of a highly active ω-3 desaturase enzyme in our crude enzyme preparation which apparently desaturated some of the ω-6 fatty acids to their ω-3 analogues.

Finally, structures of two 12-carbon long aldehydes, derived in mammalian systems from action of a 12-hydroperoxide lyase on 12-HPETE (55), were deduced using ^{1}H NMR and comparison to data already available on these compounds (55, 115). These were identified as methyl 12-oxo-5Z,8Z,10E-trienoate (**14**, 4%) and its double bond isomer methyl 12-oxo-5Z,8E,10E-trienoate (**16**, 2%). The cis, cis, trans trienal (**14**) has been reported to possess chemotactic and chemokinetic properties towards porcine PMN leukocytes. In addition, it can potentiate superoxide anion production in response to stimuli such as LTB_4 and FMLP which might suggest involvement of this substance in exacerbation of inflammation (55). The cis, trans, trans isomer of this aldehyde may have been produced as a result of non-enzymatic isomerization of **14** because this isomer is chemically more stable.

Continued studies on the mechanisms of eicosanoid biosynthesis in this algal system will enhance our understanding of the role and occurrence of eicosanoids in the marine environment and plants in general. The ensuing chapter would give some of the work performed in this area and studies aimed at enzymatically producing larger quantities of some of these oxylipins in order to further study their eicosanoid-like effects in mammalian systems.

EXPERIMENTAL

Algal material. Approximately 150 g (fr. wt) of *Gracilariopsis lemaneiformis* was obtained from an intertidal pool at Cape Perpetua, Oregon on 5 June 1989. Voucher specimens of this alga were deposited at the herbarium of the Department of Botany, University of California, Berkeley. Half of the collected material was maintained in cool sea water for transport to the laboratory and used in the enzyme preparation assay on the same day while the other half was frozen on site using dry ice and then stored at -70 °C for extraction at a later date.

Enzyme extraction and preparation. About 75 g (fr. wt) of either fresh or frozen *Gracilariopsis* was repeatedly extracted with a total of 450 ml acetone/dry ice. Solvent was removed from the algal tissue by filtration using a 10 - 20 μ sintered glass funnel. The final residue was rinsed with an additional 30 ml of acetone and dried under N_2 (113). The dry acetone powder (7 g) was divided into two nearly equal portions. From fresh *G. lemaneiformis*, these portions were used for "AA + AP" and "AP alone" treatments while fresh-frozen *G. lemaneiformis* portions were used in "AA + AP" and "AA + boiled AP" treatments. Each of these were then dissolved in 125 ml Erlynmeyer flasks with 60 ml of 0.1 M K_2HPO_4 buffer, pH 7.42. Boiling of the crude enzyme preparation was accomplished in a water bath with occasional stirring (98 - 100 °C, 10 minutes). At the end of the 10 minutes the temperature inside the flask had reached 90 °C and the crude enzyme preparation had turned green, in contrast to fresh material which was pinkish-brown. All flasks were chilled in ice for *ca.* 15 minutes before incubation.

Incubation. Arachidonic acid (Sigma) was dissolved in ethanol to a final concentration of 0.1 mg/μl. Treatments "AA + AP" and "AA + boiled AP" each received 5 mg of AA in 50 μl ethanol while the control "AA + buffer" treatment received 2 mg of AA in 20 μl ethanol. All flasks were incubated at 6 °C for 80 minutes with continuous slow bubbling of air through a pasteur pipet.

Enzyme product extraction. Following the incubation period, the enzyme preparations were filtered through cheese cloth. The pH was adjusted to 4 with 5% HCl, a few ml of saturated brine added, and the lipids repetitively extracted with 4 x 10 ml of ethyl acetate. The solvent of the combined extractions was reduced *in vacuo* and the recovered lipids dissolved in Et_2O for storage.

Thin layer chromatogram (TLC) analysis of the enzyme products. TLC of the acetone extract and various enzyme incubation treatments were made with Merck aluminum-backed TLC sheets (Silica gel 60 F_{254}) using a mixture of diethyl ether, hexane, and acetic acid (59:40:1). The developed TLC was then sprayed with a solution of cupric acetate (Sigma) to produce a characteristic blue char with most eicosanoids upon heating. Hydroperoxide detection was performed by spraying the TLC with iodine followed by a starch solution (114). Further, the crude enzyme product mixture resulting from "AA + AP" treatment was reduced using $SnCl_2$ (110) and compared by TLC for any changes relative to the starting mixture.

Low resolution mass spectra (LRMS) analysis. LRMS studies of arachidonic acid metabolites resulting from the "AA + AP" treatment were obtained on a Finnigan 4023 after methylation (diazomethane, Et_2O, 0.5 h) and silylation (trimethysilylimidazole) of the crude mixture. For **10**: LR GC-CIMS (CH_4,

fragment, rel. intensity) obs. M^+ m/z 406 (45%), 315 (M^+ - (TMSiOH + H^+), 33%), 295 (M^+ -C_8H_{15}, 9%), 193 ($C_{12}H_{17}O_2^+$, 100%), 91 (TMSiOH + H^+, 6%), 69 (9%).

Production and isolation of methyl ester derivatives **11**, **12**, **14**, **16**. In a subsequent study, incubation of 100 mg arachidonic acid with 27.8 grams *G. lemaneiformis* AP under conditions essentially as described above gave derivatives **11** (1.6 mg), **12** (1.6 mg), **14** (0.3 mg), **16** (0.8 mg). This was acheived following methylation and separation by repetitive NP-HPLC using 35% EtOAc/Hexanes and a 5 μ Nucleosil column (4.6 mm x 250 mm) and then 30% EtOAc/Hexanes and a 10 μ Versapak (2 x 4.1 mm x 250 mm).

*Methyl 12R,13S-dihydroxy-5Z,8Z,10E,14Z-eicosatetraenoate (**11**).* Derivative **11** was isolated as a colorless oil showing: IR ($CHCl_3$) 3415, 2929, 2739, 1437, 1220, 1157, 1025, 998 cm^{-1}; ^1H NMR (300 MHz, C_6D_6) δ 6.77 (1H, bdd, J = 15.3, 11.1, 1.0, 1.0 Hz, H-10), 6.03 (1H, bdd, J = 11.1, 11.1 Hz, H-9), 5.77 (1H, dd, J = 15.3, 6.3 Hz, H-11), 5.55 (1H, m, H-14), 5.46 (1H, m, H-15), 5.44 (1H, m, H-8), 5.39 (1H, m, H-6), 5.24 (1H, dtt, J = 10.8, 6.3, 1.6 Hz, H-5), 4.45 (1H, ddd, J = 7.6, 4.0, 3.8 Hz, H-13), 4.19 (1H, m, H-12), 3.33 (3H, s, OMe), 2.86 (2H, dddd, J = 6.7, 6.7, 1.8, 1.8 Hz, H_2-7), 2.21 (1H, bd, J = 4.0 Hz, H-12'), 2.06 (2H, t, J = 7.4, H_2-2), 1.95 (2H, dt, J = 7.3 Hz, H_2-16), 1.92 (2H, dt, J = 7.4 Hz, H_2-4), 1.73 (1H, bd, J = 4.0 Hz, H'=13), 1.57 (2H, tt, J = 7.4 Hz, H_2-3), 1.18 (2H, m, H_2-17), 1.18 (2H, m, H_2-19), 0.86 (3H, t, J = 6.9 Hz, H_3-20); LR GC EIMS (TMS ether, trimethylimidazole, 70 eV) obs. M^+ m/z 494 (M^+, 0.2%), 407 (M^+ - $C_4H_7O_2$, 0.9%), 404 (M+ - TMSiOH, 3.8%), 314 (M+ - 2 TMSiOH, 1.0%, 295 (M^+ - C_8H_{14}OTMSi, 18.4%), 199 (M^+ -

$C_{13}H_{18}O_2TMSiOH$, 7.1%), 73 (TMSi+, 100%).

*Methyl 12R, 13S-dihydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoate (**12**).* Derivative 12 was isolated as a colorless oil showing: IR (CHCl3) 3442, 2964, 1738, 1492, 989 cm-1; 1H NMR (300 MHz, C_6D_6) δ 6.67 (1H, dddd, J = 15.3, 11.1, 1.2, 1.2 Hz, H-10), 6.02 (1H, bdd, J = 11.1, 11.1 Hz, H-9), 5.74 (1H, dd, J = 15.3, 6.2 Hz, H-11), 5.23-5.55 (7H, m, H-5, 6, 8, 14, 15, 17, 18), 4.44 (1H, m, H-13), 4.17 (1H, m, H-12), 3.33 (3H, s, OMe), 2.85 (2H, bdd, J= 6.6, 6.6 Hz, H_2-7), 2.16 (1H, m, H-12'), 2.77 (2H, m, H_2-16), 2.06 (2H, t, J = 7.4, H_2-2), 1.95 (2H, dt, J = 7.4 Hz, H_2-4), 1.93 (2H, dq, J = 7.5, 7.5 Hz, H_2-19), 1.71 (1H, m, H'-13), 1.54 (2H, tt, J = 7.4 Hz, H_2-3), 0.88 (3H, t, J = 7.5 Hz, H_3-20); LR GC EIMS (TMS ether, trimethylimidazole, 70 eV) obs. M= m/z 492 (M^+, 1.4%), 405 (M^+ - $C_4H_7O_2$, 1.4%), 402 (M^+ - TMSiOH, 1.3%), 312 (M^+ - 2 TMSiOH, 1.8%), 295 (M^+ - $C_8H_{12}OTMSi$, 13.1%), 197 (M^+ - $C_{13}H_{18}O_2TMSiOH$, 7.1%), 73 ($TMSi^+$, 100%).

*Methyl 12-oxo-5Z,8Z,11E-dodecatrienoate (**14**) and methyl 12-oxo-5Z,8E,11E-dodecatrienoate (**16**).* Derivative **14** was isolated as a colorless oil and showed, for corresponding signals, 1H NMR bands highly comparable to that reported form the parent acid (55): (300 MHz, $CDCl_3$) δ 9.60 (1H, d, J = 8.0 Hz, H-12), 7.48 (1H, dd, J = 15.1, 11.1, Hz, H-10), 6.28 (1H, dd, J = 11.1, 10.2 Hz, H-9), 6.17 (1H, dd, J = 15.1, 8.0 Hz, H-11), 5.94 (1H, dt, J = 10.2, 7.0 Hz, H-8), 5.4 (2H, m, H-5,6), 3.68 (3H, s, OMe), 3.09 (2H, bdd, J = 7.0, 7.0 Hz, H_2-7), 2.34 (2H, t, J = 7.3, H_2-2), 2.14 (2H, dt, J = 7.3,7.3 Hz, H_2-4), 1.73 (2H, tt, J = 7.3, 7.3 Hz, H_2-3). Derivative **16** was also isolated as a colorless oil and had a 1H NMR spectrum identical to an authentic standard (115).

Chapter IV. BIOSYNTHESIS OF 12*R*,13*S*-DIHETE AND 12*R*,13*S*-DIHEPE FROM THE MARINE ALGA, *GRACILARIOPSIS LEMANEIFORMIS* FOR EVALUATION OF MECHANISM OF FORMATION AND BIOLOGICAL ACTIVITY

ABSTRACT

In the first part of this cell free biosynthetic study with the marine macrophyte *Gracilariopsis lemaneiformis,* it was demonstrated by mass spectrometry and ^{13}C NMR that molecular oxygen was the source of the two hydroxyl groups in the novel eicosanoid 12*R* ,13*S*-diHETE. In the second part of this study, labeled and unlabeled free acid forms of 12*R*,13*S*-diHEPE were produced biosynthetically from incubation of eicosapentaenoic acid (EPA) with *G. lemaneiformis* enzyme preparations. The yield for conversion of EPA to 12*R*,13*S*-diHEPE was lower than that of arachidonic acid (AA). This may indicate that one or both of algal 12-lipoxygenase or 12-hydroperoxide isomerase enzymes prefer AA to EPA as a substrate. Some of the problems which may be responsible for the reduced yield encountered in this study will be discussed.

INTRODUCTION

Previously, we showed that the acetone powder crude enzyme preparation from *G. lemaneiformis* was able to metabolize exogenously supplied arachidonic acid to the known 12-LO metabolite, 12*S*-HETE (59), as well as 12*R*,13*S*-diHETE which was a major product (ca. 8%) of the incubation experiments (112). Development of the cell free system discussed in this chapter circumvented the problem of poor cell wall penetration by exogenously supplied organic substrates, a traditional obstacle encountered in biosynthetic experiments with marine macrophytes (117). The biogenetic origin of the oxygen atom at C-13 in 12*R*,13*S*-diHETE (**3**, figure IV.1), a unique location for oxidation in eicosanoids, has been of particular interest to us ever since our first isolation of this natural product from another temperate red alga, *Farlowia mollis* (101). The possible mechanisms for introduction of the oxygen atom at C-13 are depicted in figure IV.1 and could be envisioned as a) 12-lipoxygenation of arachidonic acid (AA, **1**, figure IV.1) to 12*S*-hydroperoxyeicosatetraenoic acid (12-HPETE, **2**, figure IV.1) followed by 12,13 epoxide formation and attack by water at C-13 of the epoxide, b) inter or intramolecular rearrangement of the distal hydroperoxy oxygen of the 12-HPETE (**2**) in a manner comparable to how it has been suggested in the literature (95), or finally c) independent oxidation of C-13 by a separate catalytic activity (i.e.-cytochrome P-450). Employing this cell free preparation from *G. lemaneiformis*, we have effectively probed this mechanistic feature of 12*R*,13*S*-diHETE biosynthesis, the first time that such a detailed feature of lipid biosynthesis has been examined in a marine macrophyte.

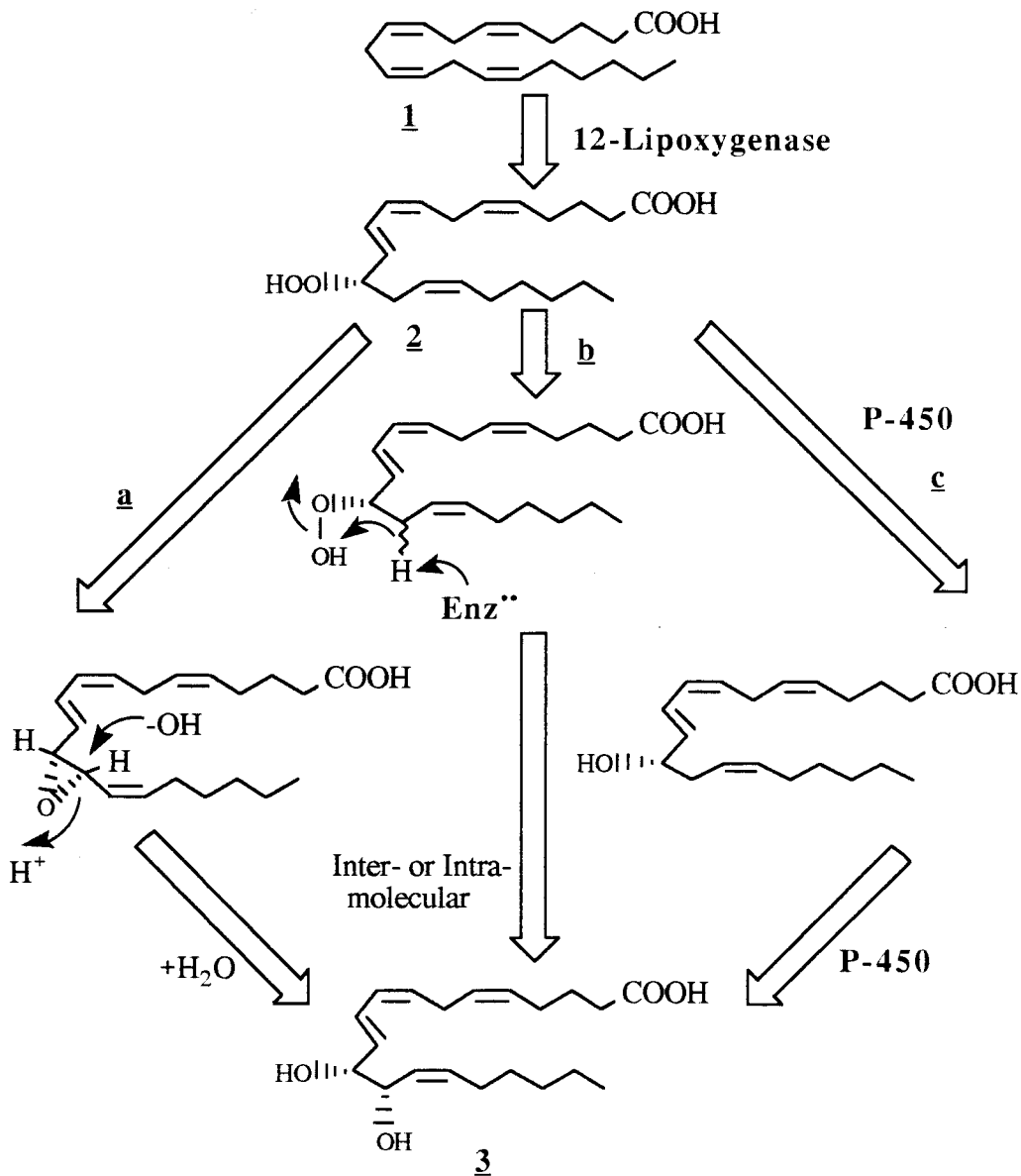


Figure IV.1. Potential mechanisms of 12*R*,13*S*-diHETE (**3**) formation.

The novel algal natural product, 12*R*,13*S*-dihydroxyeicosapentaenoic acid (12*R*,13*S*-diHEPE) was found to inhibit production of progestrone from sheep ovary. Direct injection of 12*R*,13*S* -diHEPE methyl ester into the ovarian artery of sheep after ovulation caused regression of corpus luteum (CL) and hence caused a reduction in progestrone production and led to early exhibition of estrous in the animals (118). This prostaglandin-like activity could be exploited in animal management practices for shortening the estrous cycle of the non-pregnant animals so that they could be bred again. Compounds with prostaglandin-like activity could also have medical use in humans.

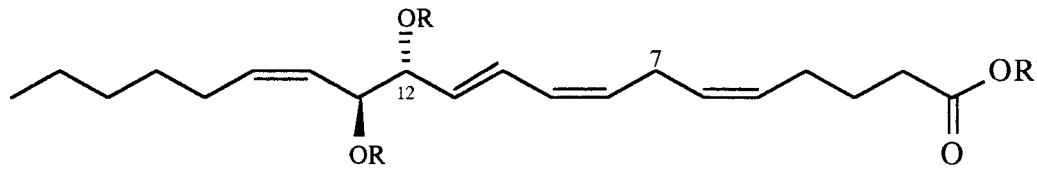
Because of the interesting *in vivo* activity of this compound an *in vitro* study was performed using sheep CL cells to study the intracellular mechanism (s) by which the production of progestrone in these cells is inhibited. In addition, it was of interest to localize the site of action of this substance within the CL cells for which 1-^{14}C 12*R*,13*S*-diHEPE was needed. While, 12*R*,13*S*-diHEPE is not commercially available in any form, its biosynthesis has become possible in our laboratory as a result of enzymatic studies with *Gracilariopsis lemaneiformis* (112). These techniques, with minor modifications, were used to produce both cold and ^{14}C-labeled 12*R*,13*S*-diHEPE for these *in vitro* studies.

The segment of this study on oxidation mechanism of AA to 12*R*,13*S*-diHETE is a part of a publication already in press (112).

RESULTS AND DISCUSSIONS

G. lemaneiformis AP was used for the production of ^{18}O-labeled 12,13-diHETE (3). The different potential origins (water versus O_2) for oxygen at C-12 and C-13 were distinguished by forming metabolite 3 in the presence a 50% $^{16}O/^{18}O$ gas mixture. Direct TLC analysis of the products of this incubation in an acidified solvent (EtOAc/hex/HOAc 64:35:1) demonstrated the existence of several oxidized AA metabolites. The crude methylated extract was repetitively chromatographed by normal phase HPLC to yield 1.0 mg of pure methyl 12*R*,13*S*-diHETE (4). The 300 MHz ^1H NMR, ^{13}C NMR, and LR GC-EIMS (of the corresponding bis-TMS ether, 5) were fully descriptive of methyl 12,13-diHETE when compared with data from our earlier work in this structural class (101). A small positive rotation for this biosynthetically produced sample of compound 4 confirmed its enzymatic origin and 12*R*,13*S* absolute stereochemistry as previously found for the natural product (3, 107, 119). That this rotation was less than that measured for authentic samples of 3 indicated either partial racemization or, more likely, a less accurate measurement given the small sample size.

A DEPT-45 ^{13}C NMR of the oxygen isotope labeled compound (4) displayed signals for all of the protonated carbons. Of primary interest were the C-12 and C-13 signals resonating at δ75.20 and δ70.62 (4), respectively, which following Gaussian resolution enhancement showed two ^{18}O-isotope shifted carbon signals at δ75.18 and δ70.60 (figure 2). The magnitude of the chemical shift dispersion between these ^{16}O-^{13}C and ^{18}O-^{13}C signals (0.02 ppm) is in good



4 R = H, R' = CH_3
5 R = TMSi, R' = CH_3

agreement with literature values for carbons with singly bonded oxygen atoms (120). The presence of ^{18}O at both sites necessarily excludes the involvement of water in these oxidations of C-12 or C-13. The biosynthetic origin of these oxygen atoms in 4 was confirmed by mass spectrometric analysis (LR GC-EIMS) of the bis-trimethylsilyl ether (5) (trimethylsilylimidazole). Ions observed at m/z 494 (M^+, 0.18%), 496 ($M^+ + 2$, 0.30%), and 498 ($M^+ + 4$, 0.25%) clearly demonstrated that the C-12 and C-13 oxygen atoms both originated from molecular oxygen.

From these experiments, it is clear that molecular oxygen is used by *G. lemaneiformis* to enzymatically oxidize C-12 and C-13 beginning with an arachidonic acid precursor. The oxidation at C-12 is most likely mediated by a 12-lipoxygenase enzyme while that at C-13 results either by separate oxidation at C-13 or by rearrangement of the proposed C-12 hydroperoxide intermediate (figure 1). Further investigations probing the nature of these unique algal lipoxygenase and oxidase enzyme systems are currently underway. To our knowledge, this is the first report of the utilization of a cell free system in biosynthetic studies with a marine macrophyte.

Natural products investigations with *G. lemaneiformis* (107) revealed the presence of ω-3 eicosanoids which logically result from lipoxygenase metabolism of eicosapentaenoic acid (EPA). However, our enzyme work had demonstrated that arachidonic acid (AA) could perhaps be desaturated by our AP and also result in production of ω-3 eicosanoids (see chapter III or reference 112). Therefore, it was not clear whether our *G. lemaneiformis* enzyme preparation would accept EPA as well as it did AA for a substrate. As a result, a small scale incubation was performed using 5 mg EPA and about 2 g *G. lemaneiformis* AP. After standard extraction and TLC, it became clear, at least qualitatively, that the enzyme preparation was able to metabolize EPA as well as AA. This conclusion was based on TLC's of the enzyme product extract which showed bands for some unmetabolized EPA, yellowish green charring aldehydes (see chapter III), blue charring 12,13-diHEPE, and some very polar material close to the origin. Next, 90 mg of EPA was incubated with AP which resulted in the production of the same spectrum of metabolites as in the small scale incubation. These products were extracted using our standard methods. The pure 12,13-diHEPE free acid obtained from this process weighed 1.1 mg, corresponding to a yield of 1.2%. In previous incubations, production yields as high as 8% have been achieved for the methyl ester of the mentioned compound when introducing AA as a substrate (see chapter III or reference 112). This lower yield may indicate that the 12-lipoxygenase and/or hydroperoxide isomerase enzymes in our AP preferred AA to EPA for a substrate. In addition, it is possible that the techniques used in this experiment to obtain 12,13-diHEPE in its free acid form (thick layer chromatography) resulted in this reduced yield.

For production of 1-^{14}C 12*R*,13*S*-diHEPE, particulate material was removed from the AP enzyme preparation in order to enhance its extraction efficiency. This was deemed necessary due to the small quantity and expense of 1-^{14}C EPA. The AP was dissolved in buffer and then centrifuged. In order to have ample enzyme activity and to achieve maximal conversion of EPA, all of the supernatant solution obtained from centrifugation of AP was utilized in the incubation. However, this time the yield for production of radiolabeled 12,13-diHEPE was even lower (0.8%) than before. Several factors may have played a role in reducing the yield. One factor could have been that the supernatant obtained after centrifugation of the AP was more dilute than desired for attaining maximum enzyme/substrate interactions. Additionally, the particulate materials may have provided AA or EPA with a substrate to which they could have attached and thus become dispersed thereby increasing enzyme/substrate interactions. In retrospect, it may have been worthwhile concentrating that enzyme preparation. Another possibility is that in order to reduce emulsion problems and to terminate the enzyme reaction, an almost equal volume of MeOH was added to the reaction mixture. It is very likely that this addition of MeOH to the aqueous layer altered free fatty acid partition coefficients such that it made $CHCl_3$ extraction of 12,13-diHEPE less efficient.

The results of the *in vitro* studies are still under analysis in collaboration with department of Animal Science at Oregon State University.

EXPERIMENTAL

General. Infrared spectra were recorded on a Nicolet 5 DXB FT 15 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ACP 300 NMR spectrometer and all chemical shifts are reported relative to an internal TMS standard. Mass spectra were obtained on a Finnigan 4023 mass spectrometer. High performance liquid chromatography (HPLC) was performed with Waters M-6000 and M-45 pumps, U6K injectors, and a Waters lambda-Max 480 lc spectrophotometer. For thin layer chromatography Merck aluminum-backed TLC sheets (silica gel 60 F254) was used and thick layer chromatography was performed on glass-backed TLC plates (KIESELGEL 60 F-254 Silica Gel). Centrifugation was performed using a Heraeus SEPATECH centrifuge 17RS. All solvents were distilled from glass prior to use.

a. Biosynthetic formation and isolation of ^{18}O-labeled 12,13-diHETE (4). An acetone powder crude enzyme preparation (17.1 g from 157 g tissue) was produced from *G. lemaneiformis* as described in chapter III (6). After confirming the presence of 12-LO activity in this preparation by incubating a small portion with arachidonic acid followed by TLC analysis, 16.8 g of the powder was used for the production of ^{18}O-labeled 12,13-diHETE (4) by $^{18}O_2$ gas mixture. Mass spectrometric analysis of this gas mixture (Cambridge Isotope) showed it to be of random speciation (23.6% $^{16}O_2$, 48.4% $^{16}O^{18}O$, 28% $^{18}O_2$), although originally sold as "Oxygen-18 O_2; ^{18}O, 50%". The incubation was conducted in a closed system containing the acetone powder stirring in 200 ml of 0.1 M phosphate

buffer (pH 7.4) containing 0.1% UCON as an anti-foaming agent. The head space (ca. 50 ml) and buffer medium were repetitively gas-stripped with N_2 alternating with water aspirated vacuum. The system was then twice charged with the $^{16}O/^{18}O$ gas mixture and then 100 mg of 99% pure AA (Sigma) dissolved in 1 ml ethanol was added. After 25 minutes at RT, the incubation was terminated by acidification to pH 4.0 (10% HCl). Organic extraction with chloroform (700 ml) was assisted by the addition of ca. 200 ml of saturated brine. Following evaporation of the chloroform, 137.1 mg of a dark green oil were recovered. Direct TLC analysis of this material in an acidified solvent (EtOAc/hex/HOAc 64:35:1) demonstrated the presence of several oxidized AA metabolites. The crude extract was methylated (ethereal CH_2N_2) and repetitively chromatographed by normal phase HPLC (a. 40% EtOAc/hex, 10 mm x 50 cm, RSIL, 10μ ; b. 25% EtOAc/hex, 2 x 3.9 mm x 25 cm, μ-Poracil, 10μ) to yield 1.0 mg of pure ^{18}O-labeled methyl 12*R*,13*S*-diHETE (**4**).

^{18}O-labeled methyl 12R,13S-diHETE (4). $[\alpha]_D$ = +10.4°, c = 0.03, acetone (for the authentic sample (**3**) obtained by extraction of the alga, $[\alpha]_D$ = +84.2°, c = 0.71, acetone). The 300 MHz ^1H NMR of **4** was the same as reported in chapter III. ^{13}C NMR (75MHz, C_6D_6) δ 134.02 (C15), 132.60 (C8), 130.19 (C10), 129.47 (C5), 128.30 (C6,tentative, obscured by solvent), 127.97 (C9), 127.64 (C11), 127.33 (C14), 75.20 (^{16}O-C12), 75.18 (^{18}O-C12), 70.62 (^{16}O-C13), 70.60 (^{16}O-C13), 51.07 (OCH_3), 33.33 (C2), 31.71 (C18), 29.61 (C17), 28.20(C16), 26.73 (C7), 26.44 (C4), 24.97 (C3), 22.87 (C19), 14.23 (C20); LR GC-EIMS of bis-trimethylsilyl ether **5** (trimethylsilylimidazole) m/z 498 (M^+ + 4, 0.25%), 496 (M^+ + 2, 0.30%), 494 (M^+, 0.18%), 408 ((M^+ + 4) -

TMSiOH), 0.25%), 407 (M^+ - $C_4H_7O_2$, 3.8%),406 ((M^+ + 2) - TMSiOH), 4.4%), 404 (M^+ - TMSiOH, 3.8%), 314 (M^+ - 2TMSiOH, 1.0%), 305 ($[C_9H_{15}{}^{18}O_2(TMSi)_2]^+$, 7.9%), 303 ($[C_9H_{15}{}^{16}O^{18}O(TMSi)_2]^+$, 11.3%), 301 ($[C_9H_{15}{}^{16}O_2(TMSi)_2]^+$, 6.8%), 297 ($[C_{13}H_{18}{}^{16}O_2{}^{18}OTMSi]^+$, 20%), 295 ($[C_{13}H_{18}O_3TMSi]^+$, 18.4%), 266 (4.2%), 265 (4.6), 263 (4.2%), 213 ($[C_9H_{15}{}^{18}OTMSi]$+, 6.4%), 211 ($[C_9H_{15}OTMSi]$+, 7.0%), 205 ($[C_{13}H_{17}O_2]^+$, 8.1%), 201 ($[C_8H_{14}{}^{18}OTMSi]^+$, 5.9%), 199 ($[C_8H_{14}OTMSi]^+$, 7.1%), 193 ($[C_{12}H_{17}O_2]^+$, 5.0%), 173 ($C_{12}H_{13}O]^+$, 9.2%), 161 (5.5%), 155 (4.4%), 149 (5.2%), 147 (7.1%), 145 (5.4%), 131 (15.6%), 129 (8.0%), 119 (7.2%), 105 (7.7%), 97 ($[C_7H_{13}]^+$, 1.4%), 91 (12.3%), 81 (5.4%), 79 (6.6%), 73 ($[C_3H_5O_2]^+$, 100%), 69 (17.5%), 67 (7.2%), 59 (7.3%), 55 (12.2%).

b. Biosynthetic formation and isolation of unlabeled 12,13-diHEPE free acid.

Enzyme extraction and preparation. G. lemaneiformis acetone powder (AP) was prepared as described in chapter III.

Incubation and enzyme product extraction. Incubation and extraction methods were as described in chapter III. To confirm the ability of the crude enzyme preparation to utilize eicosapentaenoic acid (EPA, Sigma) as a substrate, a small scale incubation was performed utilizing 5 mg of this fatty acid with about 2 g AP. For the production of pure 12,13-diHEPE free acid, 90 mg EPA was incubated with 46 mg AP dissolved to a volume of 500 ml with 0.1 M K_2HPO_4 buffer, pH 7.4. After standard extraction (see chapter III) using 3 x 300 ml $CHCl_3$, the extract was reduced under vacuum to yield 23.5 mg of a greenish oil.

Enzyme product purification. The extracted oil was observed to contain 12,13-diHEPE free acid which, as previously described in chapter III, exhibited a blue charring spot at an R_f ~ 0.1 on TLC (Et_2O-Hexanes-HOAc, 64:35:1). Subsequently, because this blue charring spot attributed to the compound of interest was well separated from other constituents of the extract on the TLC, all of this material was dissolved in Et_2O and applied to a sheet of Thick Layer Chromatography plate using the same solvent system as mentioned above. The band of interest was scraped off, dissolved in Et_2O, and passed through a 10-20 μm sintered glass funnel while rinsing with ample Et_2O. After reducing the solvent under vacuum, this material was weighed to be 2.4 mg semi-pure 12,13-diHEPE free acid. After adding 0.3 mg of similar material obtained from the small scale incubation (using 5 mg EPA) to the semi-pure compound, the combination was further purified on NP HPLC (7% IPA/hexanes with 0.1% HOAc; 2 x 4.1 mm x 250 mm, 10 μ Versapak) to yield 1.1 mg pure 12,13-diHEPE free acid.

c. Biosynthetic formation and isolation of 1-^{14}C 12,13-diHEPE free acid.

Enzyme extraction and preparation. About 6.7 g of the acetone powder was dissolved in 170 ml 0.1 M K_2HPO_4 buffer, pH 7.4 and stirred for 60 minutes. This material was then centrifuged at 12000 g and 4 °C for 30 minutes. All of the supernatant (160 ml) was removed and used for the incubation.

Incubation. 1-^{14}C EPA was purchased from NEN research products and was supplied in 0.5 ml EtOH (3.7 MBq/ml EtOH, 1 becquerel = 1 nuclear transformation/second, 3.7 x 10^{10} Bq = 1 Ci) with a specific activity of 1.5

GBq/mmol (0.1 mCi/ml). This material (0.38 mg) was mixed with an equal mass of non-labeled EPA and then incubated for 60 minutes with the enzyme preparation the activity of which was confirmed prior to use.

Labeled enzyme product extraction. At the end of incubation period, 150 ml of MeOH was added to the enzyme mixture to reduce emulsion problems during extraction and terminate the enzymatic reaction. Then, the lipids were salted out into 3 x 250 ml $CHCl_3$ which was reduced under vacuum to yield a yellowish green oil. This organic extract which was stored in Et_2O under N_2 overnight.

Labeled enzyme product purification. The only deviations from our purification scheme described above were that a simultaneous TLC of authentic 12,13-diHEPE standard was carried out to determine the R_f of this compound in the mixture, precisely. In addition, because of the minute quantity of the purified material, it was not possible to measure a yield by weighing the product. Therefore, the yield was determined by counting the radioactivity of the compound and was calculated to be 0.8%.

BIBLIOGRAPHY

1. Eisner, T. (1990) *Chemoecology* **1**, 38-40.

2. Merewood, A. (1991) *Wildlife conservation* Mar./Apr. 54-59.

3. Gilman, A.G., Rall, T.W., Nies, A.S., Taylor, P. (1990) *The Pharmacological Basis of Therapeutics*, 8th ed. Pergamon Press, INC.New York.

4. Scheuer, P.J. (1989) *Medicinal Research Reviews* **9**, 535-545.

5. Grant, P.T., and Mackie, A.M. (1977) *Nature* **267**, 786-788.

6. Bergmann, W., and Burke, D.C. (1956) *J. Org. Chem.* **21**, 226-228.

7. Kao, C.Y., and Levinson, S.R., Eds. (1986) *Ann. N.Y. Acad. Sci.* **479**, 1-448.

8. Rinehart, K.L., Gloer, J.B., Hughes, R.C., Renis, H.E., McGovern, J.P., Swynenberg, E.B., Stringfellow, D.A., Kneutzel, S.L., and Li, L.H. (1981) *Science* **212**, 933-935.

9. Rinehart, K.L, Kobayashi, J., Harbour, G.C., Hughes, R.G., Mizsak, S.A., and Scahill, T.A. (1984) *J. Am. Chem. Soc.* **106**, 1524-1526.

10. Weinheimer, A.J., and Spraggins, R.L. (1969) *Tet. lett.* 5185-5188.

11. DeSilva, E.D., and Scheuer, P.J. (1980)*Tet. lett.* **21**, 1611-1614.

12. Look, S.A., Fenical, W., Jacobs, R.S., and Clardy, J. (1986) *Proc. Natl. Acad. Sci.* (USA) **83**, 6238-6240.

13. Minale, L., Riccio, R., and Sodano, G. (1974) *Tet. Lett.* 3401-3404.

14. Sarin, P.S., Sun, D., Thornton, A., and Muller, W.E.G. (1987) *J. Nat. Cancer Inst.* **78**, 663-666.

15. Gerwick, W.H., Moghaddam, M.F., and Hamberg, M. *Arch. Biochem. Biophys.* in press.

16. Irvine, R.F., (1982) *Biochem. J.* **204**, 3-16.

17. Van Den Bosch (1980) *Biochim. Biophys. Acta* **604** 191-246.

18. Anderson J. M. (1989) *Second Messengers in Plant Growth and Development* 181-212 Alan R. Liss, INC. New York.

19. Sevanian, A., Stein, R.A., and Mead, J.F. (1981) *Lipids* **16,** 781-789.

20. Neale, M.L., Fiera, R.A., and Matthews, N. (1988) *Immunology* **64**,81-85.

21. Holmes, P.V., Hellgerg, P., and Sjöblom, P. (1988) *Experientia* **44**, 772-774.

22. Stanley-Samuelson, D.W., Jensen, E., Nickerson, K.W., Tiebel, K., Ogg, C.L., and Howard, R.W. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1064-1068.

23. Porter, N.A. (1984) *Methods in Enzymology* **105,** 273-299.

24. Gibian, M.J., and Galaway, R.A. (1977) *Bioorganic Chemistry* **1,** (Van Tamelen, E.E., ed.) pp. 117-136. New York Academic Press, Inc.

25. Jefford, C.W. and Cadby, P.A. (1981) *Fortschritte d. Chem. Org. Naturst.* **40**, 191-265.

26. Crooke, S.T., and Wong, A. (1991) *Lipoxygenases and Their Products* **1**, (Crooke, S.T. and Wong, A., ed.) pp. 105-183. Academic Press, Inc.

27. Petersson, L., Slappendel, S., and Vliegenthart, J.F.G. (1985) *Biochim. Biophys. Acta* **828**, 81-85.

28. Corey, E.J., and Nagata, R. (1987) *J. Am. Chem. Soc.* **109**, 8107-8108.

29. Corey, E.J., D'Alarcao, M., and Matsuda, P.T. (1986) *Tet. Lett.* **27**, 3585-3588.

30. Nelson, M.J., and Cowling, R.A. (1990) *J. Am. Chem. Soc.* **112**, 2820-2821.

31. Rapoport, D., Härtel, B., and Hausdorf, G. (1984) *Eur. J. Biochem.* **139**, 573-576.

32. Egmond, M.R., Vliegenthart, J.F.G., and Boldingh, J. (1972) *Biochem. Biophys. Res. Comm.* **48**, 1055-1060.

33. Hamberg, M., and Hamberg, G. (1980) *Biochem. Biophys. Res. Comm.* **95,** 1090-1097.

34. Ochi, T., and Cerutti, P.A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 990-994.

35. Hill, T.D., White, J.G., Rao, G.H.R. (1989) *Prostaglandins* **38**, 21-32.

36. Kühn, H., and Brash, A.R. (1990) *J. Biol. Chem.* **265**, 1454-1458.

37. Lax, Y., Grossman, S., Rubinstein, S., Magid, N., and Breitbart, H. (1990) *Biochim. Biophys. Acta* **1043**, 12-18.

38. Siegel, M.I., McConnell, R.T., Porter, N.A., and Cuatrecasas, P. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 308-312.

39. Siegel, M.I., McConnell, R.T., and Cuatrecasas, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3774-3778.

40. Hamberg, M., and Samuelsson, B. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3400-3404.

41. Woolard, P.M. (1986) *Biochem. Biophys. Res. Comm.* **136**, 169-176.

42. Wollard, P.M., Cunningham, F.M., Murphy, G.M., Camp, R.D.R., Derm, F.F., and Greaves, M.W. (1989) *Prostaglandins* **38**, 465-471.

43. Evans, J.F., Leblanc, Y., Fitzsimmons, B.J., Charleson, S., Nathaniel, D., and Leveille, C. (1987) *Biochim. Biophys. Acta* **917**, 406-410.

44. Capdevila, J. Yadagiri, P., Mamma, S., and Falck, J.R. (1986) *Biochem. Biophys. Res. Comm.* **141**, 1007-1011.

45. Schwartzman, M.L., Balazy, M., Masferrer, J., Abraham, N.G., McGiff, J.C., and Murphy, R.C. (1987) *Proc. Nat. Acad. Sci. USA* **84**, 8125-8129.

46. Hartmut, K., and Brash A.R., (1990) *J. Biol. Chem.* **265**, 1454-1458.

47. Antonipillai, I., Horton, R., Matarajan, R., and Nadler, J. (1989) *Endocrinology* **125**, 2028-2034.

48. Wang, J., Yuen, B.H., Leung, P.C.K. (1989) *FEBS letters* **244**, 154-158.

49. Hadjiagapiou, C. and Spector, A.A. (1986) *Prostaglandins* **31**, 1135-1144.

50. Mullane, K.M., Read, N., Salmon, J.A., and Moncada, S. (1984) *J. Pharmacol. and Exp. Ther.* **228**, 510-522.

51. Lianos, E.A. (1988) *J. Clin. Invest.* **82**, 427-435.

52. Borgeat, P., Nadeau, M., Salari, H., Poubelle, P., and Fruteau De Lactos, B. (1985) *Adv. Lipid Res.* **21**, 47-77.

53. Borgeat, P., De Laclos, B.F., Picard, S., Drapeau, J., Vallerand, P., and Corey, E.J. (1982) *Prostaglandins* **23**, 713-724.

54. Yamamoto, S., Yokoyama, C., Ueda, N., Shinjo, F., Kaneko, S., Yoshimoto,. T. (1986) *Adv. Prost. Throm. Leuk. Res.* **16**, 17-26.

55. Glasgow, W.C., Harris, T.M., and Brash, A.R. (1986) *J. Biol. Chem.* **261**, 200-204.

56. Borgeat, P., and Samuelsson, B. (1979) *J. Biol. Chem.* **254**, 7865-7869.

57. Ford-Hutchinson, A.W., Bray, M.A., Cunnigham, F.M., Davidson, E.M., and Smith, M.J.H. (1981) *Prostaglandins* **21**, 143-152.

58. Moghaddam, M.F., Gerwick, W.H., and Ballantine, D.L. (1989) *Prostaglandins* **37**, 303-308.

59. Moghaddam, M.F., Gerwick, W.H. (1990) *Phytochem.* **29**, 2457-2459.

60. Janistyn, B. (1990) *Phytochem.* **29**, 2453-2455.

61. Gerwick, W.H., Bernart, M.W. Moghaddam, M.F., Jiang, Z.D., Solem, M.L., and Nagle D.G. (1990) *Hydrobiologia* **204/205**, 621-628.

62. Vick, B.A. (1991) *Lipid* **26**, 315-320.

63. Lin, Y-L., and Wong, P., Y-K. (1989) *Adv. Prost. Throm. Leuk. Res.* **19**, 160-163.

64. Gardner, H.W., and Plattner, R.D. (1984) *Lipids* **19**, 294-299.

65. Walker, I.C., Jones, R.L., and Wilson, N.H. (1979) *Prostaglandins* **18**, 173-178.

66. Pace-Asciak, C.R., Martin, J.M., and Corey, E.J. (1986) *Prog. Lipid Res.* **25**, 625-628.

67. Pace-Asciak, C.R., Mizuno, K., and Yamamoto, S. (1983) *Prostaglandins* **25**, 79-84.

68. Piomelli, D., Feinmark, S.J., and Schwartz, J.H. (1987) *J. Neurosci.* **7**, 3675-3686.

69. Derewlany, L.O., Pace-Asciak, C.R., and Raddle, I.C. (1984) *Can. J. Physiol. Pharmacol.* **62**, 1466-1469.

70. Pace-Asciak, C.R. (1985) *Prostaglandins, Leukotrienes, and Lipoxins* 111-116.

71. Konishi, S., Hoshino, M., and Imamura, M. (1980) *J. Phys. Chem.* **84**, 3437-3440.

72. Gardner, H.W., Weisleder, D., and Nelson, E.C. (1984) *J. Org. Chem.* **49**, 508-515.

73. Pace-Asciak, C.R., Granström, E., and Samuelsson, B. (1983) *J. Biol. Chem.* **258**, 6835-6840.

74. Pace-Asciak, C.R., and Lee, W-S. (1989) *J. Biol. Chem.* **264**, 9310-9313.

75. BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, Catalog, Third Edition, 1988.

76. Garssen, G.J., Veldink, G.A., Vliegenthart, J.F.G., and Boldingh, J. (1976) *Eur. J. Biochem.* **62**, 33-36.

77. Corey, E.J., and Mehrotra, M.M. (1983) *Tet. Lett.* **24**, 4921-4922.

78. Hamberg, M. (1989) *Lipids* **24**, 249-255.

79. Hamberg, M. (1986) *Biochim. Biophys. Acta* **876**, 688-692.

80. Hamberg, M. (1988) *Chem. Phys. Lipids* **46**, 235-243.

81. Brash, A.R., Baertschi, S.W., Ingram, C.D., Harris, T.M. (1987) *J. Biol. Chem.* **262**, 15829-15839.

82. Hamberg, M. (1987) *Biochim. Biophys. Acta* **920**, 76-79.

83. Song, W-C., and Brash A.R. (1991) *Science* **253**, 781-784.

84. Fahlstadius, P., and Hamberg, M. (1990) *J. Chem. Soc. Perkin Trans.* **1**, 2027-2030.

85. Hawkins, D.J., and Brash, A.R. (1987) *J. Biol. Chem.* **262**,7629-7634.

86. Hamberg, M., and Samuelsson, B. (1974) *Biochem. Biophys. Res. Commun.* **61**, 942-949.

87. Hamberg, M. (1976) *Biochim. Biophys. Acta* **43**, 651-654.

88. Borgeat, P. and Samuelsson , B. (1979) *Proc. Nat. Acad. Sci. USA.* **76**, 2148-2152.

89. Goetzl, E. and Sun, F. (1979) *J. Exp. Med.* **150**, 406-411.

90. Cunningham, F.M., Woollard, P.M., and P.D.R. Camp. (1985) *Prostaglandins* **30,** 497-509.

91. Cunningham, F.M. and Woollard, P.M., (1987) *Prostaglandins* **34**:71-78.

92. Nakao, J., Ooyama, T., Ito, H., Chang, W.C.,and Murota., S. (1982) *Atherosclerosis* **44**,339-342.

93. Werner, E., Walenga, R.W., Dubowy, R.L.,Boone, S., and Stuart, M.J. Cancer (1985) *Res.* **45**,561-563.

94. Piomelli, D., Feinmark, S.J., and Schwartz J.H. (1987) *J. Neurosci.* **7,** 3675-3686.

95. Pace-Asciak, C.R. (1984) *J. Biol. Chem.* **259,** 8332-8337.

96. Corey, E.J., Kang, J., and Laguzza, B.C. (1983) *Tetrahedron Lett.* **24,** 4913-4916.

97. Ballantine, D.L., Gerwick, W.H., Velez, S.M., Alexander, E., and Guevara, P. (1987) *Hydrobiologia* **151/152,** 463-469.

98. Moghaddam, M.F., Gerwick, W.H., and Ballantine, D.L. (1990) *J. Biol. Chem.* **265**, 6126-6130.

99. Ballantine, D.L., and Wynne, M.J. (1985) *Phycologia* **24,** 459-465.

100. Bernart, M., and Gerwick, W.H. (1988) *Tetrahedron Lett.* **29,** 2015-2018.

101. Solem, M., Jiang, J.Z., and Gerwick, W.H. (1989) *Lipids* **24**, 256-260.

102. Lopez, A., and Gerwick, W.H. (1987) *Lipids* **22,** 190-194.

103. Lopez, A., and Gerwick, W.H. (1988) *Tetrahedron Lett.* **29**,1505-1506.

104. Rakoff, H., and Emken, E.A. (1983) *J. Am. Oil Chem. Soc.* **60,** 546-552.

105. Bax, A. and Drobny, G. (1985) *J. Mag. Res.* **61,** 306-320.

106. Meyer, B.N., Ferrigni, N.R.,Putnam, J.E., Jacobsen, L.B. Nichols, D.E.,and McLaughlin, J.L. (1982) *J. Med. Plant Res.* **45**, 31-36.

107. Jiang, Z.D., and Gerwick, W.H. (1990) *Phytochem.* **29**, 1433-1440.

108. Capdevila, J., Chacos, N., Werringloer, J., Prough, R.A. and Estabrook, R.W. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5362-5367.

109. Samuelsson, B. (1983) *Science* **220**, 568575.

110. Marcus, L., Prusky, D., and Jacoby, B. (1988) *Phytochem.* **27**, 323-327.

111. Zimmerman, D.C., and Vick, B.A. (1973) *Lipids* **8**, 264-266.

112. Moghaddam, M.F., and Gerwick, W.H. (1991) *J. Nat. Prod.* in press.

113. Bundy, G.L., Nidy, E.G., Epps, D.E., Mizsak, S.A., and Wnuk, R.J. (1986) *J. Biol. Chem.* **261**, 747-751.

114. Stahl, E. (1969) in Thin-Layer Chromatography--A Laboratory Handbook, p.881. Springer-Verlag, New York.

115. Nagle, D.G., Gerwick, W.G. (1990)*Tetrahedron lett.* **31**, 2995-2998.

116. Fredicq, S., and Hommersand, M.H. (1989) *J. Phycol.* **25**, 228-241.

117. Marchant, H.J., and Fowke, L.C. (1977) *Can. J. Bot.*, **55**, 3080-3086.

118. Leers-Sucheta, S., Slayden, O., Jiang, Z.D., Moghaddam, M., Gerwick, W.H., and Stormshak, F. (1991) *Society for the Study of Reproduction* Presentation Abstract.

119. Lumin , S.,and Falck, J.R. (1990) *Tetrahedron Lett.*, **31**, 2971-2974.

120. Vederas, J.C. (1987) *Nat. Prod. Rep.*, **4**, 277-337.

APPENDIX

Appendix. PRELIMINARY INVESTIGATIONS OF CHEMICAL CONSTITUENTS OF THE OREGON RED ALGA *GLOIOSIPHONIA VERTICILLARIS*

ABSTRACT

The organic crude extract of the red marine alga *Gloiosiphonia verticillaris* (Farlow) Smith (Gloiosiphoniaceae) showed antimicrobial activity against several bacteria pathogenic to humans. However, deactivation of the antimicrobial substances during fractionation inhibited us from isolating these compounds. Chemical studies of the organic extract were conducted to gain insights into the nature of compounds present in this alga. This effort yielded a compound in its pure form. More experiments are needed to establish the structure of this compound unequivocally. At this time, a tentative bicyclic structure is assigned to this compund.

INTRODUCTION

The red marine alga *Gloiosiphonia verticillaris* (Farlow) Smith (Gloiosiphoniaceae) was collected and evaluated for the antimicrobial potential of its crude organic extract. While this crude extract exhibited antimicrobial activity against several human pathogens, we were not able to isolate the antimicrobial natural product(s) due to the loss of this activity during fractionation process. However, in order to gain insights into the classes of chemistry present in this organism, one of the fractions was methylated and fractionated further. This resulted in isolation of a colorless oily substance in pure form. Because more data is needed to determine the structure of this compound unequivocally, only a tentative structure can be proposed at this time.

RESULTS AND DISCUSSIONS

As part of an extensive survey of the biomedicinal potential of Oregon marine algae, the crude lipid extract of the marine red alga *Gloiosiphonia verticillaris* (Farlow) Smith (Gloiosiphoniaceae) was examined for a combination of antimicrobial properties and unique compounds by TLC. The crude extract of this alga exhibited antimicrobial activity against two gram positive microorganisms, *Bacillus subtilis* (12 mm) and *Staphylococcus aureus* (12 mm) as well as two gram negative microorganisms, *Escherichia coli* (7.5 mm) and *Salmonella typhimurium* (8.0 mm). In addition, presence of several potentially unique secondary metabolites was detected by TLC. In order to isolate the antimicrobial substance the crude extract was subjected to normal phase chromatography. Unfortunately, this process led to the loss of the antimicrobial activity.

It was needed to gain insights into the nature of chemical constituents of *G. verticillaris* in order to approach this purification problem more effectively in future attempts. The fractions that had resulted from the last purification step while pursuing antimicrobial activity were still impure and present in low quantities. Therefore, to investigate the classes of chemistry present in *G. verticillaris*, another chromatography fraction present in much higher quantity was pursued (see materials and methods). Subsequently, this organic extract which weighed ca. 150 mg was methylated to improve its chemical stability and chromatographic behavior and fractionated to yield a pure compound to which a tentative structure has been assigned.

A molecular formula of $C_{13}H_{18}O_6$ (5° unsaturation) was deduced for this pure compound from observation of a M^+ ion (m/z 270) in both high resolution (1.0 mamu deviation) and low resolution (24.6% signal intensity) EIMS. The IR spectrum revealed the presence of a hydroxyl as well as one or more carbonyl groups. This compound showed a UV maxima at 262 nm indicative of an α,β unsaturated ketone. ^{13}C NMR (observed 13 ^{13}C signals) data proved consistent with the molecular formula (table A.1) and showed signals for two ketones at δ 199.8 and δ 212.9. Furthermore, two highly polarized olefinic carbons resonated at δ 134.6 and δ 170.9. A comparison between the fully decoupled ^{13}C NMR and ^{13}C DEPT revealed two quaternary carbons at δ 52.8 and δ 80.3 as well as three methyl (δ 58.3, δ 59.4, and δ 59.7) and four methylene (δ 28.5, δ 32.0, δ 32.3, and δ 71.7) groups.

^1H NMR (table A.1) and ^1H-^1H COSY (table A.2) confirmed the identities of the methylenes and showed the protons of each to be diastereotopic. The chemical shifts of the methyl groups and their sharp singlet established them as methoxy groups. Furthermore, no olefinic protons were observed in the ^1H NMR spectrum which indicated that the olefin was tetrasubstituted. The broad singlet proton present at δ 3.29 was attributed to a hydroxyl group.

A ^1H-^{13}C heteronuclear correlation spectroscopy (HETCOR) experiment was performed to establish proton-carbon connectivities. The coupling patterns in the ^1H NMR and the correlations in the ^1H-^1H COSY revealed the presence of two adjacent methylenes in addition to two isolated methylenes with only geminal couplings between their diastereotopic protons. The long range ^1H-^1H COSY provided us with virtually the same data as obtained from ^1H-^1H COSY and was

not helpful in structure elucidation of this compound.

These partial structures accounted for three degrees of unsaturation. Therefore, it was concluded that this molecule contained two rings to account for the remaining two degrees of unsaturation. Presence of six quaternary carbons in this small molecule inhibited us from accurately placing the substituents in a bicyclic system. Therefore, a nuclear overhauser enhancement correlation spectroscopy (NOESY) was employed to explore spatial relationships between the protons and perhaps allows us to establish connectivities. This experiment provided us with useful information (table A.2) and proved instrumental in the assignment of a tentative structure to the unknown compound (figure A.1).

EXPERIMENTAL

Instruments. Infrared spectra were recorded on a Nicolet 5 DXB FT 15 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ACP 300 NMR spectrometer and all chemical shifts are reported relative to an internal TMS standard. Low and high resolution ass spectra were obtained on a Finnigan 4023 and a Kratos MS 50 TC mass spectrometer. High performance liquid chromatography (HPLC) was performed with Waters M-6000 and M-45 pumps, U6K injectors, and a Waters lambda-Max 480 lc spectrophotometer. For thin layer chromatography Merck aluminum-backed TLC sheets (silica gel 60 F254) was used. Vacuum and flash chromatography was performed using Woelm 30-40 μ Silica gel. All solvents were distilled from glass prior to use.

Bioassays. Antimicrobial assays used standard antimicrobial sensitivity methodology in which compound-impregnated paper discs (6.5 mm diameter) were incubated on the surface of seeded agar. Crude extract was tested at 2 mg/disc and applied in 20 μl of diethyl ether. After evaporation of the ether (20-30 min RT) the discs were placed on Mueller-Hinton agar (Difco) seeded with one of the following: *Staphylococcus aureus* (ATCC 12600), *Candida albicans* (ATCC 14053), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 9721). The plates were incubated at 37° C for 24 hours and the zones of inhibition measured in mm.

Collection, Extraction, and Isolation. G. verticillaris was collected from exposed tide pools at Seal Rock and Strawberry Hill on the Oregon coast on May 12 and May 25, 1991. The alga was immediately frozen with dry ice until workup.

The defrosted alga (610 g dry weight) was extracted using a total of 10 L CH_2Cl_2/MeOH (2:1). The extration effort was hindered because of the high mucopolysaccharide content of the alga. The solvent was removed under vacuo to give 8.3 g of a dark green viscous oil.

Approximately 4.7 g of the crude extract underwent bioassay guided fractionation. This material was applied to a normal phase silica gel column in vacuum mode and chromatographed in a gradient of EtOAc/Hexanes. Of the resulting six fractions, the fourth showed antimicrobial activity. This fraction (ca. 1.1 g) was fractionated further using the same vacuum chromatographic techniques to yield seven fractions. Because fractions five to seven showed similar TLC and antimicrobial characteristics, they were recombined (ca. 980 mg) and fractionated to yield ten fractions. At this point, only fraction six (32 mg) showed antimicrobial activity and this fraction was only remotely antimicrobial. Normal phase HPLC (17% IPA/Hexanes , 500 x 10 mm Phenomenex 10 μm) fractionation of this material yielded four fractions none of which exhibited any antimicrobial activity.

Of the ten fractions which resulted from the last set of vacuum chromatography, the third one (152 mg) showed an acceptable separation on the TLC. In order to improve chromatographic behavior and chemical stability of the constituents, this fraction was methylated using excess ethereal CH_2N_2 for 7 minutes, followed by airing out and under vacuo evaporation of the solvent. NP HPLC (7.5% IPA/Hexanes, 500 x 10 mm Phenomenex 10 μm) of this fraction resulted in six fractions of which the fifth (21.9 mg) was enriched in a UV-absorbing, acid charring (50% H_2SO_4) compound. In addition, this fraction had an interesting ^1H NMR. Subsequently, two consecutive NP HPLC purifications

using 4% and 2% IPA/Hexanes as carrier solvents was carried out to yield 3.8 mg of a methylated compound in its pure form.

This compound was a colorless oil and showed the following characteristics: UV (CH_3CN) λ_{max} = 262 nm (ε = 11258); IR (neat) 3438, 2948, 1749, 1704, 1621, 1463, 1349, 1304, 1202, 1100, 1036 cm^{-1}; HR EIMS observed m/z 270.11138 (1.04 milliatomic mass units deviation); LR EIMS m/z (fragment, rel. intensity) 270 (M^+, 24.6%), 138 (19.7 %), 214 (43.4 %), 199 (23.2 %), 181 (10.2 %), 169 (76.0 %), 155 (100.0 %), 139 (15.5 %), 111 (10 %), 69 (12.4 %), 55 (36.3 %); ^1H and ^{13}C NMR (table A.1) as well as all other NMR experiments were performed in $CDCl_3$.

# of Hs	^{1}H (δ)	m	J (Hz)	^{13}C (δ)
1	1.99	ddd	13.0,6.0,3.4	28.50
1	2.14	d	17.1	31.99
1	2.36	m	-	32.30
1	2.41	m	-	28.48
1	2.70	ddd	19.8,9.6,6.1	32.30
1	2.97	d	17.1	31.99
1	3.29	bs	-	-
3	3.36	s	-	59.69
1	3.47	d	10.5	71.72
1	3.77	d	10.5	71.72
3	3.82	s	-	59.42
3	4.07	s	-	58.26
-	-			52.80
-	-			80.30
-	-			134.6
-	-			170.9
-	-			199.8
-	-			212.9

Table A.1. High field ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR data for the unknown compound.

^1H (δ)	^1H-^1H COSY Correlations (δ)	^1H-^1H NOESY Correlations (δ)
1.99	2.41, 2.70	-
2.14	2.97	3.77, 4.07
2.36	2.41, 2.70	-
2.41	1.99, 2.36	-
2.70	1.99, 2.36	-
2.97	1.99	3.36, 3.47, 3.82, 4.07
3.29	-	-
3.36	-	2.97, 3.47, 3.77, 4.07
3.47	3.77	2.97, 3.36, 3.82 (?)
3.77	3.47	2.14, 3.36, 3.82 (?)
3.82	-	2.97 (?), 3.47 (?), 3.77 (?)
4.07	-	2.14, 2.97, 3.36

Table A.2. ^1H-^1H COSY and ^1H-^1H NOESY correlations to each proton resonance in the unknown compound.

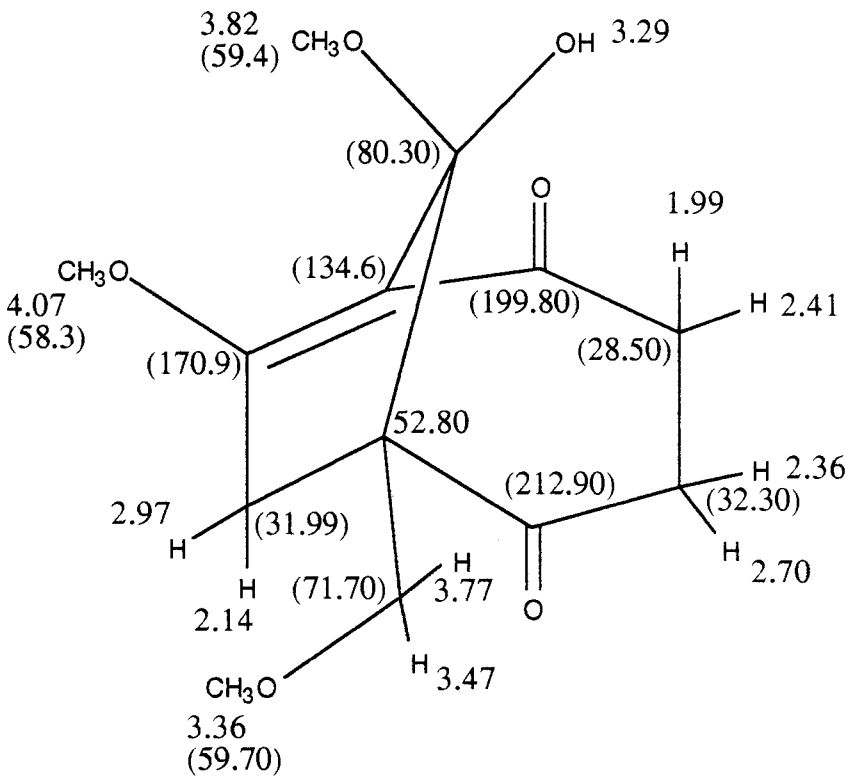


Figure A.1. A tentative structure for the unknown compound from *G. verticillaris*.