AN EVALUATION OF ANTIMICROBIAL ACTIVITY IN THE COMMON SEAWHIP, *LEPTOGORGIA VIRGULATA* (LAMARCK)

A thesis submitted in partial fulfillment of the requirements for the degree

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ERRATA

- Page 85, Lines 4-7. Statement should read: "Fractionation was monitored at 238, 248, and 272 nm. These wavelengths were chosen because they represent chromophore signatures for 11β, 12β-epoxypukalide (Ksebati *et al*. 1984), pukalide (Missakian *et al*. 1975), and homarine (Polychronopoulos *et al*. 2001; Keller *et al*. 2004), respectively."

- Page 90, Lines 13-15. Statement should read: "Elution was monitored at 238, 248, and 272 nm, wavelengths representing the chromophore signatures for 11β, 12β-epoxypukalide (Ksebati *et al*. 1984), pukalide (Missakian *et al*. 1975), and homarine (Polychronopoulos *et al*. 2001; Keller *et al*. 2004), respectively."

- Page 91, Lines 2-5. Statement should read: "ESI-MS, monitored at 238, 248, and 272 nm (the chromophoric signatures for 11β, 12β-epoxypukalide, pukalide, and homarine, respectively), detected masses in fractions of active *L. virgulata* extracts corresponding to homarine at m/z = 94, 138, and 160 amu."

- Page 121, Lines 4-7. Statement should read: "Fractionation was monitored at 238, 248, and 272 nm, wavelengths representing the chromophore signatures for 11β, 12β-epoxypukalide (Ksebati *et al*. 1984), pukalide (Missakian *et al*. 1975), and homarine (Polychronopoulos *et al*. 2001; Keller *et al*. 2004), respectively."

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

A hallmark of the cnidarian innate immune system is the rapid, nonspecific response of antimicrobial compounds to damage, stress, or pathogen exposure. Antimicrobial activity was examined in the common seawhip, *Leptogorgia virgulata* (Lamarck), from South Carolina waters. Extraction and assay protocols were developed to permit identification of antimicrobial activity in crude methanol/sterile water extracts of *L. virgulata.* Antimicrobial activity was detected by bacterial growth inhibition assays using *Escherichia coli* BL21, *Vibrio harveyii*, *Micrococcus luteus*, and a *Bacillus* sp. isolate from a scleractinian coral (*Acropora cervicornis*). This research represents the first report of antimicrobial activity in *L. virgulata* and, of equal note, in a temperate/sub-tropical coral in the Atlantic Ocean.

Antimicrobial activity in *L. virgulata* was measured at three reference and four anthropogenically impacted sites in South Carolina between May and October 2005. No obvious relationship existed between the degree of anthropogenic stress at any of the seven sites and the production of antimicrobial compounds in *L. virgulata.* Colonies of *L. virgulata* collected from the Charleston City Marina in Charleston, SC, were the only samples which did not exhibit antimicrobial activity against selected bacteria during the six-month study period. Antimicrobial activity was highly variable at the other six sites, differing significantly at Cherry Point Seafood Company, Patriots Point Marina, Kiawah River Bridge, Huntington Beach State Park–South Jetty, and upper 60 Bass Creek–North Inlet among points of collection throughout the study season.

Reverse-phase high performance liquid chromatography (HPLC), HPLC coupled to mass spectrometry (LC-MS), and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy were used in conjunction with a liquid growth inhibition assay system to isolate, purify, and characterize unknown metabolites from active antimicrobial fractions of *L. virgulata.* Corroborative MS/NMR evidence based on diagnostic molecular weights and chemical shifts validated the presence of homarine and a homarine analog in pooled fractions 14-21 and also 22-24. Homarine, a well-known emetic and antifouling metabolite, has been isolated previously from *L. virgulata.* In subsequent liquid growth inhibition assays, replicates of pooled fractions 14-21 inhibited the growth of *V. harveyii* and *M. luteus*, indicating that these partially purified fractions contained biologically active compounds responsible for the antimicrobial activity in *L. virgulata.* It was not determined whether homarine and/or a homarine analog is exclusively responsible for the antimicrobial activity in *L. virgulata.* The findings of this study suggest that homarine is, in part, an active constituent of the innate immune system in *L. virgulata*, which may act synergistically with cofactors and/or congeners in the octocoral to mount a rapid response to stressors, such as microbial invasion and disease.

PREFACE

Study Organism

Physical Distribution

The gorgonian genus *Leptogorgia* (Cnidaria: Anthozoa: Alcyonacea) has a geographical distribution encompassing the East Indies, eastern coast of the United States, the Caribbean, East and South Africa, and the Mediterranean (Beasley 1994). The regional occurrence of the common seawhip, *Leptogorgia virgulata* (Lamarck), is well documented along the western Atlantic from the "Bay of New York" (Bayer 1961) to Brazil (Dineen 2001). Although most abundant in warm, shallow water above 10 m depth (Adams 1980; Beasley 1994), *L. virgulata* has been found dominating an expansive range of depths (0-50 m), temperatures (13-31°C), and salinities (25-37 ppt) (Adams 1980; Dineen 2001). *L. virgulata* is capable of withstanding a variety of environmental stressors (Gerhart 1991) and can be quite abundant on a reef (Adams 1980), thereby providing significant habitat and resources to the ecosystem. As with other gorgonians (Dinesen 1983; Lasker and Coffroth 1983; Slattery and Bockus 1997; Slattery *et al.* 1999), the common seawhip can be vitally important to coral reef structure, biomass, and trophic dynamics.

Biology

Leptogorgia virgulata is an ahermatypic, colonial gorgonian of the family Gorgoniidae. Its irregularly-branched colonies are attached to a flexible skeletal axis of gorgonin (Beasley 1994) that provides much of the support for the octocoral (Figure 1).

Covering the axis is a thin, fleshy layer of coenenchyme composed of mesoglea, disk-spindle-shaped sclerites (spicules), and ameboid cells (Bayer 1953; Bayer *et al*. 1956, 1983; Lucas and Knapp 1997). Regularly spaced and arising from the coenenchyme are numerous, genetically-identical individuals called polyps (Adams 1980; Bayer *et al*. 1956, 1983). Each polyp, or zooid, is composed of two layers of living tissue, the ectodermis and gastrodermis, separated by a sparsely cellular, jelly-like mesogleal matrix interspersed with scleroblasts (sclerite-producing cells) and cell strings (strands of cells in the mesoglea) (Bayer *et al*. 1956, 1983; Blair 2003). Comprising the distal end of each polyp, the anthocodium, are eight pinnately-branched tentacles, eight mesenteries, and a mouth (Bayer *et al*. 1956, 1983; Beasley 1994). The mouth connects directly to the pharynx, which opens into the gastrovascular cavity, the site of food transport and digestion. *L. virgulata*, a suspension feeder, ingests plankton and other small animals that come within reach of its polyps' tentacles.

The distribution of color among *L. virgulata* colonies is quite variable. Members of a population range from near white, to magenta, orange, yellow, purple, and deep violet (Figure 2) (Bayer 1961; Patton 1972; Adams 1980; personal observations). The production of color in the coral's calcite sclerites (Figure 2) is governed by a single gene with four alleles: magenta, orange, yellow, and metachromatic (i.e., colonies that change color with age) (Adams 1980). The alleles for magenta, orange, and yellow are apparently codominant. Metachromatic alleles are dominant to magenta, while orange and yellow alleles are dominant to metachromatic (Adams 1980).

Unlike most gorgonians, *L. virgulata* does not appear to reproduce asexually through cloning or fragmentation (Gotelli 1988, 1991). It does, however, reproduce

sexually and has a reported sex ratio biased in favor of females (Adams 1980; Beasley 1994). *L. virgulata* is a gonochoristic, non-brooding, broadcast spawner with an extensive reproductive season in the northern hemisphere lasting from April to October (Adams 1980). Simultaneous release of mature eggs by female colonies (Wilson 1883) has been attributed to control by photoperiod (Adams 1980). Spawning events have been observed at or within two hours after sunrise each day of the breeding season (Wilson 1883; Adams 1980). External fertilization is followed by subsequent development of planula larvae within 24 hours, settlement from the plankton within five days, and subsequent metamorphosis within 12-19 days (Adams 1980; Gotelli 1988, 1991). Sexual maturity of *L. virgulata* colonies requires approximately 24 months of development (Grigg 1977), but elongation of the axial skeleton from the rigid anthostele (calyx) and complete development of primary and secondary polyps is accomplished in as little as 23 days after attachment (Adams 1980). Within six weeks of attachment, the typical colony will have reached 2-3 cm in height and will be comprised of 15-20 zooids.

The tissue of *L. virgulata*, like many other corals and invertebrates, is covered with a protective mucosal layer. As one of the first lines of defense in *L. virgulata*, mucus acts as a physical and chemical barrier against damage, stress, and pathogen exposure and can aid in the coral's ability to differentiate "self" from "nonself" (Bigger and Runyan 1979; Bigger and Hildemann 1982; Neigel and Avise 1983; Bigger 1988). It is well established that coral mucus is capable of harboring numerous bacterial aggregates (Sorokin 1973; Ducklow and Mitchell 1979*a*; Ducklow and Mitchell 1979*b*) that are closely attuned to the host's metabolism and that play a role in processing mucus for detritivores and suspension feeders on a reef (Ducklow and Mitchell 1979*b*). In fact,

the microbial population of a coral's mucosal layer quite often exceeds that of the surrounding water column, both numerically and metabolically (Ducklow and Mitchell 1979*a*; Paul *et al*. 1986). However, in addition to natural flora, various foreign pathogens, including bacteria in the marine environment, also form epibiotic associations with corals. These pathogens can significantly degrade coral mucus and tissue (Phillips 1963; Correra and Sanchez 1996), inhibit feeding or reproductive output, suppress the immune response, or kill the coral (Slattery *et al*. 1995).

Little is known about coral immunology or how corals interact with their environment on a molecular level to prevent microbial attacks. Yet, due to their essential role in numerous reef ecosystems, it is important to evaluate coral host defense immunity. As *L. virgulata* coexists with diverse communities of microbes in a myriad of habitats throughout the western Atlantic, it could be utilized as a sentinel species to examine the impact of invasive microbes and disease on coral defense systems.

This thesis investigates the innate immunity, specifically antimicrobial activity, of the common seawhip, *Leptogorgia virgulata,* from South Carolina waters. The goals of the study were 1) to identify antimicrobial activity in crude extracts of *L. virgulata*; 2) to determine if that activity differed between May and October 2005 at 3 reference and 4 anthropogenically impacted sites throughout Charleston and Georgetown Counties, South Carolina; and 3) to isolate, purify, and characterize the compound(s) potentially responsible for the observed activity.

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CHAPTER 1

Optimizing the Extraction of Antimicrobial Peptides from *Leptogorgia virgulata*

INTRODUCTION

Innate Immunity

Antimicrobial Peptides

The ability of a multicellular organism to defend itself against invasion by microbes (bacteria, fungi, viruses, and protozoa) depends on its ability to mount immune responses. In vertebrates, the acute recognition and suppression of pathogen infections activates an adaptive response with specific antibodies and T-cell receptors directed against microbial antigens (Hoffmann *et al*. 1999). The immune system of invertebrates, however, does not mount an acquired response to infection. It instead relies on a first-line, innate identification of the pathogen, localized inflammation of the injured area, and as one systemic response, the synthesis of specific antimicrobial compounds at the cell surface or within a cell to target the microbe (Fearon and Locksley 1996).

Important effectors of innate, non-adaptive immunity include secondary and tertiary antimicrobial metabolites, such as diterpenes and prostaglandins in certain species of octocorals (to be discussed in Chapter 2), as well as gene-encoded peptides with antimicrobial activity (Ganz and Lehrer 1999). These antimicrobial peptides (AMPs) of varied size and amino acid composition are found in bacteria, protozoa, plants, invertebrates, and vertebrates (Bulet *et al*. 1999), suggesting their participation in the evolutionary success of target organisms (Zasloff 2002). Due to the easily-defined secondary structural features of AMPs, more than 900 have been isolated and described (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm), over half of which have been observed in insects (Bulet *et al*. 1999). AMPs are secreted not only by epithelial surfaces, but are

also present in invertebrate mucus, hemocytes, and granules of phagocytic cells (Kimbrell and Beutler 2001), as well as the fat body (comparable to the mammalian liver) in *Drosophila* (Ganz and Lehrer 1999).

AMPs can be organized into four major classes: (1) proline-rich peptides; (2) glycine-rich peptides; (3) cysteine-rich peptides with intramolecular disulfide bridges forming hairpin-like ß-sheets or a-helical/ß-sheet combined structures; and (4) linear peptides, forming a-helices and lacking in cysteine residues (reviewed by Hétru *et al*. 1998; Bartlett *et al*. 2002). Generally of small size, low molecular weight, and with a net positive charge (Bartlett *et al*. 2002; Boman 2003), AMPs can adopt an amphipathic design of clustered cationic and hydrophobic amino acids (Zasloff 2002) ideal for affixing to and potentially killing microorganisms with negatively charged membranes. AMPs most frequently target and bind to carbohydrates or lipopolysaccharides (LPSs) of an invading microbial cell membrane (Fearon and Locksley 1996) in a non-receptor-mediated fashion, although certain AMPs require receptor recognition for antimicrobial function (Shai 2002). Bacterial membranes are often organized such that their outermost layer has a high preponderance of lipids with anionic phospholipid groups but lacks cholesterol. LPSs in the outer membrane and anionic phospholipids of the inner membrane of Gram-negative bacteria, as well as acidic polysaccharides and LPSs in the single membrane of Gram-positive bacteria, confer a net negative charge to the surface of both kinds of bacteria (Shai 2002). Conversely, cell membranes of animals are rich in neutral phospholipids and cholesterol, substances that inhibit the incorporation of AMPs into membranes. Cationic regions of AMPs thus can interact electrostatically with or displace anionic components of the bacterial membrane without harming animal cell

membranes or components (Andreu and Rivas 1998; Bartlett *et al*. 2002; Shai 2002; Zasloff 2002).

Modes of Action of Antimicrobial Peptides

To interface with and insert into the target microbial membrane by non-receptor-mediated actions, AMPs must undergo substantial modifications in conformation (Shai 2002). It is here that the amphipathic characteristic of many peptides becomes important. In solution, AMPs generally maintain a hydrophilic structure. Yet, in order to interface with a microbial membrane, they must reverse their organization and present only hydrophobic regions to the lipid constituents of the membrane. This is thought to be accomplished via a hinge mechanism, which exposes the hydrophobic regions to the membrane for attachment but consequently draws the hydrophilic regions into the lumen of the peptide oligomer (Shai 2002; Zasloff 2002).

There are two proposed models elucidating non-receptor-mediated membrane disruption following a conformational change on the part of the peptide: the barrel-stave and carpet models. The barrel-stave model (Figure 3) suggests that AMPs of a certain size and structure self-associate on the microbial membrane surface and then, via hydrophobic interactions, penetrate the lipidic core of the membrane and form bundles of transmembrane pores resulting in cell lysis. The carpet model (Figure 3), however, is a nonspecific mechanism, permitting many different peptides to fulfill its criteria (Shai 2002; Zasloff 2002). Utilizing this method, AMPs actually bind to the microbial membrane surface and reorient themselves with their hydrophobic regions toward lipids

and their hydrophilic regions toward phospholipid groups. Via subsequent disruption of bilayer curvature by the peptides, the membrane is disintegrated.

Within an organism, there are numerous AMPs with different chemical and physical properties and functions. These peptides can act in different combinations to bind to, disrupt, or even enter the microbial membrane, as well as eventually inhibit the growth of or kill a foreign microbe. Many hypotheses have been presented as to the specific defensive mechanisms by which families of AMPs disrupt the functions of or penetrate the microbial membrane once bound to it. These include, but are not limited to, depolarization of the bacterial membrane or inhibition of protein import and respiration by insect cecropins; interaction with and relocation of lipids or divalent cations of LPSs within the outer cell membrane bilayers by indolicidin and analogs, gloverin, bactenecin, a-defensins, cecropin B, and cecropin A-melittin hybrids; inhibition of outer membrane protein synthesis by attacins and gloverin; the creation of pores or transmembrane channels in the microbial membrane, permitting peptide permeabilization or leakage of cellular contents, by cecropins and defensins; and the stereospecific recognition and entry of peptides into the target cell resulting in damage to intracellular targets by apidaecin, drosocin, and the metalnikowins (reviewed by Andreu and Rivas 1998; Bartlett *et al*. 2002; Zasloff 2002). Once inside the microbial cell, different proposed defensive mechanisms of AMPs include inhibition of ACTH-induced steroidogenesis by corticostatic peptides (Zhu *et al*. 1989; Zhu and Solomon 1992); induction of hydrolases to degrade bacterial peptidoglycan resulting in lysis of the microbe; breakage of single-stranded microbial DNA by defensins; arrest of DNA synthesis by PR-39; histamine

release; and production of hydrogen peroxide (reviewed by Andreu and Rivas 1998; Bartlett *et al*. 2002; Zasloff 2002).

In contrast to the vast number of AMPs isolated from higher invertebrates, none have been characterized in corals. Yet, due to the importance of scleractinian and soft corals in numerous environments, especially as the foundation of patch, atoll, barrier, and fringing reef systems, it is critical to determine whether they can synthesize one or more of a variety of AMPs active against invasive microbes found on or in the corals or in their surrounding environment.

Study Objectives

Numerous approaches have been taken in the successful extraction of crude antimicrobial fractions, including extractions in ethanol (Koh 1997; Encarnación *et al*. 2000; Koh and Sweatman 2000), citric acid and ethanol (Kasahara and Bosch 2003), acetonitrile and trifluoroacetic acid (Clark *et al*. 1994), chloroform and water (personal communication, Peter Moeller, NOAA), ethyl acetate and water (Targett *et al*. 1983; Iguchi *et al*. 2004), dimethyl sulfoxide (Shoaf and Lium 1976; Filbin and Hough 1984), Tris-HCl, and methanol and water (Rittschof *et al*. 1985; Kim 1994; Slattery *et al*. 1995; Slattery *et al*. 1997). The goal of the current study was to examine variations of each extraction procedure and optimize a protocol by which to extract antimicrobial agents, specifically peptides, from the common seawhip, *Leptogorgia virgulata*, from South Carolina waters.

Two methods were used to assay antimicrobial activity of extracts from *L. virgulata*: the standard disc diffusion assay (Boman *et al*. 1972) and the liquid growth

inhibition assay (Bulet *et al*. 1993). Both Gram-negative (*Escherichia coli* BL21 and *Vibrio harveyii*) and Gram-positive (*Micrococcus luteus* and a *Bacillus* sp. isolate from a scleractinian coral, *Acropora cervicornis*) strains were used in the assays. The various methods of extraction used to isolate AMPs from *L. virgulata* were compared *in vitro* to controls to validate assay conditions.

MATERIALS AND METHODS

Field Collections

Branches from several *Leptogorgia virgulata* colonies were collected between May 2004 and March 2005 at three sites in Charleston, South Carolina (Department of Natural Resources Scientific Permit # 0325). These locations included the docks at Cherry Point Seafood Company (32º35'57.56"N, 80º10'37.19"W), Patriots Point Marina (32º47'17.31"N, 79º54'29.42"W), and Oak Creek Bridge (32º40'07.98"N, 79º56'50.12"W) (Figure 4). Collections were made directly from docks or by snorkel at depths of 0.5-4.0 m. All *L. virgulata* colonies were recognized on the basis of conventional taxonomic criteria (morphological characteristics such as shape, as well as color). Samples were transported in ambient sea water to Hollings Marine Laboratory, Charleston, SC, where they were stored at -80°C. Prior to extraction, entire branches of *L. virgulata* were pulverized under liquid nitrogen in a 6850 cryogenic mill (SPEX CertiPrep, Inc.) and returned to -80°C.

Screening Methods of Extraction of Antimicrobial Peptides from *L. virgulata*

Ethanol Extraction

Between 0.2 and 3.0 grams wet tissue weight of *L. virgulata* was extracted in 7 volumes (w/v) of 80% ethanol (Koh 1997; Encarnación *et al*. 2000; Koh and Sweatman 2000) at 4°C for 24 hours. Crude extracts were centrifuged at 10,000 rpm (12,096 x g) at 4°C for 30 minutes using a Beckman-Coulter centrifuge (JA-25.50 fixed angle rotor). Aliquots of 1.5 mL of supernatant were portioned in 2.0 mL pre-weighed microcentrifuge

tubes (Fisher Scientific, New Jersey) and evaporated to dryness under vacuum on low heat (Thermo-Savant SC110A Speed Vac® Plus). Each 500 µg of extract remaining (Koh 1997) was dissolved in 20 µL of 10 mM Tris-HCl (pH 8.0) and stored at -20°C (Haug *et al*. 2004) until it could be assayed for antimicrobial activity.

Citric Acid / Ethanol Extraction

Between 0.6 and 3.0 g wet tissue weight of coral was extracted in 7 vol (w/v) of 8% citric acid/100% ethanol (50:50, v/v) at 4°C for 24 hrs (Kasahara and Bosch 2003). Crude extracts were centrifuged at 10,000 rpm (12,096 x g) at 4°C for 30 min using a Beckman-Coulter centrifuge (JA-25.50 fixed angle rotor). Due to the antimicrobial properties of citric acid (Gundidza and Gaza 1993; Eswaranandam *et al*. 2004; Fite *et al*. 2004), the extract supernatant was removed and dialyzed (MWCO 1,000) against 1 L of 10 mM Tris-HCl (pH 8.0) at 4°C for 24 hrs with four changes. The purpose of dialysis was to permit the diffusion of smaller citric acid ions into the surrounding buffer, while retaining the larger *L. virgulata* peptides within the dialysis tubing. Aliquots of 1.5 mL of the supernatant were evaporated under vacuum on low heat (ThermoSavant SC110A Speed Vac® Plus). Each 500 µg of extract remaining (Koh 1997) was resuspended in 20 µL of 10 mM Tris-HCl (pH 8.0) and stored at -20°C (Haug *et al*. 2004) until it could be assayed for antimicrobial activity.

Acetonitrile / Trifluoroacetic Acid Extraction

Between 1.0-3.0 g wet weight of *L. virgulata* was extracted in 5 vol (w/v) of 60% acetonitrile/1% trifluoroacetic acid (TFA) (50:50, v/v) at 4°C for 24 hrs (Clark *et al*.

1994). Crude extracts were centrifuged at 10,000 rpm (12,096 x g) at 4°C for 30 min using a Beckman-Coulter centrifuge (JA-25.50 fixed angle rotor). The extract supernatant was dried partly under nitrogen for 1 hr in a Zymark Turbovap LV Evaporator® and dialyzed (MWCO 1000), as previously described. Post-dialysis, the sample was dried completely under nitrogen in a Zymark Turbovap LV Evaporator® for 24 hrs, lyophilized (FTS Systems, Inc. Multi-Trap Lyophilizer) for 48 hrs, and resuspended in 6 mL of 0.1% TFA (Clark *et al*. 1994). After an additional centrifugation at 12,096 x g at 4°C for 10 minutes, the supernatant was removed, portioned into 1.5 mL aliquots, and evaporated to dryness under vacuum on low heat (Thermo-Savant SC110A Speed Vac® Plus). Each 500 µg of extract remaining (Koh 1997) was dissolved in 20 µL of 10 mM Tris-HCl (pH 8.0) and stored at -20°C (Haug *et al*. 2004) until it could be assayed for antimicrobial activity.

Chloroform / Water Extraction

Between 8.0-10.0 g wet weight of *L. virgulata* was extracted in 50 mL of chloroform/sterile water (50:50, v/v) at 4°C for 24 hrs (personal communication, Peter Moeller, NOAA). Extracts were filtered through a sand and Sealite column (personal communication, Peter Moeller, NOAA) and washed with solvents of decreasing polarity, including sterile water, methanol, and chloroform. The water-soluble and lipophilic portions of the coral extract were partitioned by a glass separatory funnel and washed either with sterile water or methanol and chloroform, respectively. The crude lipophilic extract was dried both under vacuum in a cooling rotary evaporator (Thermo NESLAB® RTE-7 Digital Plus Refrigerated Bath) and under nitrogen in a Zymark Turbovap LV

Evaporator®. Lipophilic extracts were resuspended in 100 µL methanol and stored at -20°C (Haug *et al*. 2004) until they could be assayed for antimicrobial activity.

Crude water-soluble extracts were frozen, lyophilized (FTS Systems, Inc. Multi-Trap Lyophilizer) to dryness for 48 hrs, and resuspended in 100 µL sterile water. In order to isolate, concentrate, and semi-purify antimicrobial peptides from the water-soluble portions of *L. virgulata*, a solid phase extraction was performed with a Waters Sep-Pak® C_{18} cartridge (1 cc, 50 mg). The Sep-Pak was conditioned with 2 mL of methanol and 2 mL of sterile water prior to extraction. The coral filtrate was eluted from the Sep-Pak® with 1.5 mL sterile water and dried under nitrogen in a Zymark Turbovap LV Evaporator®. The water-soluble extract was then dissolved in 100 µL sterile water and stored at -20°C (Haug *et al*. 2004) until it could be assayed for antimicrobial activity.

Ethyl Acetate / Water Extraction

Between 0.25 and 2.0 g wet weight of *L. virgulata* was extracted in 3 vol (w/v) of ethyl acetate/sterile water (50:50, v/v) at 4°C for 24 hrs (Targett *et al*. 1983; Iguchi *et al*. 2004). Crude coral extracts were filtered through a sand and Sealite column and washed with ethyl acetate and sterile water. The water-soluble and lipophilic layers of the sample were partitioned by a glass separatory funnel and washed with sterile water or ethyl acetate, respectively. The lipophilic extract was dried under nitrogen in a Zymark Turbovap LV Evaporator® and resuspended in 150 µL methanol. The water-soluble extract was frozen, lyophilized (FTS Systems, Inc. Multi-Trap Lyophilizer) to dryness for 48 hrs, and dissolved in 150 µL sterile water. Both crude extracts were stored at -20°C (Haug *et al*. 2004) until they could be assayed for antimicrobial activity.

Dimethyl Sulfoxide and Tris-HCl Extractions

One gram wet tissue weight of coral was extracted in 10 volumes (w/v) of either 100% dimethyl sulfoxide (DMSO) (Shoaf and Lium 1976; Filbin and Hough 1984) or 10 mM Tris-HCl. Each crude extract was shaken for 24 hrs and centrifuged at 10,000 rpm (12,096 x g) at 4°C for 30 min using a Beckman-Coulter centrifuge (JA-25.50 fixed angle rotor). Extract supernatants were then removed and stored at -20ºC (Haug *et al*. 2004) until they could be assayed for antimicrobial activity.

Antimicrobial Assay – Disc Diffusion Assay

Antimicrobial activity, used as a general indicator of bioactivity in coral extracts, was measured using a standard disc diffusion assay (Boman *et al*. 1972). Assays were performed with the Gram-negative *Escherichia coli* BL21 (culture collection of Paul Gross, MUSC), the Gram-positive *Micrococcus luteus* (ATCC 4698), and a *Bacillus* sp. isolate from a scleractinian coral, *Acropora cervicornis* (culture collection of Cheryl Woodley, NOAA). Those assays which included extracts in 100% DMSO or 10 mM Tris-HCl also were performed using the Gram-negative *Vibrio harveyii* (culture collection of Cheryl Woodley, NOAA). Bacteria were cultured in Luria (LB) broth (Fisher Scientific, New Jersey) at 31°C for 24 hrs, at which time 1 mL of bacteria was inoculated with 4 mL poor-broth nutrient medium (1% bacto-tryptone, 0.5% NaCl w/v, pH 7.5) (Fisher Scientific, New Jersey) and grown for an additional 5 hrs at 31°C. Cultures were then diluted with poor-broth to an initial OD_{600} of 0.001 (Destoumieux *et al*. 1999; Lamberty *et al*. 1999). Aliquots of 100 µL of bacteria were spread onto LB agar plates (Fisher Scientific, New Jersey). Ten microliters of each coral extract were

added to 5 mm sterile Whatman filter disks (Fisher Scientific, New Jersey) placed atop the agar, and plates were incubated at 31°C for 24 hrs to permit bacterial lawn growth. Control disks included 10 µL of 10 mM Tris-HCl (pH 8.0), methanol, or 100 µM $Ala^{[8,13,18]}$ magainin-II-amide (Sigma M8155) (Mystkowska *et al*. 2001). Additional control disks were extraction-solvent-dependent and contained 10 µL of either 80% ethanol, 8% citric acid and ethanol, 60% acetonitrile and 1% TFA, chloroform and sterile water, ethyl acetate and sterile water, or 100% DMSO. Zones of inhibition, defined as the distance from each edge of a disk to the lawn of bacteria, verified the relative ability of a crude *L. virgulata* extract to inhibit bacterial growth.

Antimicrobial Assay – Liquid Growth Inhibition Assay

Determination of antimicrobial activity in *L. virgulata* also was analyzed by the liquid growth inhibition assay (Bulet *et al*. 1993). Assays were performed with the Gram-negative *E. coli* BL21, as well as the Gram-positive *M. luteus* and *Bacillus* sp. Those assays which included extracts in 100% DMSO or 10 mM Tris-HCl again were executed using the Gram-negative *V. harveyii*. Bacteria were cultured in LB broth at 31°C for 24 hrs, at which time 1 mL of bacteria was inoculated with 4 mL poor-broth nutrient medium and grown for an additional 5 hrs at 31°C. Cultures were then diluted with poor-broth to an initial OD_{600} of 0.001 (Destoumieux *et al*. 1999; Lamberty *et al*. 1999). In sterile 96-well microtiter plates (Fisher Scientific, New Jersey), 90 µL of a suspension of logarithmic phase bacteria (OD_{600} of 0.001) were added to 10 µL aliquots of each crude *L. virgulata* extract (Bulet *et al*. 1993; Destoumieux *et al*. 1999; Lamberty *et al*. 1999). Blank wells were included that contained 100 µL of sterile water or poor-

broth. Control wells contained 90 µL of bacteria and 10 µL of 10 mM Tris-HCl (pH 8.0), methanol, or 100 µM Ala$^{[8,13,18]}$ magainin-II-amide (Sigma M8155) (Mystkowska *et al*. 2001). Additional control wells were extraction-solvent-dependent and contained 90 µL of bacteria and 10 µL of either 80% ethanol, 8% citric acid and ethanol, 60% acetonitrile and 1% TFA, chloroform and sterile water, ethyl acetate and sterile water, or 100% DMSO. All treatments were performed in triplicate. Microbial growth was measured by an increase in optical density (OD) after incubation at 31ºC for 24 hrs (Bulet *et al*. 1993). Absorbance was read at 600 nm using a microtiter plate reader (Spectramax Plus® MN03749) (Bulet *et al*. 1993; Destoumieux *et al*. 1999; Lamberty *et al*. 1999). The mean absorbance (OD) of three replicates of each *L. virgulata* extract, as compared to controls, was used as a measure of antimicrobial activity in the coral.

Optimizing the Isolation of Antimicrobial Secondary Metabolites from *L. virgulata*

Methanol / Water Extraction

Ground tissue samples of *L. virgulata* weighing 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0, and 8.0 g were extracted in 0.2, 0.5, 0.7, 0.8, 1.4, 3.0, 4.0, and 7.0 mL (w/v), respectively, of methanol/sterile water (50:50, v/v) at 4°C for 24 hrs. Crude extracts were centrifuged at 10,000 rpm (12,096 x g) at 4°C for 30 min using a Beckman-Coulter centrifuge (JA-25.50 fixed angle rotor). The extract supernatant was analyzed for antimicrobial activity using the liquid growth inhibition assay, as previously described and including *V. harveyii*. Blank wells contained 100 µL of sterile water or poor-broth. Control wells contained 90 µL of bacteria and 10 µL of methanol or 100 µM Ala$^{[8,13,18]}$ magainin-II-amide (Mystkowska *et al*. 2001). All treatments were performed in triplicate. Microbial

growth was measured by an increase in OD after incubation at 31ºC for 24 hrs (Bulet *et al*. 1993). Absorbance was read at 600 nm using a microtiter plate reader (Spectramax Plus® MN03749) (Bulet *et al*. 1993; Destoumieux *et al*. 1999; Lamberty *et al*. 1999). The mean absorbance (OD) of three replicates of each *L. virgulata* extract, as compared to controls, was used as a measure of antimicrobial activity in the coral.

RESULTS AND DISCUSSION

Antimicrobial activity was not observed in *Leptogorgia virgulata* extracts in ethanol (Koh 1997; Encarnación *et al*. 2000; Koh and Sweatman 2000), 8% citric acid and ethanol (Kasahara and Bosch 2003), acetonitrile and 1% trifluoroacetic acid (Clark *et al*. 1994), chloroform and sterile water (personal communication, Peter Moeller, NOAA), ethyl acetate and sterile water (Targett *et al*. 1983; Iguchi *et al*. 2004), dimethyl sulfoxide (Shoaf and Lium 1976; Filbin and Hough 1984), or Tris-HCl using the standard disc diffusion and liquid growth inhibition assay systems. Assay results suggest that antimicrobial peptides (AMPs) were either absent from the water-soluble and lipophilic portions of *L. virgulata* or, if present, were not specific to the strains of bacteria utilized in the assay: *E. coli* BL21, *V. harveyii*, *M. luteus*, and *Bacillus* sp. Increasing the w/v ratio of the extract (from 0.03 to 0.67) did not improve the ability to isolate AMPs.

The selected combination of solvents and protocol may not have been ideal for the isolation of AMPs from *L. virgulata* but are commonly used by researchers studying these compounds. Although antimicrobial activity was not detected in *L. virgulata* extracts using the aforementioned conditions, it is impossible to state that AMPs are not present.

Optimizing the Isolation of Antimicrobial Secondary Metabolites from *L. virgulata*

Methanol / Water Extraction

In an effort to demonstrate antimicrobial activity, a generalized extraction protocol, not specific for AMPs, was employed. The solvent protocol, more polar than

the solvents used to extract AMPs, was designed to isolate polar secondary metabolites. The liquid growth inhibition assay successfully identified antimicrobial activity in replicates of the crude aqueous methanol extract of *L. virgulata.* Extraction of greater than 0.5 grams of tissue was required to inhibit growth of *V. harveyii* and *M. luteus* and 2.0 g of tissue to inhibit *E. coli* BL21. The effectiveness of this extraction protocol at identifying antimicrobial activity in numerous *L. virgulata* samples suggested that secondary metabolites, not peptides as previously sought, were responsible for the activity observed. To our knowledge, this represents the first report of antimicrobial activity in *Leptogorgia virgulata* and, of equal note, in a temperate/sub-tropical coral in the Atlantic Ocean. Antimicrobial activities of secondary metabolites, especially terpenoids and fatty acids, have been identified previously in tropical soft corals of the Atlantic Ocean (Burkholder and Burkholder 1958; Ciereszko *et al*. 1960; Ksebati *et al*. 1984; Jensen *et al*. 1996; Carballeira *et al*. 1997; Roussis *et al*. 2001), as well as in corals of the temperate, subtropical, tropical, and polar zones of the Pacific (Fenical *et al*. 1981; Gunthorpe and Cameron 1990; Kim 1994; Goh *et al*. 1995; Fusetani *et al*. 1996; Seo *et al*. 1996; Koh 1997; Aceret *et al*. 1998; Slattery *et al*. 1999; Encarnación *et al*. 2000; Koh *et al*. 2002; Harder *et al*. 2003; Marquis *et al*. 2005), Indian (Badria *et al*. 1998; Kelman *et al*. 1998; Bala Show Reddy *et al*. 1999; Wilsanand *et al*. 1999; Geffen and Rosenberg 2005) and Arctic Oceans (Slattery *et al*. 1995; Slattery *et al*. 1997). Furthermore, this study outlines a successful methodology, including the extraction of coral tissue in methanol and water, coupled to a liquid growth inhibition assay system, which may be used to isolate antimicrobial compounds, specifically secondary metabolites, from soft corals.

Conclusions

It was the goal of the present study to compare and optimize protocols for extracting and isolating antimicrobial compounds, specifically peptides, from the common seawhip, *Leptogorgia virgulata.* The assay systems tested (i.e., the standard disc diffusion and liquid growth inhibition assays) did not detect antimicrobial activity in *L. virgulata* extracts in ethanol, 8% citric acid and ethanol, acetonitrile and 1% trifluoroacetic acid, chloroform and sterile water, ethyl acetate and sterile water, dimethyl sulfoxide, or Tris-HCl. However, when a general extraction protocol designed to isolate polar secondary metabolites rather than antimicrobial peptides was utilized, the liquid growth inhibition assay successfully identified antimicrobial activity in crude methanol/sterile water extracts of *L. virgulata* using *V. harveyii*, *E. coli* BL21, and *M. luteus*.

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CHAPTER 2

Secondary Metabolites and a Study of Antimicrobial Activity in *Leptogorgia virgulata* in Charleston and Georgetown Counties, South Carolina

INTRODUCTION

Innate Immunity

Secondary Metabolites

Secondary metabolites are those chemical compounds in an organism that are not directly involved in its growth, development, or reproduction (Devon and Scott 1972; Van Alstyne and Paul 1988). Like antimicrobial peptides, secondary metabolites have been isolated from a number of organisms, including bacteria, fungi, plants, invertebrates, and vertebrates (Coll and Sammarco 1988). These metabolites are often species-specific and can play a major role in the survival and colonization success of an organism (Maida *et al*. 1993). A lack of secondary metabolites or variation in the composition and concentration of these compounds would have significant implications for an organism and could impair its chemical defense, survival, and fecundity, promote aesthetic differences, or inhibit the development of its phenotype (Maida *et al*. 1993; http://en.wikipedia.org/wiki/Secondary_metabolites).

Secondary metabolites vary in chemical structure and size and can be classified in marine organisms as glycosides, brominated phenols, polyphenolics, polyketides, nonribosomal peptides, ribosomal peptides (peptide, polypeptide, and protein toxins), alkaloids, fatty acids, and terpenoids (Devon and Scott 1972; Van Alstyne and Paul 1988; Blunt *et al*. 2003, 2004). In corals, secondary metabolites, such as terpenoids and fatty acids, have been shown to possess diverse pharmacological activities and can be vital components of the host chemical defense system (Coll and Sammarco 1988; Van Alstyne and Paul 1988; Maida *et al*. 1993; Paul and Puglisi 2004). These bioactive compounds

can function in predator deterrence or toxicity (Fenical *et al*. 1981; Gerhart 1984, 1985, 1986; Bakus *et al*. 1986; La Barre *et al*. 1986*b*; Pawlik *et al*. 1987; Sammarco *et al*. 1987; Harvell *et al*. 1988; Fenical and Pawlik 1991; Goh *et al*. 1995; Slattery and McClintock 1995; Aceret *et al*. 1998; Epifanio *et al*. 1999; Kelman *et al*. 1999; Slattery *et al*. 1999; Koh *et al*. 2000; Paul and Puglisi 2004), surficial fouling inhibition (Keifer *et al*. 1986; Coll *et al*. 1987; Rittschof *et al*. 1994; Slattery *et al*. 1995; Harder *et al*. 2003; Rittschof *et al*. 2003), interspecies competition (Hildemann *et al*. 1979; Sammarco *et al*. 1983; Sammarco *et al*. 1985; La Barre *et al*. 1986*a*; Gunthorpe and Cameron 1990; Salter-Cid and Bigger 1991; Koh and Sweatman 2000), and chemical signaling to coordinate physiological processes such as osmoregulation, spawning, recruitment, and settlement (Coll and Sammarco 1988; Van Alstyne and Paul 1988; Coll *et al*. 1989; Gerhart *et al*. 1990). Coral secondary metabolites also have been shown to possess anti-inflammatory (Look *et al*. 1985, 1986; Fenical 1987; Groweiss *et al*. 1988; Shin *et al*. 1989; Pordesimo *et al*. 1991) and antiviral activities (Groweiss *et al*. 1988; Bala Show Reddy *et al*. 1999; Marrero *et al*. 2004). Additionally, secondary metabolites can function as antimicrobial agents, which inhibit the growth of or kill foreign, invasive microbes.

Antimicrobial activity was first documented in a gorgonian octocoral by Burkholder and Burkholder (1958) off the southern coast of Puerto Rico. Since that time, numerous antimicrobial compounds, specifically secondary metabolites, have been isolated from scleractinian and soft corals in the Caribbean (Ciereszko *et al*. 1960; Ksebati *et al*. 1984; Jensen *et al*. 1996; Carballeira *et al*. 1997; Roussis *et al*. 2001), Red Sea (Badria *et al*. 1998; Kelman *et al*. 1998; Geffen and Rosenberg 2005), Indian Ocean (Bala Show Reddy *et al*. 1999; Wilsanand *et al*. 1999), Indo-Pacific (Goh *et al*. 1995;

Aceret *et al*. 1998; Koh *et al*. 2002), China (Harder *et al*. 2003), Japan (Fusetani *et al*. 1996; Seo *et al*. 1996), Australia (Gunthorpe and Cameron 1990; Koh 1997; Marquis *et al*. 2005), Guam (Slattery *et al*. 1999), the Antarctic (Slattery *et al*. 1995; Slattery *et al*. 1997), and the eastern Pacific (Fenical *et al*. 1981; Kim 1994; Encarnación *et al*. 2000). Yet, to our knowledge, antimicrobial activity has yet to be identified in a temperate or sub-tropical coral in the Atlantic Ocean.

Chemical Defense System of *Leptogorgia virgulata*

Production of marine antifoulants by the colorful seawhip, *Leptogorgia virgulata* (Lamarck) in response to predation and fouling has been studied intensively due to the notable paucity of epibionts associated with this octocoral (Gerhart *et al*. 1988) and because it is not fed upon by fishes (Gerhart 1991). *L. virgulata* does occasionally harbor a small number of symbiotic animals, including the snail *Neosimnia uniplicata*; the Atlantic pearl oyster, *Pteria colymbus*; a tissue-feeding nudibranch *Tritonia wellsi*; the seawhip shrimp, *Neopontonides beaufortensis*; two unidentified copepod species (Patton 1972; Fox and Ruppert 1985; Ruppert and Fox 1988); the seawhip barnacle, *Conopea galeata*; skeleton shrimp, *Caprella equilibra*; a gammarid amphipod *Erichthonius brasiliensis*; and a bryozoan *Alcyonidium hauffi* (Fox and Ruppert 1985; Ruppert and Fox 1988). Yet, even with sporadic digestion of host mucus and tissue by these symbionts, they appear to associate solely in a commensal relationship with the coral, one which does not influence nor appear to be influenced by *L. virgulata*'s defense systems.

Pukalide and 11ß, 12ß-epoxypukalide (Missakian *et al*. 1975), specific novel diterpenes isolated from *L. virgulata* (Gerhart *et al*. 1988), have been shown to inhibit

swimming, reversible attachment (Rittschof *et al*. 1985; Clare *et al*. 1999), and settlement of the barnacle, *Balanus amphitrite* (Standing *et al*. 1984; Gerhart *et al*. 1988; Clare *et al*. 1999), whereas other high-molecular-weight substances within the soft coral tissue actually induce *B. amphitrite* settlement (Standing *et al*. 1984). Laboratory experiments have shown that emetic properties of *L. virgulata* mimic the activity of eicosanoids from barnacle-hatching assays, producing learned aversions in several species of fish (Gerhart 1991). The combination of calcite sclerites, pukalide, and epoxypukalide in *L. virgulata* also has proven effective against fish predation (Gerhart 1991). Gerhart and Coll (1993) recorded vomiting in the killifish, *Fundulus heteroclitus*, when it was fed artificial pellets containing the approximate concentrations of pukalide found naturally in the coenenchyme of *L. virgulata.* In addition, homarine and water-soluble extracts of *L. virgulata* containing homarine were effective at inhibiting the growth of the potentially-fouling, benthic diatom *Navicula salinicola* (Targett *et al*. 1983).

Although there is a body of literature available describing the chemical defense system of *L. virgulata,* to date, the host defense immunity of this species, specifically the potential for antimicrobial activity, has not been reported. The goal of the current study was to isolate antimicrobial agents, specifically secondary metabolites, from crude extracts of *L. virgulata.*

Field Study of *Leptogorgia virgulata,* Site Selection

Sites of collection along the eastern coast of South Carolina were chosen on the basis of availability of *Leptogorgia virgulata,* as well as the intensity of anthropogenic stressors present. Although greater than forty sites were surveyed, only seven contained

a significant number of *L. virgulata* colonies to permit sufficient, repeated collections to support the amount of tissue required for analyses of antimicrobial activity. In Charleston County, these locations included the floating docks at Cherry Point Seafood Company (32º35'57.56"N, 80º10'37.19"W), Kiawah River Bridge (32º36'12.58"N, 80º07'56.41"W), Oak Creek Bridge (32º40'07.98"N, 79º56'50.12"W), the Charleston City Marina (32º46'50.40"N, 79º57'17.36"W), and the docks at Patriots Point Marina (32º47'17.31"N, 79º54'29.42"W). Sites in Georgetown County included upper 60 Bass Creek–North Inlet estuary (33º19'38.12" N, 79º10'30.80"W) and Huntington Beach State Park–South Jetty (33º31'35.22"N, 79º01'59.08"W), located 110 and 140 km northeast of Charleston, SC, respectively (Figure 4). Reference sites selected for this study were Cherry Point Seafood Company, Kiawah River Bridge, and upper 60 Bass Creek–North Inlet.

Historical documentation of water and sediment quality was assessed at each site according to standards established by the South Carolina Department of Health and Environmental Control (SCDHEC). These standards serve as a basis for determining National Pollutant Discharge Elimination System (NPDES) effluent limitations for point source dischargers (U.S. Department of Commerce NOAA 1984). Water and sediment quality were used to classify all seven locations as either reference or anthropogenically impacted sites (Table 1).

Reference Sites

Cherry Point Seafood Company (CP)

Located on Bohicket Creek in Charleston County, South Carolina, the floating docks at Cherry Point Seafood Company are bordered creek-side by shrimping boats and land-side by power boats and *Spartina* salt marsh. The artificial substrate provided by the floating dock at CP is common to marinas in tidal coastal waters of South Carolina. The dock supports a diverse community of subtidal organisms, including the common seawhip, sponges, hydroids, bryozoans, tunicates, macroalgae, worms, and crustaceans, especially amphipods and the seawhip barnacle, *Conopea galeata* (Ruppert and Fox 1988). Seawhip colonies at CP were observed interspersed along the creek-side length of the dock at depths of 0.17 to 2.0 m. Due to the positioning of *L. virgulata* colonies, they typically experience a steady-flow regime (Blair 2003) from the tidal creek. Water quality at CP has been classified as "SA" (Table 1), indicating that tidal salt waters are suitable for primary and secondary recreational purposes and harvesting of fish and shellfish for human consumption, that they are devoid of deleterious substances and waste, that dissolved oxygen levels are maintained at or above 5.0 mg/L (with a low of 4.0 mg/L), that fecal coliform and enterococci levels do not exceed a geometric mean of 200/100 mL and 35/100 mL, respectively, that the pH ranges no more than 0.3 units between 6.5 and 8.5, and that turbidity does not exceed 25 NTUs (Table 1) (U.S. Department of Commerce NOAA 1984; South Carolina Department of Health and Environmental Control 2004).

Kiawah River Bridge (KRB)

Located on the Kiawah River and adjacent to Kiawah Island in Charleston County, Kiawah River Bridge is bordered on both sides by *Spartina* salt marsh. Colonies of *L. virgulata* were attached to the middle of the bridge at its base and to the river bed at a depth of 10-11 m. No light is visible at the depth of the corals. The highly-sedimented environment observed at depth may be explained by the steady flow of water provided by the bi-directional tidal currents of the Kiawah River, coupled with the significant increasing trend in turbidity documented in the river by SCDHEC (1999). Although land use and suburbanized development have increased substantially on Kiawah Island in the last 20 years, waterways associated with the island have not exceeded the recommended enterococci geometric mean (< 35 bacteria per 100 mL of water) in the surf during dry or wet weather conditions (South Carolina Department of Health and Environmental Control 2000). Consequently, the Kiawah River is still grouped with "SA" quality waterways (U.S. Department of Commerce NOAA 1984).

Upper 60 Bass Creek (60 Bass)

Upper 60 Bass Creek is located in the North Inlet estuary system, an area with less than 6% urban development (Blood and Smith 1996; South Carolina Department of Health and Environmental Control 2000) in Georgetown County, South Carolina. The upper portion of 60 Bass is flanked on both sides by *Spartina* salt marsh and can be identified easily by the large oyster mounds interspersed along the creek's center. *L. virgulata* colonies are sparse in 60 Bass, numbering only five in the 200 m of the creek surveyed. The depth of the octocoral colonies and the creek bed is 2 m. Colonies, which

were observed attached to individual oyster shells on the sandy bottom, are subjected to a strong steady-flow regime, which increases daily during the flood tides. Although no documentation exists classifying the water quality of 60 Bass, it is regarded as a pristine body of water that can be ranked with other "SA" quality waterways (personal communication, Paul Kenny, Senior Research Specialist, Belle W. Baruch Institute for Marine and Coastal Sciences). Discharge from Winyah Bay to the south does impact water and sediment characteristics in portions of the North Inlet estuary. However, North Inlet overall has been recognized for its superior quality and is considered to be a relatively undisturbed, well-mixed estuary with minimal anthropogenic impacts (Blood and Vernberg 1992; South Carolina Department of Health and Environmental Control 2000).

Anthropogenically Impacted Sites

Patriots Point (PP)

Colonies of *L. virgulata* were collected from the floating docks owned by the College of Charleston Sailing Team at Patriots Point Marina. PP is located at the mouth of the Wando River in the Charleston Harbor estuary, SC, and supports a diverse community of subtidal organisms, similar to that observed at CP. The College of Charleston floating docks are flanked on both sides by boats and the Charleston Harbor. Seawhip colonies are interspersed the entire length of both sides of the dock at depths of 0.17-1.50 m. Due to the positioning of *L. virgulata* colonies, they typically experience a steady-flow regime (Blair 2003) from the bi-directional tidal currents of the Wando River. Water quality at PP has been categorized as "SB" (Table 1) (U.S. Department of

Commerce NOAA 1984). Class "SB" waterways are tidal waters devoid of deleterious substances and waste, with dissolved oxygen levels greater than 4.0 mg/L, fecal coliform and enterococci concentrations at or below a geometric mean of 200/100 mL and 35/100 mL, respectively, a pH that ranges no more than 0.5 units between 6.5 and 8.5, and turbidity that does not exceed 25 NTUs (Table 1). Crabbing, fishing, and primary and secondary contact recreation are permissible in "SB" waters, except within buffer zones specified by SCDHEC (U.S. Department of Commerce NOAA 1984; South Carolina Department of Health and Environmental Control 2004). The harvesting of shellfish for market sale or human consumption, however, is forbidden.

Huntington Beach State Park–South Jetty (HBJ)

Huntington Beach State Park–South Jetty is located in the Murrells Inlet estuary in Georgetown County, SC. The western half of HBJ is bordered by the Atlantic Ocean and Murrells Inlet estuary. The northeastern portion of the jetty, from which *L. virgulata* colonies were collected, is surrounded by the Atlantic Ocean, although waters from the mouth of Murrells Inlet estuary also impact the site. The community composition and physical environment of the northeastern portion of HBJ appear analogous to that of a patch reef located nearshore South Carolina, making the ecosystem at HBJ unique to this study. Hundreds of thickly-branched, healthy *L. virgulata* colonies are anchored directly to the jetty boulders at depths of 3-7 m. Due to the location of the corals in this high-stress environment (Ruppert and Fox 1988), they are subjected to multidirectional, semi-diurnal tidal and wind driven currents, as well as wave surge.

The Murrells Inlet estuary, of which HBJ is a part, is an area with greater than 50% urban development in Georgetown County (Blood and Smith 1996; South Carolina Department of Health and Environmental Control 2000). Water quality of the entire estuary system has been classified as "SB", as at PP. Subsurface flow and advection from the bottom of tidal creeks connected to Murrells Inlet have been recognized as important mechanisms by which inorganic nutrient loads from rapid surface runoff, septic tank leakage, and seepage below impoundments in upland creeks are transported into Murrells Inlet (Blood and Smith 1996). Residential communities, like those surrounding HBJ, provide high levels of inorganic nitrogen and orthophosphate to the land and ocean regions of Murrells Inlet, both at low tide and continuously during summer and autumn, when tourist season is at its peak. During the rest of the year and at high tide, however, inorganic nutrient levels within Murrells Inlet are not significantly different from North Inlet estuary, an estuary with an "SA" water quality classification (Blood and Smith 1996).

Oak Creek Bridge (OCB)

Oak Creek is a small, shallow creek that empties into the confluence of Long Island River and Folly River at Folly Beach, Charleston County, SC. At Oak Creek Bridge (a.k.a. Oak Island Bridge), the creek is bordered by *Spartina* salt marsh, oyster beds, and Anchor Line Restaurant. Colonies of *L. virgulata* were observed attached to the creek bed and the base of bridge pilings located along the middle of the creek at depths of 1.5-3.3 m. The steady-flow regime (Blair 2003) provided by the bi-directional tidal currents of the creek appeared to create a moderate level of turbidity. Oak Creek

has been categorized with "SC" (Table 1) waterways, the lowest ranking water quality classification assigned by SCDHEC (U.S. Department of Commerce NOAA 1984). Class "SC" waterways are tidal waters with dissolved oxygen levels above 4.0 mg/L, fecal coliform concentrations at or below a geometric mean of 1000/100 mL, and a pH that ranges no more than 1 unit between 6.5 and 8.5 (Table 1). The same restrictions that pertain to uses within "SB" waterways also apply to "SC" classified waters. In addition, primary contact recreational activities, such as swimming, water skiing, and surfing, are prohibited (U.S. Department of Commerce NOAA 1984). Although no documentation exists assessing the origins of the creek's poor water quality, a number of sources were observed that may have exacerbated the situation. These included a moderate level of slow-moving boat traffic, sediment loading from Long Island and Folly Rivers, and land run-off from Folly Road, Anchor Line Restaurant, and the housing communities and docks on the creek.

Charleston City Marina (CCM)

Located midway along the South Carolina coastline at the junction of the Cooper, Ashley, and Wando Rivers, the Charleston Harbor estuary is the second largest watershed in the state (Davis and Van Dolah 1992). Within Charleston Harbor, the Charleston City Marina is positioned on the Ashley River, approximately 3 km northwest of the confluence of the Ashley and Cooper Rivers. Colonies of *L. virgulata* are located in the section of CCM directly beneath the end of the James Island Connector (SC-30), before its convergence with US-17. This site is bordered by a small span of *Spartina* salt marsh, the city of Charleston, and numerous floating docks of CCM and the Ashley Marina.

Corals were observed attached at a depth of 5-10 m to the base of the bridge piling and to the oyster bed within 2 m of the piling. No light was visible at the depth of the corals. The steady flow of water provided by the bi-directional tidal currents of the Ashley River was seen to create a highly-sedimented environment.

Charleston Harbor has been documented as the second largest container port along the eastern coast of the United States (Davis and Van Dolah 1992) and continues to expand. The area has a large number of transportation centers that facilitate commercial and recreational vessel traffic (Kelly 1973; Davis and Van Dolah 1992). Lands surrounding Charleston Harbor and CCM are largely developed, highly populated, and support a thriving metropolitan area and tourist industry. There are a number of point and nonpoint sources of pollution to the lower Ashley River, including sewage discharges; runoff from municipal, suburban, industrial, and military areas; mining activities; septic tank overflows; industrial outfalls; flooding of drainage systems; groundwater contamination from surface impoundments, above-ground and underground storage tanks, spills, and illegal disposals; leaching of organics and inorganics from solid waste landfills, such as the Charleston County and Lockwood Boulevard dumps; and discharges from commercial and recreational boats (Kelly 1973; Davis and Van Dolah 1992; South Carolina Department of Health and Environmental Control 1999). In addition, high levels of pollutants, such as mercury, copper, zinc, nickel, chromium, PCBs, DDTs, and fecal coliform bacteria, have been measured in the sediments of the lower Ashley River (Davis and Van Dolah 1992; South Carolina Department of Health and Environmental Control 1999). Significant decreases in dissolved oxygen and five-day biochemical oxygen demand, as well as significant increasing trends in turbidity have

also been reported for this area (South Carolina Department of Health and Environmental Control 1999). Consequently, SCDHEC has classified the water quality of the lower Ashley River, including CCM, as "SC" (Davis and Van Dolah 1992; South Carolina Department of Health and Environmental Control 1999).

Study Objectives

The goal of the present field study was to evaluate the presence of antimicrobial activity in crude methanol/sterile water extracts of the common seawhip, *Leptogorgia virgulata*, from South Carolina waters. Colonies of *L. virgulata* were collected between May and October 2005 from reference and anthropogenically impacted sites along the eastern coast of South Carolina. An assay protocol was developed using the liquid growth inhibition assay (Bulet *et al*. 1993) to analyze *L. virgulata* antimicrobial activity against general strains of the Gram-negative *Escherichia coli* BL21 (culture collection of Paul Gross, MUSC) and *Vibrio harveyii* (culture collection of Cheryl Woodley, NOAA), as well as the Gram-positive *Micrococcus luteus* (ATCC 4698) and a *Bacillus* sp. isolate from a scleractinian coral, *Acropora cervicornis* (culture collection of Cheryl Woodley, NOAA). The antimicrobial activity in *L. virgulata* was tested using known antimicrobial agents to validate assay conditions. Statistical analyses were performed to compare the variability in mean *L. virgulata* antimicrobial activity among reference and anthropogenically stressed sites in Charleston and Georgetown counties. Additionally, the variability in mean coral antimicrobial activity was compared statistically at each site among three periods of collection from May to October 2005.

MATERIALS AND METHODS

Field Collections

Five branches each from five, previously-selected *Leptogorgia virgulata* colonies were collected every other month between May and October 2005 at seven sites along the coast of South Carolina. In Charleston County, these sites included Cherry Point Seafood Company (CP, 32º35'57.56"N, 80º10'37.19"W), Kiawah River Bridge (KRB, 32º36'12.58"N, 80º07'56.41"W), Oak Creek Bridge (OCB, 32º40'07.98"N, 79º56'50.12"W), the Charleston City Marina (CCM, 32º46'50.40"N, 79º57'17.36"W), and Patriots Point Marina (PP, 32º47'17.31"N, 79º54'29.42"W) (Figure 4). In Georgetown County, study locations included upper 60 Bass Creek–North Inlet (60 Bass, 33º19'38.12" N, 79º10'30.80"W) and Huntington Beach State Park–South Jetty (HBJ, 33º31'35.22"N, 79º01'59.08"W) (Figure 4). Collections were made directly from docks, by snorkel, or using SCUBA at depths of 5-11 m. All *L. virgulata* colonies were recognized on the basis of conventional taxonomic criteria (morphological characteristics such as shape, as well as color). Samples were frozen immediately in a dry shipper and transported to Hollings Marine Laboratory, Charleston, SC, where they were stored at -80°C.

Screening of Antimicrobial Secondary Metabolites in *Leptogorgia virgulata*

Methanol / Water Extraction

Whole branches of *L. virgulata* were pulverized under liquid nitrogen in a 6850 cryogenic mill (SPEX CertiPrep, Inc.) and returned to -80°C until just prior to extraction.

Two grams wet tissue weight of each coral sample was extracted in 3 volumes (w/v) of methanol/sterile water (50:50, v/v). Each crude extract was shaken for 2 minutes and centrifuged at 10,000 rpm (12,096 x g) at 4°C for 30 min using a Beckman-Coulter centrifuge (JA-25.50 fixed angle rotor). The extract supernatant was removed and stored at 4°C until it could be assayed for antimicrobial activity.

Antimicrobial Assay

Antimicrobial activity in *L. virgulata* was identified using the liquid growth inhibition assay (Bulet *et al*. 1993). Assays were performed with two Gram-negative bacteria (*Escherichia coli* BL21 (culture collection of Paul Gross, MUSC) and *Vibrio harveyii* (culture collection of Cheryl Woodley, NOAA)) and two Gram-positive bacteria (*Micrococcus luteus* (ATCC 4698) and a *Bacillus* sp. isolate from *Acropora cervicornis* (culture collection of Cheryl Woodley, NOAA)). Bacteria were cultured in Luria (LB) broth (Fisher Scientific, New Jersey) at 31°C for 24 hrs, at which time 1 mL of bacteria was inoculated with 4 mL poor-broth nutrient medium (1% bacto-tryptone, 0.5% NaCl w/v, pH 7.5) (Fisher Scientific, New Jersey) and grown for an additional 5 hrs at 31°C. Cultures were then diluted with poor-broth to an initial OD_{600} of 0.001 (Destoumieux *et al*. 1999; Lamberty *et al*. 1999). In sterile 96-well microtiter plates (Fisher Scientific, New Jersey), 90 µL of a suspension of logarithmic phase bacteria (OD_{600} of 0.001) were added to 10 µL aliquots of each soft coral crude extract (Bulet *et al*. 1993; Destoumieux *et al*. 1999; Lamberty *et al*. 1999). Blank wells were included that contained 100 µL of sterile water or poor-broth. Control wells contained 90 µL of bacteria and 10 µL of methanol or 100 µM $Ala^{[8,13,18]}$ magainin-II-amide (Sigma M8155) (Mystkowska *et al*.

2001). All treatments were performed in triplicate. Microbial growth was measured by an increase in optical density (OD) after incubation at 31ºC for 24 hrs (Bulet *et al*. 1993). Absorbance was read at 600 nm using a microtiter plate reader (Spectramax Plus® MN03749) (Bulet *et al*. 1993; Destoumieux *et al*. 1999; Lamberty *et al*. 1999). The mean absorbance (OD) of three replicates of each *L. virgulata* extract, as compared to controls, was used as a measure of antimicrobial activity in the coral.

Statistical Analysis

All statistical tests were modeled using R, a language for statistical computing (R Development Core Team 2005). Variability in mean coral antimicrobial activity was compared among reference and anthropogenically impacted sites in SC between May and October 2005 using a one-tailed t-test ($p = 0.05$) (Sokal and Rohlf 1995). Variability in mean coral antimicrobial activity at each of the seven sites was evaluated between May and October 2005 using a pair-wise t-test ($p = 0.05$) (Sokal and Rohlf 1995).

RESULTS

Antimicrobial activity was detected with a liquid growth inhibition assay in crude methanol/sterile water extracts of *L. virgulata.* The activity contained in extracts of more than 2.0 grams of coral tissue was effective in inhibiting the growth of *Escherichia coli* BL21, *Vibrio harveyii*, *Micrococcus luteus*, and *Bacillus* sp.

The level of antimicrobial activity in crude methanol/water extracts of *L. virgulata*, as inferred by optical density, varied among reference and anthropogenically impacted sites in South Carolina between May and October 2005. Antimicrobial activity in *L. virgulata* colonies sampled at each site every other month also varied among the three points of collection from May to October 2005. There was an apparent lack of antimicrobial activity in samples of *L. virgulata* collected from CCM against the selected bacteria in this study (Figures 5-8, Table 2). Inhibition of *M. luteus* growth was detected only in extracts from one colony of *L. virgulata* at CCM in June 2005. Therefore, against *M. luteus*, mean antimicrobial activity in *L. virgulata* at CCM differed significantly from the average activity measured in seawhip samples from each of the other six sites (one-tailed t-test, $p = 0.05$) (Figure 5, Table 2). Extracts from colonies of *L. virgulata* collected between May and October 2005 from CP, 60 Bass, PP, and CCM did not inhibit *Bacillus* sp. (Figure 6, Table 2). Against *E. coli* BL21, mean antimicrobial activity in *L. virgulata* colonies from KRB was significantly different from the mean coral antimicrobial activity documented at the other sites (one-tailed t-test, $p = 0.05$) (Figure 7, Table 2). In addition, tested with *V. harveyii*, the mean antimicrobial activity in extracts of *L. virgulata* obtained from KRB and OCB was significantly different from the mean

activity at the other five study sites (one-tailed t-test, p= 0.05) and different from each other at the level of $p < 0.1$ (Figure 8, Table 2).

Tested with *E. coli* BL21, *M. luteus*, and *Bacillus* sp., the mean antimicrobial activity in extracts of *L. virgulata* from CP did not differ temporally among the three collection points in May, July, and September 2005 (Figures 9-11). However, against *V. harveyii*, the average antimicrobial activity in colonies of *L. virgulata* collected from CP in May differed significantly from the mean activity documented in the same corals again in July and September 2005 (pair-wise t-test, p= 0.001) (Figure 12).

Against *Bacillus* sp., *E. coli* BL21, and *V. harveyii*, mean antimicrobial activity in the five seawhip colonies collected from KRB did not differ significantly during the 2005 study season (Figures 10-12). When tested with *M. luteus*, however, the mean antimicrobial activity of colonies from KRB differed significantly between the second and third collections in August and October 2005 (pair-wise t-test, p= 0.01) (Figure 9).

Using *M. luteus* and *Bacillus* sp., the mean antimicrobial activity in *L. virgulata* from 60 Bass was not significantly different among the three collection points during the study period (Figures 9-10). When tested with *E. coli* BL21, the mean antimicrobial activity in *L. virgulata* differed significantly between June and August 2005 (pair-wise t-test, p= 0.05) (Figure 11). In addition, the average antimicrobial activity against *V. harveyii* in *L. virgulata* from 60 Bass differed significantly among June, August, and October 2005 (pair-wise t-test, p= 0.05) (Figure 12).

Tested with *M. luteus* and *Bacillus* sp., the mean antimicrobial activity in *L. virgulata* samples from PP did not differ significantly during the six-month study period (Figures 9-10). Against *E. coli* BL21, antimicrobial activity in extracts of *L. virgulata*

differed significantly between the second and third collections in July and September 2005 (pair-wise t-test, p= 0.05) (Figure 11). Mean coral antimicrobial activity against *V. harveyii* from the first collection in May also differed significantly from July and September 2005 (pair-wise t-test, p= 0.0001) (Figure 12).

The mean antimicrobial activity in colonies of *L. virgulata* at HBJ varied throughout the summer season against the four strains of bacteria used in the assay system. Average inhibition of *M. luteus*, *Bacillus* sp., and *V. harveyii* by colonies in October differed significantly from the mean coral antimicrobial activity recorded previously in June and August 2005 (pair-wise t-test, p= 0.0001) (Figures 9-10 and 12). In addition, against *E. coli* BL21, the average antimicrobial activity in *L. virgulata* differed significantly among June, August, and October 2005 (pair-wise t-test, p= 0.05) (Figure 11).

Using all test bacteria, the mean antimicrobial activity in seawhip colonies collected from OCB did not differ significantly (pair-wise t-test, p= 0.05) between May and October 2005 (Figures 9-12).

Although antimicrobial activity against *M. luteus* was documented in replicates of one colony of *L. virgulata* at CCM in June 2005, mean activity of seawhip samples against *M. luteus* did not differ significantly among the first, second, and third collections in June, August, and October 2005, respectively (pair-wise t-test, p= 0.05) (Figure 9). In extracts from *L. virgulata* at CCM, average antimicrobial activity against *Bacillus* sp. and *V. harveyii* also did not differ significantly (pair-wise t-test, p= 0.05) during the study period (Figures 10 and 12). A slight depression of *E. coli* BL21 growth was detected in assays containing crude methanol/sterile water extracts of *L. virgulata* collected from

CCM in October 2005. This observation could explain the significant difference in mean *L. virgulata* antimicrobial activity against *E. coli* BL21 measured in June and October 2005 (pair-wise t-test, p= 0.001) (Figure 11).

Strong levels of antimicrobial activity against *M. luteus* (a terrestrial bacterium) and *V. harveyii* (a marine bacterium) were observed in *L. virgulata* colonies at no fewer than 3 sites at each point of collection during the 2005 study season. Colonies of *L. virgulata* collected from CP, 60 Bass, PP, and CCM were not active against the *Bacillus* sp. isolate between May and October 2005. Similarly, antimicrobial activity against *E. coli* BL21 was detected in seawhip samples only at KRB and OCB until September 2005, when activity was observed in at least one colony at CP, KRB, 60 Bass, PP, HBJ, and OCB against the bacterium. Although *V. harveyii* has been reported as an ecologically relevant bacterium in the marine environment of soft corals (Kelman *et al*. 1998; Slattery *et al*. 1999), *M. luteus* has yet to be isolated from a marine habitat (Bultel-Poncé *et al*. 1998). However, due to the apparent sensitivity of *V. harveyii* and *M. luteus* to *L. virgulata* in the assay system, they were selected as reference organisms for use in all subsequent discussions of antimicrobial activity in the common seawhip.

DISCUSSION

Antimicrobial activity was detected in crude methanol/water extracts of *Leptogorgia virgulata*, as determined by suppression of growth of four general strains of bacteria: the Gram-negative *Escherichia coli* BL21 and *Vibrio harveyii* and the Gram-positive *Micrococcus luteus* and *Bacillus* sp. To our knowledge, this represents the first report of antimicrobial activity in *Leptogorgia virgulata* and, of equal note, in a temperate/sub-tropical coral in the Atlantic Ocean.

Variability in the average antimicrobial activity in *L. virgulata* was evaluated at and among three reference and four anthropogenically impacted sites in South Carolina between May and October 2005. Although antimicrobial activity was identified in at least 1 of the 5 seawhip colonies collected at each site at some point in the summer season, the level and specificity of antimicrobial activity against the four strains of bacteria in the assay system varied among coral colonies at each site and among sites. These observations are not surprising, as intraspecific variability in the defensive chemistry of gorgonians has been well-documented at the level of branches within a clonal individual to individuals separated by a few meters or hundreds of kilometers (Harvell *et al*. 1993; Maida *et al*. 1993; Kelman *et al*. 2000; Slattery *et al*. 2001).

A Comparison of Antimicrobial Activity in L. virgulata among Study Sites

The average *L. virgulata* antimicrobial activity at CCM against *M. luteus* differed significantly from the mean activity measured in extracts of the coral from each of the other six study sites (one-tailed t-test, p= 0.05). The lack of antimicrobial activity in *L.*

virgulata colonies collected from CCM may be explained by the condition of the study site and the corals located there. CCM resides in the lower Ashley River, which has been classified as an "SC" waterway, the lowest ranking water quality category (U.S Department of Commerce NOAA 1984). It is feasible that poor water and sediment quality from a number of point and non-point sources of pollution, as well as sediment and nutrient loading in the marina (Kelly 1973; Davis and Van Dolah 1992; South Carolina Department of Health and Environmental Control 1999), are affecting the health, innate immune response, and overall abundance of *L. virgulata.* Between May and October 2005, all of the seawhip colonies at CCM appeared unhealthy. Branches of colonies were pallid with a visible deficiency of coenenchyme, and many branch tips had been stripped entirely of coenenchyme, revealing the gorgonin skeleton beneath. By October 2005, axial skeletons of most *L. virgulata* colonies were either predominantly or completely exposed. Fleshy macroalgae had begun to dominate not only the base and exposed areas of *L. virgulata,* but also the substrate to which the colonies were attached, beginning a potential phase shift in community structure (McCook 1999) from *L. virgulata* to macroalgae. Observations made at CCM throughout the collection season would suggest that physical and anthropogenic stressors, when coupled with high sediment and nutrient input (McCook 1999; McCook *et al*. 2001), triggered the loss of coral mucus and protective tissue surrounding the gorgonin axis, the overall deterioration of *L. virgulata* health and abundance, and the subsequent increase in macroalgal fouling. These changes could explain the lack of antimicrobial activity observed in *L. virgulata* at CCM. It is possible that colonies of *L. virgulata* at CCM produce antimicrobial compounds in response to microbial fouling and infection; otherwise the coral probably

would not exist at this heavily-impacted site. However, when subjected to heavy, constant stress and/or damage from a variety of environmental sources, the antimicrobial activity in *L. virgulata* may be sufficiently spent in most colonies as to appear absent when measured subsequently in an assay system. An example of this exists in the sponge *Aplysina fistularis*, which in response to a simulated injury, released secondary metabolites aerothionin and homoaerothionin into the water in quantities 10 to 100 times greater than intact specimens (Walker *et al*. 1985). It is also possible that corals at CCM are highly active against microbes in their ambient environment but were not specific to the panel of bacteria utilized in the liquid growth inhibition assay system. Furthermore, there may be temporal or seasonal variability in the antimicrobial activity in *L. virgulata,* such that the immune response is elevated during the winter months but depressed in the warmer months when physical and anthropogenic pressures may become too much for the coral to combat.

V. harveyii was inhibited strongly by extracts of *L. virgulata* collected from KRB and OCB between May and October 2005. Although mean antimicrobial activity in *L. virgulata* at KRB and OCB differed at the level of $p < 0.1$, when compared to the average antimicrobial activity against *V. harveyii* at the other five study sites during the summer season, a significant difference was documented (one-tailed t-test, $p = 0.05$). It is puzzling that the mean antimicrobial activity in *L. virgulata* at KRB and OCB differed significantly from the mean antimicrobial activity at the other five sites but not from each other. Colonies of *L. virgulata* at KRB and OCB differ in their depth in the water column by as much as 8 m, they are subjected to a different intensity of anthropogenic stressors, and the natural characteristics of their surrounding ecosystems appear to differ, although

competition, predation, and availability of nutrients were not measured in this study. Similar findings in the literature which would explain the observations at these two sites could not be substantiated. In fact, Harvell *et al*. (1993) and Kelman *et al*. (2000) demonstrated significantly elevated levels of diterpenoid production by soft corals located on deeper reefs as compared to shallow reef sites. If the immune response of the dominant color morph of *L. virgulata* in SC (yellow) is more effective at inhibiting bacterial growth than other color morphs, then it is noteworthy that a predominance of yellow seawhip colonies existed in Kiawah River and Oak Creek. However, the dominant color morph of *L. virgulata* at CP, PP, and CCM also was yellow, suggesting that it is unlikely that color plays a role in the intensity of antimicrobial activity in *L. virgulata.* It is possible that on average, *L. virgulata* at KRB and OCB exhibits a broad spectrum of antimicrobial activity in comparison to corals at the other five sites. Additionally, *L. virgulata* at the other sites, on-average, may not be specific to the panel of bacteria utilized in the assay system. Further evaluation of the antimicrobial activity in *L. virgulata* at these sites would require the use of a larger sample size and a broader panel of ecologically relevant microbes in the assay system.

Antimicrobial Activity in L. virgulata within each Study Site

KRB and HBJ

Mean antimicrobial activity in *L. virgulata* against two reference bacteria, *M. luteus* and *V. harveyii,* was compared at each of the seven study sites among three collections between May and October 2005. Though the relationship between health and the production of antimicrobial compounds in *L. virgulata* was not evaluated in this

study, it is interesting to note that colonies at OCB, KRB, and HBJ maintained a healthy appearance throughout the study period. Against *V. harveyii* and *M. luteus*, average antimicrobial activity in *L. virgulata* from OCB did not differ significantly among the three collections in May, July, and September 2005 (Figure 9, 12). At KRB, a sizeable difference was measured between the average *L. virgulata* antimicrobial activity against *M. luteus* in May 2005 and the mean activity in August and October 2005 (Figure 9). The large degree of variation in mean activity was not statistically significant, though, due to the presence of an outlier in the dataset—one coral that did not inhibit the growth of *M. luteus* in May 2005. Removing the outlier from data analysis also did not change the lack of statistical significance between the mean antimicrobial activity in *L. virgulata* in May 2005 and the mean activity measured in August and October 2005. In contrast, using *M. luteus*, the average antimicrobial activity in colonies of *L. virgulata* collected from KRB was significantly greater in October than in August 2005. *L. virgulata* samples collected at HBJ also exhibited a significant increase in mean antimicrobial activity against *M. luteus* and *V. harveyii* in October 2005, as compared to the mean activity measured in June and August 2005.

The substantial increase in *L. virgulata* antimicrobial activity against *M. luteus* and *V. harveyii* for samples collected at KRB and HBJ in October 2005 may be associated with the conclusion of the reproductive season, which, for the common seawhip, has been reported to occur in early October (Adams 1980). There are many trade-offs between the chemical defense system of an organism and its different fitness components, such as growth rate, reproductive output, timing of reproduction, and survivorship (Harvell 1990). The production of defensive metabolites can be costly to an

organism at certain times during the year because it temporarily limits energy available for somatic or reproductive processes (Slattery *et al*. 2001). For example, Lively (1986) showed a significant reduction in growth and reproduction of barnacles with inducible defenses, as compared to those without. Variability in the production of secondary metabolites in association with reproductive periodicity also has been documented in a marine polychaete (Goerke and Weber 1991), Red Sea soft coral (Green *et al*. 1992), sea cucumber (Bandaranayake and Des Rocher 1999), and microalgae (reviewed by Cembella 2003). In addition, significant seasonality differences in the production of pukalide (Coll *et al*. 1989; Slattery *et al*. 2001), epoxypukalide (Coll *et al*. 1989), and 11ß-acetoxypukalide (Slattery *et al*. 2001) were reported for species of sinularian soft corals on the Great Barrier Reef and at other locations in the Indo-Pacific. It is possible for colonies of *L. virgulata* collected at KRB and HBJ that the production of a broad spectrum of antimicrobial compounds may be costly to reproduction. Therefore, although low levels of antimicrobial activity could be induced in the coral at any point in the season in response to certain stimuli, such as microbial fouling or infection, it may be beneficial for colonies at these two study sites to divert most of their energy toward chemical defense and the production of antimicrobial metabolites at the conclusion of the reproductive season in October.

There is strong evidence supporting the correlation between reproductive periodicity and the production of secondary metabolites in many marine invertebrates. Thus, it is possible that there is an association between the production of antimicrobial compounds in *L. virgulata* and its reproductive season, though the current study did not evaluate this relationship. Slattery *et al*. (2001) found no relationship between the

production of pukalide and 11ß-acetoxypukalide in the soft corals *Sinularia maxima* and *S. polydactyla* and the reproductive season. Betancourt-Lozano *et al*. (1998) reported the continuous presence of antimicrobial activity in the sponge *Aplysina fistularis*, independent of reproductive period but dependent on the physical stimuli in the marine environment. The plasticity of antimicrobial activity in *L. virgulata* may be explained by a number of environmental characteristics, including temperature, salinity, competition, sedimentation, nutrient input, and anthropogenic stressors. In support of this concept, Leone *et al*. (1995) and Fleury *et al*. (2000) documented temporal changes in octocoral terpenoids in response to competitive and nutritional stressors, respectively. Additionally, colonies of *L. virgulata* at KRB and HBJ may exhibit a broad spectrum of antimicrobial activity at certain times of the year and be bacterial species-specific in their activity the rest of the year. This temporal variation in specificity could account for the dramatic increase in antimicrobial activity against *M. luteus* and *V. harveyii* in the October 2005 samples.

CP and PP

The mean antimicrobial activity in extracts of the common seawhip collected at CP and PP in May 2005, tested with the marine bacterium *V. harveyii*, was significantly greater than the average antimicrobial activity observed in extracts of the same colonies in July and September 2005. The health of all of the *L. virgulata* colonies at both sites, including those utilized in the study, appeared to deteriorate between May and July 2005, which could explain the significant depression in antimicrobial activity from the beginning of the season. In fact, by the final point of collection in September 2005, many

of the colonies at both sites were either dead or heavily fouled with macroalgae and epibionts that commonly associate with the coral, including the seawhip shrimp (*Neopontonides beaufortensis*) (Patton 1972; Fox and Ruppert 1985; Ruppert and Fox 1988), skeleton shrimp (*Caprella equilibra*), barnacle (*Conopea galeata*), and a bryozoan (*Alcyonidium hauffi*) (Fox and Ruppert 1985; Ruppert and Fox 1988). Although the association of the common seawhip and its symbionts appears to be a stable one (Patton 1972; Fox and Ruppert 1985; Ruppert and Fox 1988), any substantial changes to the overall protective coenenchyme, chemical defense, and/or health of *L. virgulata* may offset the relationship between host and symbiont, favoring the considerable fouling that was observed with colonies at CP and PP.

Coral colonies at CP and PP were attached to docks at or within 2 m of the surface and thus should have been accustomed to rapid changes in current strength, sunlight exposure, and salinity of 5-7 ppt with the falling tides or during a rain event. However, the floating docks at PP where collections were made were cleaned thoroughly each morning with a freshwater hose (personal communication, Colin Bentley, Dockmaster, Patriots Point Marina). The large, daily input of freshwater may have lowered the salinity of the ambient seawater to such a degree that colonies of *L. virgulata*, some of which were located directly under the hoses and all of which were within 1.5 m of the surface, experienced transient hyposalinity stress. Goreau (1964) and Egaña and DiSalvo (1982) attributed localized mass coral bleaching events in Jamaica and off the coast of Easter Island to large inputs of freshwater during tropical storm events. Although the input of freshwater at PP is minimal in comparison to the freshwater input from a tropical storm, its impact on the surrounding coral community,

specifically *L. virgulata*, may have been similar. It is possible that in response to daily hyposalinic stress, the coenenchyme of the coral was sloughed off, exposing the gorgonin skeleton beneath, the immune response of *L. virgulata* weakened, and it became susceptible to fouling by microbes, macroalgae, and epibionts in the surrounding community. Over time, the substantial loss of surface area, increased fouling, and pulsed salinity stress could have resulted in the unhealthy appearance, apparent loss of antimicrobial activity against *V. harveyii*, and mortality of *L. virgulata* by July 2005. Alternatively, as was suggested for colonies of *L. virgulata* collected at KRB and HBJ, temporal variation in the concentration or specificity of antimicrobial compounds in *L. virgulata* may exist at PP, depending on the intensity of different environmental stimuli, such as temperature, ultraviolet radiation, competition, predation, sedimentation, nutrient input, or anthropogenic stress.

It is difficult to explain the unhealthy appearance, substantial fouling, and the apparent loss of antimicrobial activity against *V. harveyii* observed in colonies of *L. virgulata* at CP in July and September 2005. There is no source of significant salinity stress at the site, although transient summer rain storms between June and August can add large amounts of freshwater to the surface of Bohicket Creek, lowering the local salinity (30-35 ppt) by as much as 10 ppt. However, as was previously suggested, coral colonies at CP are located within 2 m of the surface and are likely accustomed to rapid changes in salinity with the falling tides or during rain events. In addition, no oxidative or thermal stress is apparent at the site, the water and sediment have been recorded to be high-quality, and sources of anthropogenic impact are few. Again, temporal or seasonal variation in the intensity or specificity of antimicrobial compounds in *L. virgulata* may

exist at CP, accounting for the coral's inability to inhibit growth of *V. harveyii* in July and September 2005.

60 Bass

Approximately 200 m of 60 Bass were surveyed completely in April 2005 to locate colonies of the common seawhip for use in this study. Within that distance, only five *L. virgulata* colonies were documented, the closest of which were located at a distance of 5 m from each other. Only two of the five colonies conformed to the highly-branched morphology typical to *L. virgulata* in estuarine tidal creeks of South Carolina (Blair 2003). The other three colonies appeared similar in morphology to coastal ocean seawhips, which have stiff, upright axial skeletons and possess few branches or branching levels (Blair 2003). Colonies of *L. virgulata* at 60 Bass were affixed to individual oyster shells in the sediment 2 m from the surface and were subjected to a strong, steady-flow of water in the creek, especially during the two flood tides each day. The drag force exerted by the bi-directional tidal currents in the creek appeared extreme, streamlining the coral colonies with the water flow until they contacted the sandy substrate. Thus, although the waters of 60 Bass have been recognized for their pristine quality and sources of anthropogenic impact are few (personal communication, Paul Kenny, Senior Research Resource Specialist, Belle W. Baruch Marine Field Lab), this high-flow area appears to support only a few colonies of *L. virgulata* and a sparse community of macroalgae in the summer. The lack of benthic organisms in the creek may be explained by an inability of marine larvae to attach to and settle on the soft, sandy bottom, due to the shortage of solid substrate and the harsh environment provided by the creek's current. Additionally, even

if benthic organisms could become established, without a firm attachment to the shallow creek bed, an organism could be easily damaged or uprooted by the current, as with colonies of *L. virgulata.*

The physical stresses of the upper creek environment may have been responsible for the significant depression in antimicrobial activity in *L. virgulata* and the apparent deterioration in the outward appearance (health) of the coral throughout the study season. Tested with *V. harveyii*, the average antimicrobial activity in samples of *L. virgulata* collected from 60 Bass differed significantly among the three collections in June, August, and October 2005. In addition, against *M. luteus*, antimicrobial activity in extracts of *L. virgulata* collected from the creek differed statistically between June and October 2005 at the level of $p < 0.1$. By October 2005, only one, six-branched colony remained intact among the five colonies observed and collected from the upper creek. Of the other four colonies, two colonies were completely dead. The other two were partially stripped of their coenenchyme and heavily fouled with macroalgae. This suggests a weakening of the coral's chemical defense system and response to fouling and may also account for the significant decrease in antimicrobial activity observed in the coral. It is possible that the strong current and high influx of sediment damaged the coral colonies, exposing the gorgonin skeleton and permitting fouling by epibionts and macroalgae in the surrounding community. Over time, the substantial loss of surface area and increased fouling may have resulted in the unhealthy appearance and apparent loss of *L. virgulata* antimicrobial activity against *V. harveyii* and *M. luteus* by October 2005.

Alternatively, as was previously suggested for a number of collection sites, temporal or seasonal variation in the intensity or specificity of antimicrobial compounds

in *L. virgulata* may exist at 60 Bass, accounting for the depression in seawhip antimicrobial activity against *V. harveyii* observed in August and October 2005. Additionally, statistical analyses of variability in mean antimicrobial activity in colonies at this site must be interpreted with some hesitancy due to the small sample size (n=3) compared throughout this study. Further evaluation of antimicrobial activity in *L. virgulata* from any of the creeks in North Inlet, including 60 Bass, would require a larger sample size of the coral.

It is interesting to note that during extreme low spring tides in February and March 2006, about 50 adult colonies of *L. virgulata* were identified by Brock Renkas (Research Technician, Belle W. Baruch Institute for Marine and Coastal Sciences) approximately 0.125 km downstream from the location in 60 Bass (33º19'38.12" N, 79º10'30.80"W) where this study was conducted in 2005. The colonies, most of which appeared to be quite healthy, were located in an embayment of 60 Bass, well-protected from the strong, steady flow typical to the creek. The attachment of corals to this location, instead of the center of the creek, would have removed them from the physical stresses of the flow regime and may have increased their health, fitness, and chances of survival, in comparison to colonies analyzed in this study. Color morphologies of *L. virgulata* within the embayment included yellow, orange, and white, and branching morphologies varied greatly. Colonies were grouped in clusters throughout the embayment (33º19'33.90-35.30" N, 79º10'30.10-31.70"W) and were either partially or fully exposed to the air during the extreme low spring tides. Aerial exposure of corals during extreme low tides, for as much as 3 hours at a time, and during both the night and daytime, has been reported on reefs in Malaysia, New Caledonia, Hawaii, Australia,

Madagascar, the Red Sea, and the Mediterranean (Hong and Sasekumar 1981; Daumas *et al*. 1982; Krupp 1984; Romaine *et al*. 1997; Wild *et al*. 2005).

Conclusions

The goal of the 2005 field study was to evaluate the presence of antimicrobial activity in extracts of the common seawhip, *Leptogorgia virgulata,* from South Carolina waters. Antimicrobial activity was identified in extracts of *L. virgulata* against *E. coli* BL21, *Bacillus* sp., and two reference bacteria, *V. harveyii* and *M. luteus*. Variability in antimicrobial activity was compared among three reference and four anthropogenically impacted sites throughout Charleston and Georgetown Counties, SC, between May and October 2005. Additionally, variability in seawhip antimicrobial activity at each of the seven sites was compared among three points of collection between May and October 2005. This report expands upon the existing body of literature analyzing the chemical defense system of *L. virgulata,* which has been shown to exhibit antifouling (Targett *et al*. 1983; Standing *et al*. 1984; Rittschof *et al*. 1985; Gerhart *et al*. 1988; Clare *et al*. 1999) and anti-predator (Gerhart 1991; Gerhart and Coll 1993) properties.

It was originally hypothesized that *L. virgulata* located at study sites with high levels of anthropogenic stressors, such as CCM, would produce the greatest concentration of antimicrobial compounds in response to the stress. The results revealed that the situation is far more complicated than predicted. There was no obvious relationship between the degree of anthropogenic stress at any of the seven sites and the production of antimicrobial compounds in *L. virgulata* located there. The level and specificity of seawhip antimicrobial activity, tested against two reference strains of bacteria in the

assay system, varied among coral colonies at each site and among all seven sites. Evidence did suggest that if a colony of *L. virgulata* was impacted so severely by osmotic stress (PP), high current flow (60 Bass), or a combination of anthropogenic stresses and sediment and nutrient loading (CCM) as to show overt signs of biological deterioration, it apparently lost or was greatly reduced in its ability to produce antimicrobial compounds. Additionally, as the health of the colony deteriorated and its gorgonin axis was exposed, surface area was created that permitted heavy fouling by macroalgae and epibionts in the surrounding environment. As an end result, many colonies eventually died. At CP, a site with no apparent sources of physical or anthropogenic stressors, many colonies still experienced a loss of coenenchyme and an observed depression in antimicrobial activity throughout the study season. This observation was attributed to temporal or seasonal variability in the intensity or specificity of antimicrobial compounds in *L. virgulata*, which could influence the overall health of the coral and subsequent macroalgal or epibiont fouling.

Though the relationship between health and the production of antimicrobial compounds in *L. virgulata* was not directly evaluated in this study, it is interesting to note that colonies at OCB, KRB, and HBJ maintained a healthy appearance throughout the six-month study period. In samples of *L. virgulata* from OCB, antimicrobial activity against *V. harveyii* and *M. luteus* did not differ significantly among the three collections from May to October 2005. In contrast, a significant increase in antimicrobial activity was observed in extracts of *L. virgulata* samples collected from KRB (against *V. harveyii*) and HBJ (against *V. harveyii* and *M. luteus*) in October 2005. This significant

increase may be explained by a trade-off between reproductive periodicity (April-October) and the production of antimicrobial metabolites in the seawhip.

It would appear that a variety of factors, including osmotic stress, high current flow, point and non-point sources of pollution, sediment and nutrient loading, temporal or seasonal variation, reproductive periodicity, and possibly even colony health influence the level and specificity of antimicrobial activity in *L. virgulata* along the coast of South Carolina. At each of the seven sites in this study, one or a combination of these factors may explain the variability in *L. virgulata* antimicrobial activity observed between May and October 2005.

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CHAPTER 3

Isolation and Structural Elucidation of Biologically Active Secondary Metabolites from *Leptogorgia virgulata*

INTRODUCTION

Host Defense in *Leptogorgia virgulata*

The common seawhip, *Leptogorgia virgulata,* is documented to possess a strong chemical defense system capable of inducing emesis in fishes, deterring fish predation (Gerhart 1991; Gerhart and Coll 1993), and preventing attachment, settlement, and fouling by common epibionts (Targett *et al*. 1983; Standing *et al*. 1984; Rittschof *et al*. 1985; Gerhart *et al*. 1988; Clare *et al*. 1999). Potent defensive metabolites previously isolated from *L. virgulata* include two novel diterpenoids, pukalide and 11ß, 12ß-epoxypukalide (Gerhart *et al*. 1988), and a polar, nitrogen-containing compound, homarine, which comprises 0.3% of the weight of *L. virgulata* (Targett *et al*. 1983).

Pukalide

Pukalide ($C_{21}H_{24}O_6$) is a furanocembranolide diterpene originally isolated from the octocoral *Sinularia abrupta* (Missakian *et al*. 1975). Pukalide and its derivatives are widespread among the Octocorallia (Faulkner 1984, 1986, 1987; Clare *et al*. 1999) and are recognized as some of the most highly oxygenated terpenoids known (Coll *et al*. 1989). At nontoxic concentrations, pukalide is a potent natural product and defensive metabolite, which can exhibit feeding deterrent, anti-attachment, anti-settlement, antifouling, anti-tumor, anti-inflammatory, narcotic, and cytotoxic properties (Coll *et al*. 1989; Gerhart and Coll 1993; Clare *et al*. 1999). Slattery *et al*. (1999) demonstrated the antimicrobial activities of pukalide, isolated from the soft coral *Sinularia polydactyla*, in disc spot assays using a sympatric *Vibrio* sp. Coll *et al*. (1989) suggested that pukalide

also may play a role in invertebrates in chemotaxis, chemical release for ovulation, or as a juvenile hormone. Figure 13 illustrates the complex structure of pukalide, including the 14-membered ring typical to furanocembranolides. Although the total synthesis of furanocembranolides was described by Paquette *et al*. (1992) and Rayner *et al*. (1992), the final steps elucidating the biochemical synthesis of pukalide have yet to be described in the literature. Wipf and Soth (2002), however, did elucidate the synthesis of the C_1-C_{18} fragment of pukalide in 11 steps.

Epoxypukalide

11ß, 12ß-epoxypukalide ($C_{21}H_{24}O_7$) is a furanocembranolide diterpene originally isolated from the gorgonian *Leptogorgia setacea* (Ksebati *et al*. 1984). Since it is a derivative of pukalide, the structure (Figure 13), its chemical shifts for ^{1}H and ^{13}C-NMR (Ksebati *et al*. 1984), and the roles 11ß, 12ß-epoxypukalide plays in the marine environment (Coll *et al*. 1989) are analogous to pukalide. The final steps of the biochemical synthesis of 11ß, 12ß-epoxypukalide also have not been described in the literature.

Homarine

Homarine ($C_7H_7NO_2$) (a.k.a N-methyl picolinic acid or N-methyl-2-carboxypyridine) (Targett *et al*. 1983) was first isolated and characterized from the American lobster, *Homarus americanus*, by Hoppe-Seyler (1933). It is a polar, nitrogen-containing compound of low molecular weight. Homarine is common in numerous marine invertebrates and freely diffuses into the water column. It has been reported to

function as a predator deterrent and antifoulant (Welsh and Prock 1958; Baker and Murphy 1976; Netherton and Gurin 1982; Targett *et al*. 1983; Davis *et al*. 1989; McClintock *et al*. 1994; Polychronopoulos *et al*. 2001). Slattery *et al*. (1997) revealed that ecologically relevant concentrations of homarine, isolated from the soft coral *Gersemia antarctica*, exhibit antimicrobial activities in response to the growth of sympatric *Alteromonas* sp., *Moraxella* sp., and *Psychrobacter* sp. Additionally, it has been suggested that homarine functions as a cellular osmotic pressure regulator, as it is present in numerous marine invertebrates but absent from freshwater invertebrates (Gasteiger *et al*. 1955, 1960). Netherton and Gurin (1982) presented evidence that homarine also may function as a transmethylating agent in shrimp. They suggested that homarine acts as a methyl group donor to appropriate receptors and serves as a significant methyl reservoir in crustacean tissues. Furthermore, homarine has been documented to act as a morphogen, which inhibits pattern formation and metamorphosis in the marine hydroid *Hydractinia echinata* (Berking 1986, 1987).

The structure of homarine is depicted in Figure 13. Netherton and Gurin (1982) described the biosynthetic pathway of homarine in shrimp muscle homogenates. They provided strong evidence that homarine is formed from glycine without the intermediate formation of picolinic acid. In addition, they demonstrated the reversible pathways permitting the methylation of picolinic acid to homarine and the demethylation of homarine to picolinic acid.

Isolating Antimicrobial Metabolites

Homarine, pukalide, and 11ß, 12ß-epoxypukalide exhibit antimicrobial activities in a number of marine organisms and are present as defensive metabolites in *L. virgulata.* Accordingly, one might suspect that one or a combination of these compounds may be responsible for the antimicrobial activity documented in the crude methanol/water extracts of *L. virgulata* (Chapter 2). Solid phase extractions, which separated crude aqueous methanol extracts into 1% TFA, methanol, and acetone fractions, proved unsuccessful at isolating compounds from *L. virgulata* that exhibited antimicrobial activity. Therefore, a different strategy was taken to separate and purify candidate compounds with antimicrobial activity in order to determine structural features.

Reverse-phase high performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (NMR) are universal, analytical chemical techniques, which have been used to isolate, purify, and elucidate the structure of numerous marine natural products (Shimizu 1985; Rinehart *et al*. 1990; Riguera 1997; Cunico *et al*. 1998; Bringmann and Lang 2003). In the present study, the use of C-8 and C-18 columns in reverse-phase HPLC and HPLC coupled to mass spectrometry (HPLC-MS) effectively retained the analytes of interest. Coupled to the appropriate assay system, a bioassay-guided fractionation scheme was developed to isolate and purify candidate antimicrobial metabolites from *L. virgulata.* Enough material was isolated using this methodology to characterize the unknown biologically active compounds using LC-MS and NMR.

The primary objective of this research was to develop reverse-phase HPLC-MS, reverse-phase HPLC, and NMR methods to isolate and unambiguously characterize

antimicrobial fractions from *L. virgulata.* MS and NMR spectra of *L. virgulata* fractions were compared to MS spectra of analytically pure samples of homarine, as well as NMR spectra of homarine, pukalide, and 11ß, 12ß-epoxypukalide to determine if these known metabolites were present in fractions of crude extracts with documented antimicrobial activity.

MATERIALS AND METHODS

Isolation and Structural Elucidation of Biologically Active Secondary Metabolites

Methanol Extraction

Samples of whole branches were collected from *Leptogorgia virgulata* representing two groups of four colonies, those exhibiting high levels of activity and those exhibiting no activity based on results from previous bacterial liquid growth inhibition assays (Chapter 2). Tissues were pulverized under liquid nitrogen in a 6850 cryogenic mill (SPEX CertiPrep, Inc.) and stored at -80°C until extraction. Depending upon the amount of available tissue, between 4.0 and 14.0 grams wet tissue weight of each coral was extracted in 1.25 volumes (w/v) of 100% methanol. Crude extracts were shaken for 24 hrs and centrifuged at 10,000 rpm (12,096 x g) at 4°C for 30 min using a Beckman-Coulter centrifuge (JA-25.50 fixed angle rotor). The supernatant was removed, dried under nitrogen in a Zymark Turbovap LV Evaporator®, and lyophilized to dryness (FTS Systems, Inc. Multi-Trap Lyophilizer) for 24 hrs.

High Performance Liquid Chromatography–Mass Spectrometry of Crude Extracts

For initial separation of crude *L. virgulata* extracts, dried residues were dissolved in 500 µL methanol and sonicated to enhance solubility. Injections of 5 µL of each extract were separated by reverse-phase high performance liquid chromatography and characterized via mass spectrometry (HPLC-MS) on an Agilent 1100 Series HPLC fitted with a photodiode array detector (PDA) and mass spectrometer. Samples were run at 35°C on a Gemini C_{18} (100 x 2.00 mm, 5 µM) 110A column (Phenomenex 00D-4435-

B0). The gradient mobile phase included solvent A: 99.9% HPLC grade water + 0.1% acetic acid and solvent B: 99.9% HPLC grade methanol + 0.1% acetic acid. A flow rate of 0.4 mL/min using the initial conditions of 95A:5B was held for 2 min, increased to 95% B over 13 min, held for 5 min, decreased to 5% B over 0.1 min, and held at 5% B for 4.9 min. Fractionation was monitored at 254 nm. Electrospray ionization-mass spectrometry (ESI-MS) detected diagnostic masses corresponding to homarine at m/z = 94.3 and 138.3 amu ($C_7H_7NO_2$, M^+, 94.1, 138.2, Polychronopoulos *et al*. 2001), pukalide at m/z = 372.6 amu ($C_{21}H_{24}O_6$, M^+, 372.1564, Missakian *et al*. 1975), and 11ß, 12ß-epoxypukalide at m/z = 389.9 amu ($C_{21}H_{24}O_7$, M^+, 388.15221, Ksebati *et al*. 1984). The masses analogous to homarine in the crude *L. virgulata* extracts were identical to mass peaks reported for analytically pure homarine using fast atom bombardment mass spectrometry (FAB-MS) (Polychronopoulos *et al*. 2001) (Figure 14).

High Performance Liquid Chromatography

In an effort to semi-purify active compounds from *L. virgulata*, HPLC-MS samples with documented antimicrobial activity from previous bioassays (Chapter 2) were combined and dried under nitrogen in an LV Evaporator®. The pooled sample was then lyophilized for 24 hrs, resuspended in 2.5 mL of 10% methanol/sterile water (v/v), and sonicated to enhance solubility. Eighteen injections of 65 µL of the pooled extracts were partitioned by reverse-phase HPLC on an Agilent 1100 Series HPLC fitted with a PDA. The LC was operated at 28°C using two, sequential Inertsil® C_8 (150 x 4.6 mm, 5 µM) columns (Alltech 89874). The gradient mobile phase used was solvent A: water, solvent B: 100% MeOH, and solvent C: 100% acetonitrile (all HPLC grade solvents). A

flow rate of 0.5 mL/min under the initial conditions of 100A:0B:0C was held for 5 min, increased to 100% B over 40 min, held for 5 min, adjusted to 100% C, and held for 10 min. One-minute fractions (50 fractions in total) were collected at 0.5 mL/min with a subsequent 10 min wash. Fractionation was monitored at 238, 248, and 272 nm. These wavelengths were chosen because they represent chromophore signatures for homarine (Polychronopoulos *et al*. 2001), pukalide (Missakian *et al*. 1975), and 11ß, 12ß-epoxypukalide (Ksebati *et al*. 1984), respectively. Figure 15 illustrates the three HPLC chromatograms of pooled active crude *L. virgulata* extracts.

HPLC-MS of Fractionated Samples

Strong UV chromophore signals correlating to activity were isolated during the separation of the pooled crude *L. virgulata* extracts. These signals were monitored at 238, 248, and 272 nm and were recorded on the HPLC chromatograms between minutes 10 and 49 (Figure 15). Consequently, individual fractions 11-50 were dried under nitrogen in an LV Evaporator®, lyophilized to dryness for 24 hrs, dissolved in 100 µL methanol/sterile water (50:50, v/v), and sonicated to increase solubility. Injections of 5 µL of each fraction were partitioned and characterized according to atomic mass by reverse-phase HPLC-MS on an Agilent 1100 Series HPLC fitted with a PDA and mass spectrometer. Samples were run at 35°C on a Gemini C_{18} (100 x 2.00 mm, 5 µM) 110A column (Phenomenex 00D-4435-B0). The gradient mobile phase used was solvent A: 99.9% HPLC grade water + 0.1% acetic acid and solvent B: 99.9% HPLC grade methanol + 0.1% acetic acid. A flow rate of 0.4 mL/min under the initial conditions of 95A:5B was held for 2 min, increased to 95% B over 13 min, held for 5 min, decreased to

5% B over 0.1 min, and held at 5% B for 4.9 min. Extract fractionation again was monitored at 238, 248, and 272 nm. ESI-MS detected masses corresponding to homarine at m/z = 94, 138, and 160 amu (Polychronopoulos *et al*. 2001), which were identical to mass peaks identified by FAB-MS analysis of pure homarine (Polychronopoulos *et al*. 2001) (Figure 14).

Nuclear Magnetic Resonance Spectroscopy

Fractions exhibiting diagnostic mass signatures corresponding directly to homarine at m/z = 94, 138 amu (fractions 14-21), m/z = 94, 160 (22-24), m/z = 94, 138, 160 (25, 27-28), m/z = 138 (30, 33, 37, 39-41), and m/z = 94 (36, 38, 42) were combined. These pooled fractions were dried under nitrogen in an LV Evaporator® for 24 hrs, lyophilized for 48 hrs to remove all traces of solvent, and then dissolved in 0.5 mL d_4-methanol (CD_3OD). Determinations of ^{1}H- and ^{13}C-NMR spectra were made at 28ºC. Spectra were measured on a Bruker Digital DMX 500 MHz NMR spectrometer (^{1}H and ^{13}C). Chemical shifts (in ppm) of pooled HPLC fractions of biologically active extracts of *L. virgulata* were compared to reference ^{1}H- (Figure 16, Table 3) and ^{13}C-NMR spectra (Figure 17, Table 3) of pure homarine (courtesy of Prokopios Magiatis, Department of Pharmacy, University of Athens, Greece, unpublished data) and ^{1}H-NMR spectra of pure pukalide (Figure 18) and 11ß, 12ß-epoxypukalide (Figure 19) (courtesy of Paul Keifer, Eppley Institute for Research in Cancer, University of Nebraska Medical Center, unpublished data).

Antimicrobial Assay of Homarine-Containing Fractions of L. virgulata

Verification of antimicrobial activity in fractions 14-21, which exhibited ^{1}H-NMR and ^{13}C-NMR fingerprints for homarine, was accomplished using the liquid growth inhibition assay, as previously described. Prior to analysis, pooled fractions 14-21 were dried under nitrogen in an LV Evaporator® for 6 hrs, lyophilized to dryness for 48 hrs, and then dissolved in 200 µL methanol/sterile water (50:50, v/v). Assays were performed with two reference bacteria, the Gram-negative *Vibrio harveyii* (culture collection of Cheryl Woodley, NOAA) and the Gram-positive *Micrococcus luteus* (ATCC 4698). Bacteria were cultured in LB broth at 31°C for 24 hrs, at which time 1 mL of bacteria was inoculated with 4 mL poor-broth nutrient medium and grown for an additional 5 hrs at 31°C. Cultures were then diluted with poor-broth to an initial OD_{600} of 0.001 (Destoumieux *et al*. 1999; Lamberty *et al*. 1999). In sterile 96-well microtiter plates (Fisher Scientific, New Jersey), 90 µL of a suspension of logarithmic phase bacteria (OD_{600} of 0.001) were added to 10 µL aliquots of pooled fractions 14-21 (Bulet *et al*. 1993; Destoumieux *et al*. 1999; Lamberty *et al*. 1999). Blank wells contained 100 µL of sterile water or poor-broth. Control wells contained 90 µL of bacteria and 10 µL of methanol or 100 µM Ala$^{[8,13,18]}$ magainin-II-amide (Sigma M8155) (Mystkowska *et al*. 2001). All treatments were performed in triplicate. Microbial growth was measured by an increase in optical density (OD) after incubation at 31ºC for 24 hrs. Absorbance was read at 600 nm using a microtiter plate reader (Spectramax Plus® MN03749) (Bulet *et al*. 1993; Destoumieux *et al*. 1999; Lamberty *et al*. 1999). The mean absorbance (OD) of three replicates of the *L. virgulata* sample (pooled fractions 14-21) was compared to controls and used as a measure of antimicrobial activity in the coral fractions.

A summary of the methodology used to isolate, partially purify, characterize, and identify potential antimicrobial activities of secondary metabolites in *L. virgulata* is detailed in Table 4. These methods include extractions in aqueous methanol, LC-MS, HPLC, NMR, and bacterial growth inhibition assays.

RESULTS AND DISCUSSION

Isolation and Structural Elucidation of Biologically Active Secondary Metabolites

High Performance Liquid Chromatography–Mass Spectrometry of Crude Extracts

Mass spectral analysis of eight crude aqueous methanol extracts of *L. virgulata,* four of which demonstrated consistently no antimicrobial activity and four of which exhibited high levels of activity between May and October 2005 was performed. The data revealed MS peaks corresponding to the molecular weights of homarine at m/z = 94.3, 138.3 amu ($C_7H_7NO_2$, M^+, 94.1, 138.2, Polychronopoulos *et al*. 2001), pukalide at m/z = 372.6 ($C_{21}H_{24}O_6$, M^+, 372.1564, Missakian *et al*. 1975), and 11ß, 12ß-epoxypukalide at m/z = 389.9 ($C_{21}H_{24}O_7$, M^+, 388.15221, Ksebati *et al*. 1984). These MS peaks gave retention times of 0.9-3.5 minutes for homarine, 18.5-19.0 minutes for pukalide, and 19.0-19.5 minutes for 11ß, 12ß-epoxypukalide (Figures 20-22). Of note is a peak on each UV chromatogram (254 nm) associated with the MS peaks of interest, suggesting the presence of a chromophore associated with pukalide, 11ß, 12ß-epoxypukalide, and homarine in the uncharacterized seawhip compounds (personal communication, Peter Moeller, NOAA). However, identical molecular weights to the signals of pukalide and 11ß, 12ß-epoxypukalide were undetectable when the UV chromatogram and MS spectra of the eight uncharacterized crude extracts were time-averaged in five-minute increments (Figure 23). This suggested that the concentrations of pukalide and 11ß, 12ß-epoxypukalide in the crude extracts were minute in concentration compared to homarine and other uncharacterized compounds resolved by the HPLC matrix. Peaks corresponding to the molecular weights of homarine were

present still after time-averaging in both the four active and four non-active crude extracts of *L. virgulata* (Figure 24). It is interesting to note that the relative intensity of MS spectral peaks associated with homarine was up to 10-fold greater in magnitude in all biologically active extracts as compared to non-active extracts (Figure 24). This suggested that homarine and/or the cofactors which may act with homarine to produce an antimicrobial response in *L. virgulata* were present in a greater quantity in the four crude extracts exhibiting antimicrobial activity than in the four extracts displaying no activity as assayed.

High Performance Liquid Chromatography

Crude aqueous methanol extracts exhibiting antimicrobial activity and analyzed by HPLC-MS were separated into 50 sequential fractions by reverse-phase HPLC. HPLC fractionation was monitored at 238, 248, and 272 nm, wavelengths representing the chromophore signatures for homarine (Polychronopoulos *et al*. 2001), pukalide (Missakian *et al*. 1975), and 11ß, 12ß-epoxypukalide (Ksebati *et al*. 1984), respectively. Figure 15 depicts the HPLC chromatograms of the pooled active crude extracts of *L. virgulata.* Strong chromophoric signals correlating to activity were isolated during separation of the crude extracts. These signals were recorded on the three HPLC chromatograms between minutes 10 and 49 (Figure 15). Accordingly, individual fractions 11-50 were subjected to additional separation and characterization by HPLC-MS.

HPLC-MS of L. virgulata Fractions

ESI-MS, monitored at 238, 248, and 272 nm (the chromophoric signatures for homarine, pukalide, and 11ß, 12ß-epoxypukalide, respectively), detected masses in fractions of active *L. virgulata* extracts corresponding to homarine at m/z = 94, 138, and 160 amu. These masses were identical to mass peaks reported for analytically pure homarine using FAB-MS (Polychronopoulos *et al*. 2001) (Figure 14). The intensity of MS spectral peaks associated with mass signatures of homarine was greatest in fractions 14-21 [m/z 94, 138]; examples (fractions 17-19) are depicted in Figures 25-27. Those fractions exhibiting identical diagnostic mass signatures to homarine were combined, dried, and prepared for NMR analysis. Pooled fractions included 14-21 [m/z 94, 138]; 22-24 [m/z 94, 160]; 25, 27, and 28 [m/z 94, 138, 160]; 30, 33, 37, 39-41 [m/z 138]; and fractions 36, 38, and 42 [m/z 94].

Nuclear Magnetic Resonance Spectroscopy

Chemical shifts (in ppm, d_4-methanol) of the pooled fractions of active extracts of *L. virgulata* were referenced to ^{1}H- (Figure 16, Table 3) and ^{13}C-NMR spectra (Figure 17, Table 3) of analytically pure homarine and ^{1}H-NMR spectra of authentic samples of pukalide (Figure 18) and 11ß, 12ß-epoxypukalide (Figure 19). Of the five sets of pooled fractions, spectra consistent with homarine were observed in fractions 14-21 and, although the signal was weaker, in fractions 22-24. Diagnostic chemical shifts representative of homarine were not identified in the ^{1}H-NMR spectra of fractions 25-50. Although signals corresponding to homarine were identified previously in the LC-MS spectra of individual fractions 25, 27, 28, 30, 33, and 36-42, it is unlikely that homarine

was present in those fractions due to the polar nature and low affinity of the metabolite for the C-18 column in HPLC. In addition, ^{1}H-NMR signals representative of pukalide and/or 11ß, 12ß-epoxypukalide were not isolated from any of the pooled fraction sets. This evidence suggests either that pukalide and 11ß, 12ß-epoxypukalide were present in the pooled *L. virgulata* fractions in concentrations too low for ^{1}H-NMR detection or that they were not present at all, even though mass signatures similar to those reported for the two diterpenes were detected by LC-MS of the crude extracts.

Signals in the ^{1}H- (Figures 28-29) and ^{13}C-NMR (Figures 30-31) spectra of fractions 14-21 were identical to the chemical shifts of analytically pure homarine (Figures 16-17, Table 3) and a homarine analog. A tight singlet at 4.43, triplet at 7.96, doublet at 8.10, triplet at 8.54, and doublet at 8.79 ppm in the ^{1}H-NMR spectrum of the uncharacterized fractions (Figures 28-29), coupled with ^{13}C-NMR resonances at 46.8, 126.14, 145.05, 145.60, 154.2, and 163.95 ppm (Figures 30-31) indicated the presence of homarine in fractions 14-21. In addition, the singlet at 4.43 (Figure 28), when coupled with a triplet at 8.17, doublet at 8.28, triplet at 8.70, and doublet at 8.84 ppm in the ^{1}H-NMR spectrum indicated the presence of a homarine analog in fractions 14-21 (Figure 29). Additional signals in the ^{1}H-NMR and ^{13}C-NMR spectra of fractions 14-21 can be explained by the semi-purified nature of the mixture and the presence of congeners.

Chemical shifts (in ppm, d_4-methanol) in the ^{1}H-NMR spectrum of combined fractions 22-24, although less intense than the spectra of fractions 14-21, also were identical to analytically pure homarine (Figures 32-33). Signals included a singlet at 4.44, triplet at 7.94, doublet at 8.09, triplet at 8.53, and doublet at 8.76. This evidence again revealed the presence of homarine in fractions 22-24. Additional signals in the ^{1}H-

NMR spectrum of fractions 22-24 can be explained by the semi-purified nature of the mixture and the presence of congeners. In addition, due to a lack of available sample size, we could not obtain clean ^{13}C-NMR analyses nor verify the antimicrobial properties of fractions 22-24 in subsequent assays.

Antimicrobial Assay of Homarine-Containing Fractions

When tested with two reference bacteria, *V. harveyii* and *M. luteus*, the liquid growth inhibition assay successfully identified antimicrobial activity in replicates of combined fractions 14-21. This evidence demonstrated that partially purified fractions 14-21 contained the compound(s) responsible for the antimicrobial activity in *L. virgulata.*

Conclusions

It was the primary objective of this study to develop reverse-phase HPLC-MS, reverse-phase HPLC, and NMR methods to isolate and unambiguously characterize antimicrobial fractions from *L. virgulata.* Corroborative LC-MS, ^{1}H-, and ^{13}C-NMR evidence demonstrated the molecular weights and chemical shifts of homarine and a homarine analog in fractions 14-21 and, to a lesser degree, fractions 22-24 from *L. virgulata* extracts with documented antimicrobial activity. Homarine is a recognized antifouling metabolite in *L. virgulata.* In subsequent liquid growth inhibition assays, fractions 14-21 inhibited the growth of *V. harveyii* and *M. luteus*, indicating that these fractions contained the compound(s) responsible for the antimicrobial activity in *L. virgulata.* Homarine and/or a homarine analog could not be directly confirmed as the

agent(s) responsible for the antimicrobial activity in *L. virgulata.* However, evidence in this study suggests that homarine is, in part, an active component of the innate immune system of the common seawhip. Further work into the antimicrobial activity and innate immunity of *L. virgulata* is necessary to determine if homarine acts alone or synergistically with cofactors and/or congeners, such as pukalide, 11ß, 12ß-epoxypukalide, or a suite of yet-uncharacterized compounds, to mount a rapid response to microbial invasion and disease in the common seawhip.

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SUMMARY

Extraction and assay protocols were developed to purify and characterize antimicrobial compounds from the soft coral *Leptogorgia virgulata* (common seawhip), collected from South Carolina waters. Antimicrobial activity was identified in crude methanol/sterile water extracts of *L. virgulata* using a liquid growth inhibition assay system with four general strains of bacteria: *Escherichia coli* BL21, *Vibrio harveyii*, *Micrococcus luteus*, and a *Bacillus* sp. isolate. This represents the first report of antimicrobial activity in *L. virgulata* and, of equal note, in a temperate/sub-tropical coral in the Atlantic Ocean.

Variability in the antimicrobial activity in *L. virgulata* was compared among three reference and four anthropogenically impacted sites in South Carolina between May and October 2005. The Gram-negative *V. harveyii* and Gram-positive *M. luteus* were selected as reference bacteria for use in all discussions of statistical analyses. No obvious relationship existed between the degree of anthropogenic stress at any of the seven sites and the production of antimicrobial compounds by *L. virgulata.* Of the seven field sites, only *L. virgulata* collected at the Charleston City Marina did not exhibit antimicrobial activity against selected bacteria in the liquid growth inhibition assay. The apparent lack of antimicrobial activity in and deterioration of health of *L. virgulata* at the City Marina may be explained by the poor water quality, the large number of point and non-point sources of pollution, and the high sediment and nutrient input at the site. Average inhibition of *M. luteus* by samples collected at the City Marina was significantly lower than the mean antimicrobial activity in *L. virgulata* samples at the other six study sites.

In addition, although mean antimicrobial activity against *V. harveyii* in *L. virgulata* colonies collected from Kiawah River and Oak Creek Bridges was not statistically different ($p < 0.1$), when compared to the average antimicrobial activity against the bacterium at the other five study sites, a significant difference was observed ($p = 0.05$). It was suggested that colonies of *L. virgulata* collected at Kiawah River and Oak Creek Bridges may exhibit a broad spectrum of antimicrobial activity or may have produced activity specific to the selected bacteria in this study in comparison to seawhip colonies at the other sites.

Variability in the antimicrobial activity in *L. virgulata* also was compared at each site among three points of collection during the six-month study period. With *M. luteus*, the average antimicrobial activity in *L. virgulata* collected at Kiawah River Bridge was significantly greater in October than in August 2005. Samples of *L. virgulata* collected from Huntington Beach State Park–South Jetty also exhibited a significant increase in mean antimicrobial activity against *V. harveyii* and *M. luteus* in October 2005, as compared to samples collected in June and August 2005. The apparent increase, in October, of antimicrobial activity in seawhip colonies at these two sites may be attributed to a trade-off between reproductive periodicity (April-October in *L. virgulata*) and the production of antimicrobial metabolites in the coral.

Mean inhibition of *V. harveyii* by *L. virgulata* colonies collected at Cherry Point Seafood Company and Patriots Point Marina in May 2005 was significantly greater than the average activity of samples collected in July and September 2005. The unhealthy appearance, substantial fouling, and apparent loss of antimicrobial activity in colonies at these two sites by July 2005 may be explained by temporal or seasonal variation in the

production or specificity of antimicrobial compounds in the coral. Seawhip colonies at Patriots Point also may have experienced osmotic stress and subsequent deterioration in health and antimicrobial metabolite production due to the large, daily input of freshwater used to clean the docks.

The average antimicrobial activity against *V. harveyii* in *L. virgulata* colonies obtained from upper 60 Bass Creek–North Inlet differed significantly among the three collections in June, August, and October 2005. The physical stresses created by the high-flow, bi-directional tidal currents of the creek may have influenced the significant depression in antimicrobial activity observed in *L. virgulata*, the apparent decline in coral health, subsequent fouling by macroalgae, and death of certain colonies by October 2005.

It would appear that a variety of factors, including osmotic stress, high current flow, point and non-point sources of pollution, sediment and nutrient loading, temporal or seasonal variation, reproductive periodicity, and possibly even colony health influence the level and specificity of antimicrobial activity in *L. virgulata* along the coast of South Carolina. At each of the seven sites in this study, one or a combination of these factors could explain the variability in *L. virgulata* antimicrobial activity observed during the 2005 study season.

Reverse-phase HPLC, LC-MS, ^{1}H-NMR, and ^{13}C-NMR, coupled to the bacterial assay system, permitted the development of a bioassay-guided fractionation scheme to isolate, purify, and characterize metabolites from antimicrobial fractions of *L. virgulata.* LC-MS, ^{1}H-NMR, and ^{13}C-NMR spectral analyses of fractions 14-21 and, to a lesser degree, fractions 22-24 of active *L. virgulata* extracts revealed strong signals corresponding to the molecular weights and chemical shifts of homarine and a homarine

analog. These compounds are well-known antifouling metabolites in the common seawhip. In subsequent liquid growth inhibition assays, replicates of fractions 14-21 inhibited the growth of *V. harveyii* and *M. luteus*, indicating that partial purification of the crude extract yielded fractions containing the compound(s) responsible for the antimicrobial activity in *L. virgulata.* It was not determined whether homarine and/or a homarine analog is exclusively responsible for the antimicrobial activity in *L. virgulata,* although the antimicrobial properties of homarine have been reported previously in another species of soft coral *Gersemia antarctica*.

The findings of this study suggest that homarine is, in part, an active constituent of the innate immune system in *L. virgulata.* Further investigation into the antimicrobial activity and innate immunity of *L. virgulata* would require attaining pure samples of homarine, homarine analog(s), pukalide, and 11ß, 12ß-epoxypukalide to use as standards in the liquid growth inhibition assay system. A standardized range of concentrations for each pure compound would be determined and tested individually and in conjunction with the other metabolites. This would help validate whether homarine, homarine analog(s), pukalide, and/or 11ß, 12ß-epoxypukalide have a similar ability to inhibit the growth of *E. coli* BL21, *V. harveyii*, *M. luteus*, and *Bacillus* sp. In addition, these studies would help confirm whether homarine acts alone or synergistically with cofactors and/or other defensive metabolites in the seawhip to mount a rapid response to stressors in the marine environment.

Table 1. **Water quality standards for all streams, rivers, and water bodies in Charleston County, South Carolina** (adapted from U.S. Department of Commerce NOAA 1984; South Carolina Department of Health and Environmental Control 2004).

ITEM	CLASS SA	CLASS SB	CLASS SC
Garbage, cinders, ash, sludge, other wastes	None Allowed	None Allowed	None Allowed
Treated wastes, toxic wastes, deleterious substances, colored, or other wastes except as in above	None alone or in combination with other substances or wastes in sufficient amounts: to adversely affect the taste, color, odor or sanitary condition of clams, mussels, or oysters for human consumption; or to impair the waters for any other best usage	None alone or in combination with other substances or wastes in sufficient amounts: to make the waters unsafe or unsuitable for primary contact recreation; or to impair the waters for any other best usage	None alone or in combination with other substances/wastes in sufficient amounts: to be harmful to the survival of marine fauna or flora or the culture or propagation thereof; to adversely affect taste, color, odor, or sanitary condition of fish for human consumption; to make the waters unsafe or unsuitable for secondary contact recreation; to impair the waters for any other best usage
Dissolved oxygen	Daily average not less than 5.0 mg/L, with a low of 4.0 mg/L	Not less than 4.0 mg/L	Not less than 4.0 mg/L
Fecal coliform	Not to exceed a geometric mean of 200/100 mL, based on 5 consecutive samples during any 30 day period; nor shall more than 10% of the total samples examined during any 30 day period exceed 400/100 mL	Not to exceed a geometric mean of 200/100 mL, based on 5 consecutive samples during any 30 day period; nor shall more than 10% of the total samples examined during any 30 day period exceed 400/100 mL	Not to exceed a geometric mean of 1000/100 mL during any 30 day period; nor exceed 2000/100 mL in more than 20% of the samples examined during such period
Enterococci	Not to exceed a geometric mean of 35/100 mL based on at least 4 samples collected from a given sampling site over a 30 day period; nor shall samples exceed a single sample maximum of 104/100 mL	Not to exceed a geometric mean of 35/100 mL based on at least 4 samples collected from a given sampling site over a 30 day period; nor shall samples exceed a single sample maximum of 501/100 mL	Not Examined
pH	Range 6.5-8.5, but not vary more than 0.3 pH units	Range 6.5-8.5, but not vary more than 0.5 pH units	Range 6.5-8.5, but not vary more than 1 pH unit
Temperature	Refer to Appendix C.7 of →	U.S. Department of Commerce →	NOAA (1984) →
Turbidity	Not to exceed 25 NTUs provided existing uses are maintained	Not to exceed 25 NTUs provided existing uses are maintained	Not Examined

Table 2. **Average antimicrobial activity in *L. virgulata* samples collected between May and October 2005** (as measured by bacterial growth inhibition, determined by optical density change).

	Bacteria			
Site Comparison	***E. coli* BL21**	***V. harveyii***	***M. luteus***	***Bacillus* sp.**
Cherry Point Seafood Company	0.3256 (0.0185)[e]	0.2433 (0.0377)	0.0940 (0.0187)[e]	0.3424 (0.0132)
Kiawah River Bridge	0.1615 (0.0271)*[e]	0.0763 (0.0159)*[d e]	0.0672 (0.0219)[e]	0.1562 (0.0348)[e]
Upper 60 Bass Creek	0.2663 (0.0414)	0.2534 (0.0528)	0.0974 (0.0256)[e]	0.3362 (0.0300)
Patriots Point Marina	0.3501 (0.0227)[e]	0.2589 (0.0388)	0.1702 (0.0411)[e]	0.3345 (0.0209)
Huntington Beach State Park–South Jetty	0.2387 (0.0273)	0.2629 (0.0383)	0.1774 (0.0353)[e]	0.2712 (0.0348)
Oak Creek Bridge	0.2439 (0.0310)	0.1346 (0.0298)*[d e]	0.0665 (0.0104)[e]	0.2299 (0.0342)[e]
Charleston City Marina	0.3511 (0.0189)[e]	0.3534 (0.0156)[e]	0.3447 (0.0311)*	0.3305 (0.0093)
Positive Control[a]	0.0370 (0.0000)	0.0360 (0.0000)	0.0360 (0.0000)	0.0370 (0.0000)
Negative Control[b]	0.2790 (0.0023)	0.3020 (0.0302)	0.3200 (0.0049)	0.3380 (0.0127)
Blank[c]	0.0350 (0.0000)	0.0350 (0.0000)	0.0360 (0.0000)	0.0350 (0.0000)

Values are mean (± SEM) absorbances (optical densities) read at 600 nm after 24 hours of incubation at 31ºC.
[a] The positive control consisted of 90 µL of bacteria and 10 µL of 100 µM Ala[8,13,18] magainin-II-amide.
[b] The negative control consisted of 90 µL of bacteria and 10 µL of methanol.
[c] Blank wells contained 100 µL of poor broth (OD_{600} = 0.001).
* Mean antimicrobial activity (inferred by OD) was significantly different from all of the other study sites against the specific strain of bacteria.
[d] Average antimicrobial activity at Kiawah River Bridge and Oak Creek Bridge differed statistically at the level of $p < 0.1$.
[e] Mean antimicrobial activity was significantly different from the negative control.

Table 3. **^{1}H-NMR** (CD_3OD/TMS, 400 MHz, d ppm, *J* in Hz) **and ^{13}C-NMR** (CD_3OD/TMS, 50 MHz, d ppm) **of pure homarine** (adapted from Polychronopoulos *et al*. 2001, reproduced with permission from Prokopios Magiatis, Department of Pharmacy, University of Athens, Greece).

Position	*^{1}H-NMR*	*^{13}C-NMR*
2	---	155.2
3	8.07, d, 8	127.6
4	8.52, t, 8	147.0
5	7.93, dd, 8, 6.5	127.6
6	8.76, d, 6.5	146.6
N-CH_3	4.42, s	47.5
CO	---	165.2

Table 4. Summary flowchart depicting methodology of Chapter 3.

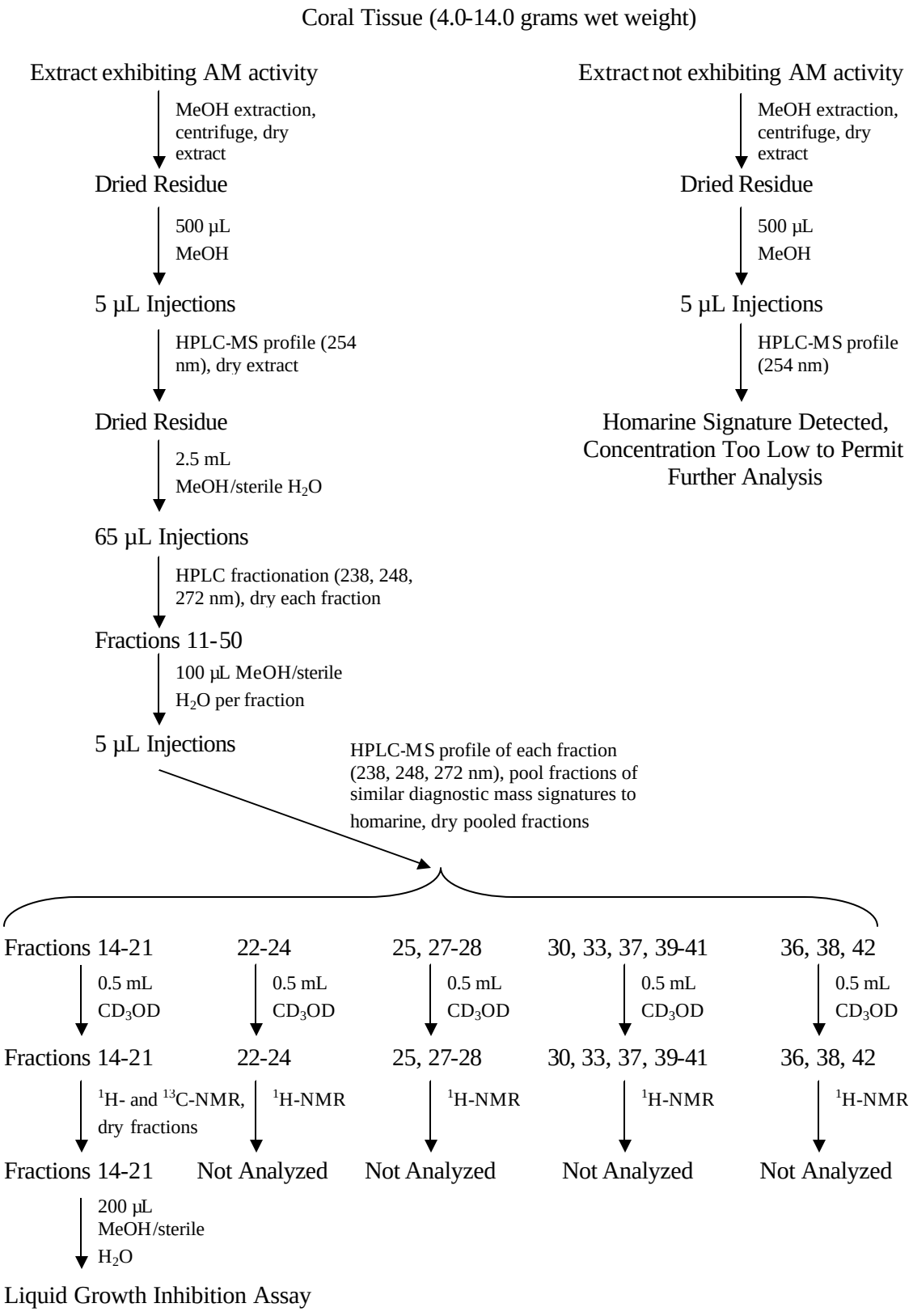
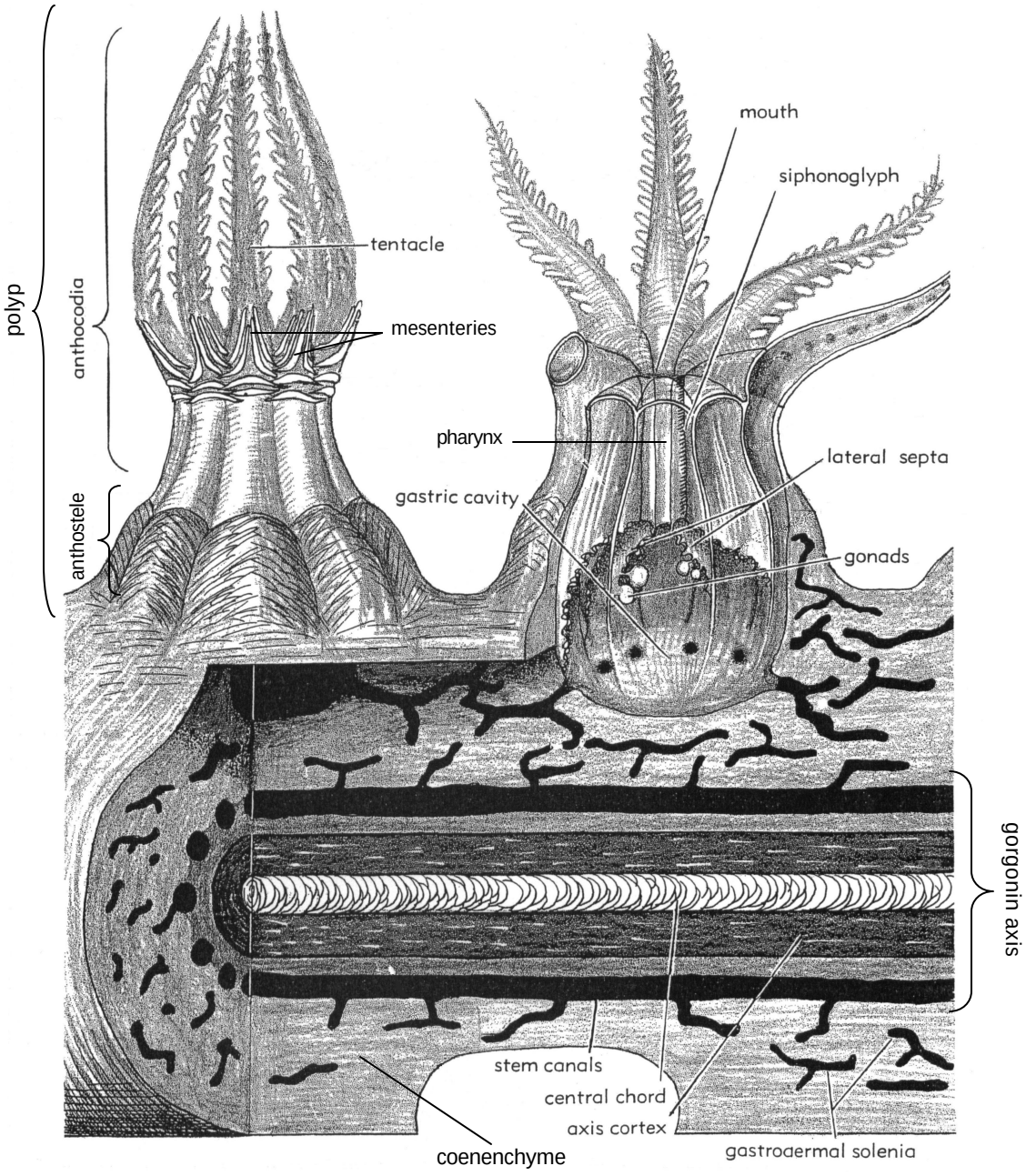


Figure 1. **General diagram of gorgonian anatomy** (after and with permission from Bayer *et al*. 1956). Illustrated is a cross-section of a colony branch, including the gorgonin axis, fleshy coenenchyme, and regularly spaced polyps arising from the coenenchyme. Each polyp is composed of two regions: the anthocodium and the anthostele. Comprising the anthocodium are eight pinnately-branched tentacles, eight mesenteries, and a mouth. The mouth connects directly to the pharynx, which opens into the gastrovascular cavity, the site of food transport and digestion.


polyp
anthocodia
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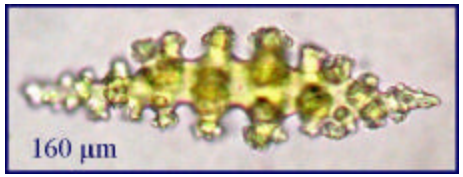
mouth
siphonoglyph
pharynx
lateral septa
gastric cavity
gonads
gorgonin axis
stem canals
central chord
axis cortex
gastrodermal solenia
coenenchyme

Figure 2. ***Leptogorgia virgulata* colonies and spicules**. The top row of images depicts examples of the different color morphologies of the common seawhip, including yellow, near white, orange, and purple, in South Carolina waters (photos courtesy of Anne Blair, NOAA). The production of color in *L. virgulata* in the disk-spindle-shaped calcite sclerites is controlled by a single gene with four alleles. Examples of sclerites are displayed below their associated color morphs, yellow and purple (photos courtesy of Anne Blair, NOAA).


160 µm

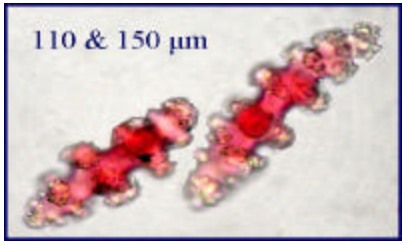
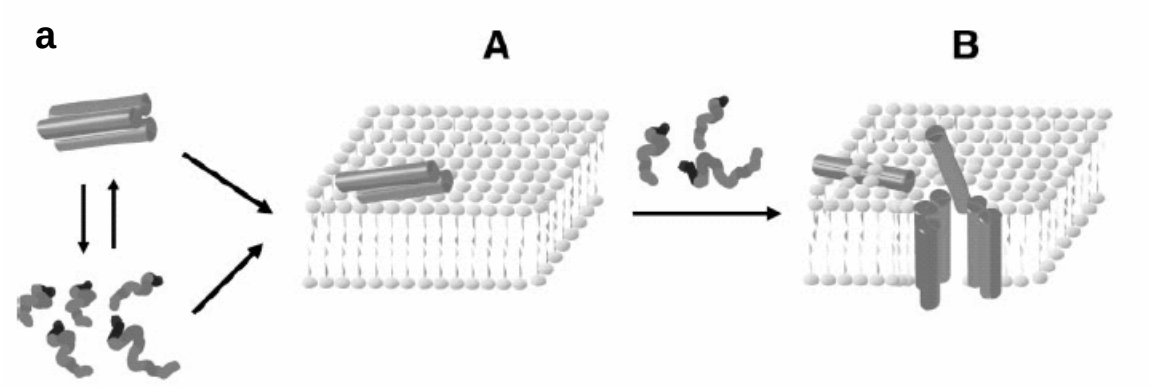

110 & 150 µm

Figure 3. **Barrel-Stave and Carpet models of membrane disruption**. There are two proposed models elucidating non-receptor-mediated microbial membrane disruption following a conformational change on the part of an antimicrobial peptide: the barrel-stave and carpet models. The barrel-stave model (a) suggests that antimicrobial peptides of a certain size and structure self-associate on the microbial membrane surface (A). These peptides then, via hydrophobic interactions, penetrate the lipidic core of the membrane and form bundles of transmembrane pores (B), resulting in cell lysis. Alternatively, the carpet model (b) suggests that antimicrobial peptides actually bind to the microbial membrane surface and reorient themselves with their hydrophobic regions toward lipids and their hydrophilic regions toward phospholipid groups (A). Via subsequent disruption of bilayer curvature by the peptides (B), the membrane is disintegrated (C).

Figure 3 (a, b) was reproduced, with permission from Yechiel Shai and the Taylor and Francis Group [http://www.tandf.co.uk], from Shai 2002.


a
A
B

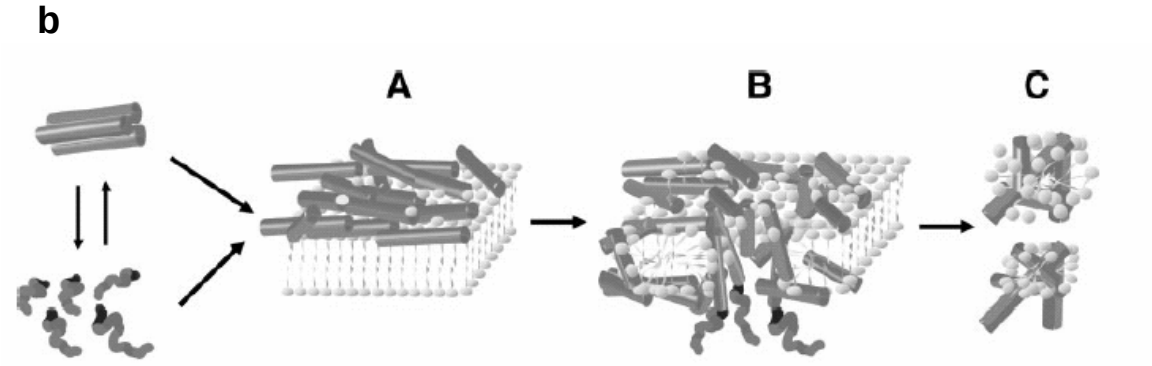

b
A
B
C

Figure 4. **Research field sites in South Carolina**. Locations of study sites along the eastern coast of SC are marked with a red dot. In Charleston County, these locations include Cherry Point Seafood Company (32º35'57.56"N, 80º10'37.19"W), Kiawah River Bridge (32º36'12.58"N, 80º07'56.41"W), Oak Creek Bridge (32º40'07.98"N, 79º56'50.12"W), the Charleston City Marina (32º46'50.40"N, 79º57'17.36"W), and Patriots Point Marina (32º47'17.31"N, 79º54'29.42"W). In Georgetown County, sites include upper 60 Bass Creek–North Inlet (33º19'38.12" N, 79º10'30.80"W) and Huntington Beach State Park–South Jetty (33º31'35.22"N, 79º01'59.08"W).

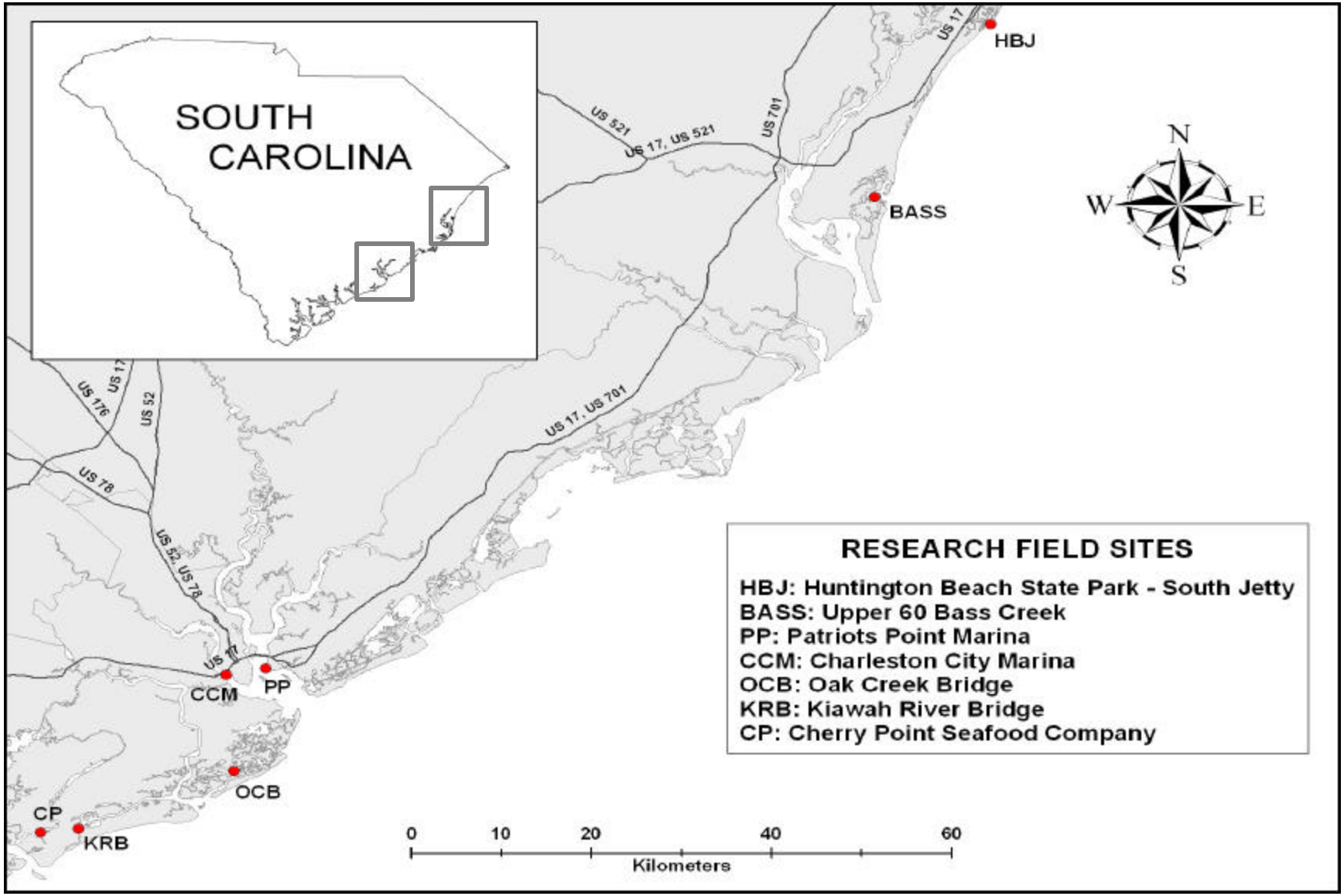

SOUTH CAROLINA
HBJ
BASS
CCM
PP
OCB
CP
KRB
US 17
US 521
US 17, US 521
US 701
US 17, US 701
US 176
US 52
US 78
US 52, US 78
N
W
E
S
RESEARCH FIELD SITES
HBJ: Huntington Beach State Park - South Jetty
BASS: Upper 60 Bass Creek
PP: Patriots Point Marina
CCM: Charleston City Marina
OCB: Oak Creek Bridge
KRB: Kiawah River Bridge
CP: Cherry Point Seafood Company
0
10
20
40
60
Kilometers

Figure 5. **Variation in mean optical density (O.D.) of *M. luteus* among sites**. The average antimicrobial activity (± SEM) against *M. luteus*, inferred by optical density, in crude methanol/water extracts of *L. virgulata* is presented for each field site. Colonies of *L. virgulata* collected from all of the study sites, excluding Charleston City Marina, demonstrated strong antimicrobial activity against *M. luteus* between May and October 2005. The dotted line (•••••) at 0.320 represents the negative control (90 µL of bacteria and 10 µL of methanol), i.e., microbial growth. The dashed line (-----) at 0.036 represents the positive control (90 µL of bacteria and 10 µL of 100 µM $Ala^{[8,13,18]}$ magainin-II-amide), i.e., inhibited growth. (a) Mean antimicrobial activity in *L. virgulata* collected at the City Marina differed significantly from the average *L. virgulata* activity at each of the other sites (one-tailed t-test, p= 0.05).

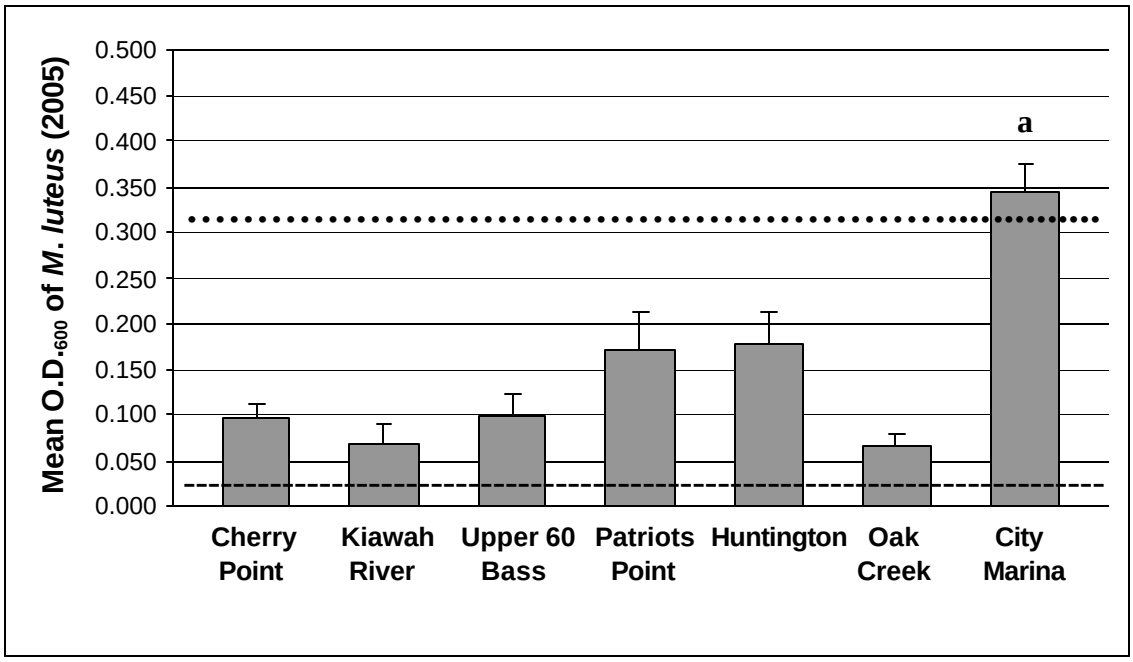

Mean O.D.600 of M. luteus (2005)
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
a
Cherry Point
Kiawah River
Upper 60 Bass
Patriots Point
Huntington
Oak Creek
City Marina

Figure 6. **Variation in mean O.D. of *Bacillus* sp. among sites**. The average antimicrobial activity (± SEM) against *Bacillus* sp., inferred by optical density, in crude methanol/water extracts of *L. virgulata* is presented for each field site. The dotted line (·····) at 0.338 represents the negative control (90 µL of bacteria and 10 µL of methanol), i.e., microbial growth. The dashed line (-----) at 0.037 represents the positive control (90 µL of bacteria and 10 µL of 100 µM $Ala^{[8,13,18]}$ magainin-II-amide), i.e., inhibited growth. Note the location of the dotted line (·····, negative control) as compared to the height of the bars at Cherry Point Seafood Company, upper 60 Bass Creek–North Inlet, Patriots Point Marina, and the Charleston City Marina. Colonies of *L. virgulata* collected at these four sites between May and October 2005 did not inhibit *Bacillus* sp. growth.

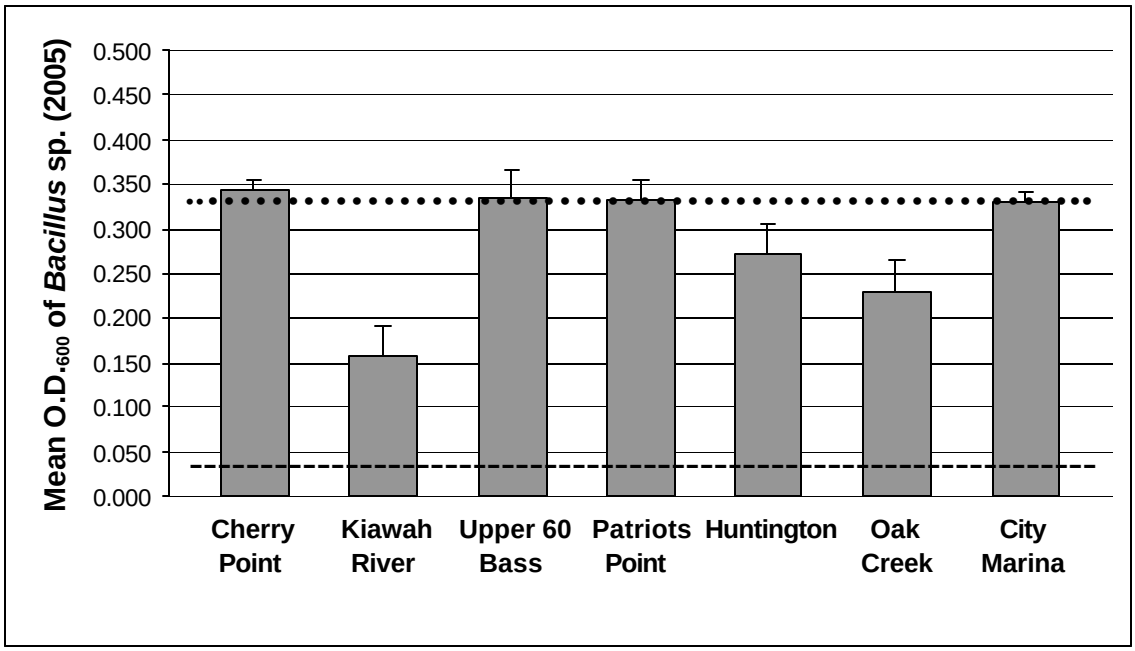

Mean O.D.600 of Bacillus sp. (2005)
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
Cherry Point
Kiawah River
Upper 60 Bass
Patriots Point
Huntington
Oak Creek
City Marina

Figure 7. **Variation in mean O.D. of *E. coli* BL21 among sites**. The average antimicrobial activity (± SEM) against *E. coli* BL21, inferred by optical density, in crude methanol/water extracts of *L. virgulata* is presented for each field site. The dotted line (·····) at 0.279 represents the negative control (90 µL of bacteria and 10 µL of methanol), i.e., microbial growth. The dashed line (-----) at 0.037 represents the positive control (90 µL of bacteria and 10 µL of 100 µM $Ala^{[8,13,18]}$ magainin-II-amide), i.e., inhibited growth. Note the location of the dotted line (negative control) as compared to the height of the bars at Cherry Point Seafood Company, upper 60 Bass Creek–North Inlet, Patriots Point Marina, and the Charleston City Marina. Between May and October 2005, only one colony of *L. virgulata* out of five collected at Cherry Point, upper 60 Bass Creek, and Patriots Point demonstrated antimicrobial activity against *E. coli* BL21. This activity was observed in September (Cherry Point and Patriots Point) and October (upper 60 Bass) 2005. Antimicrobial activity against *E. coli* BL21 was not documented in extracts of *L. virgulata* colonies collected from the Charleston City Marina during the six-month collection period. (a) Mean antimicrobial activity in *L. virgulata* at Kiawah River Bridge was significantly different from the mean coral activity documented at the other sites (one-tailed t-test, $p = 0.05$).

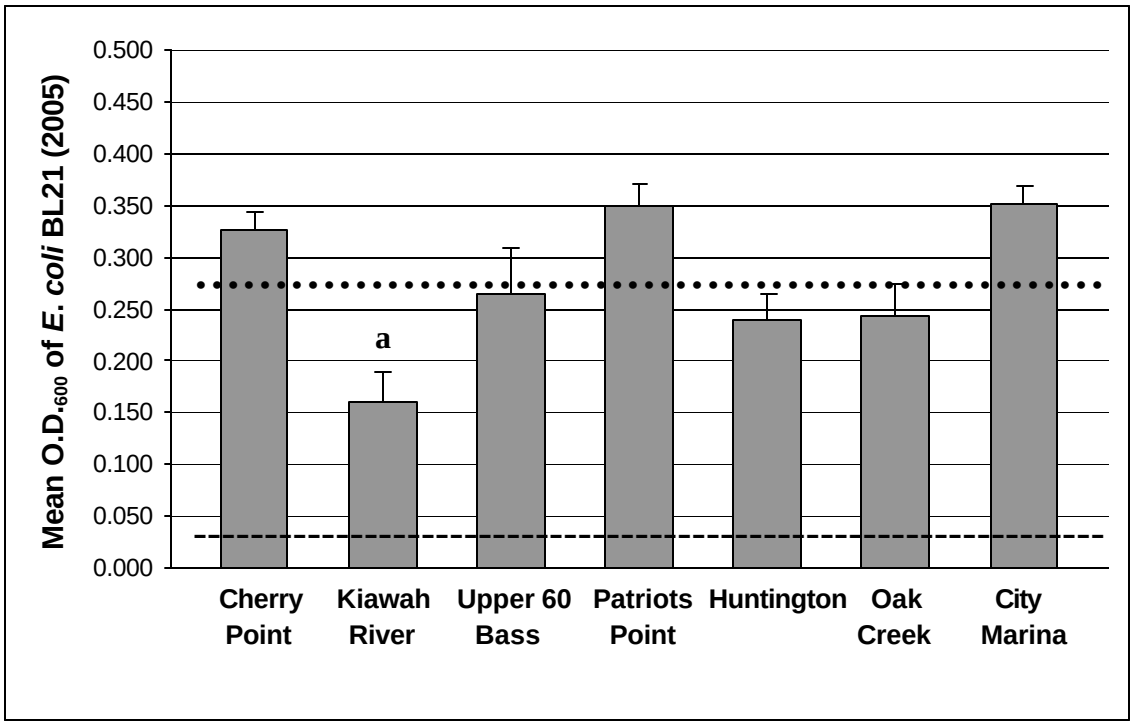

Mean O.D.600 of E. coli BL21 (2005)
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
a
Cherry Point
Kiawah River
Upper 60 Bass
Patriots Point
Huntington
Oak Creek
City Marina

Figure 8. **Variation in mean O.D. of *V. harveyii* among sites**. The average antimicrobial activity (± SEM) against *V. harveyii,* inferred by optical density, in crude methanol/water extracts of *L. virgulata* is presented for each field site. Colonies of *L. virgulata* collected from Kiawah River Bridge and Oak Creek Bridge demonstrated strong antimicrobial activity against *V. harveyii* throughout the six-month study period. The dotted line (·····) at 0.302 represents the negative control (90 µL of bacteria and 10 µL of methanol), i.e., microbial growth. The dashed line (-----) at 0.036 represents the positive control (90 µL of bacteria and 10 µL of 100 µM $Ala^{[8,13,18]}$ magainin-II-amide), i.e., inhibited growth. (a) Mean antimicrobial activity in *L. virgulata* collected at Kiawah River Bridge and Oak Creek Bridge differed at the level of $p < 0.1$, but was significantly different from the mean activity at the other five field sites (one-tailed t-test, $p = 0.05$).

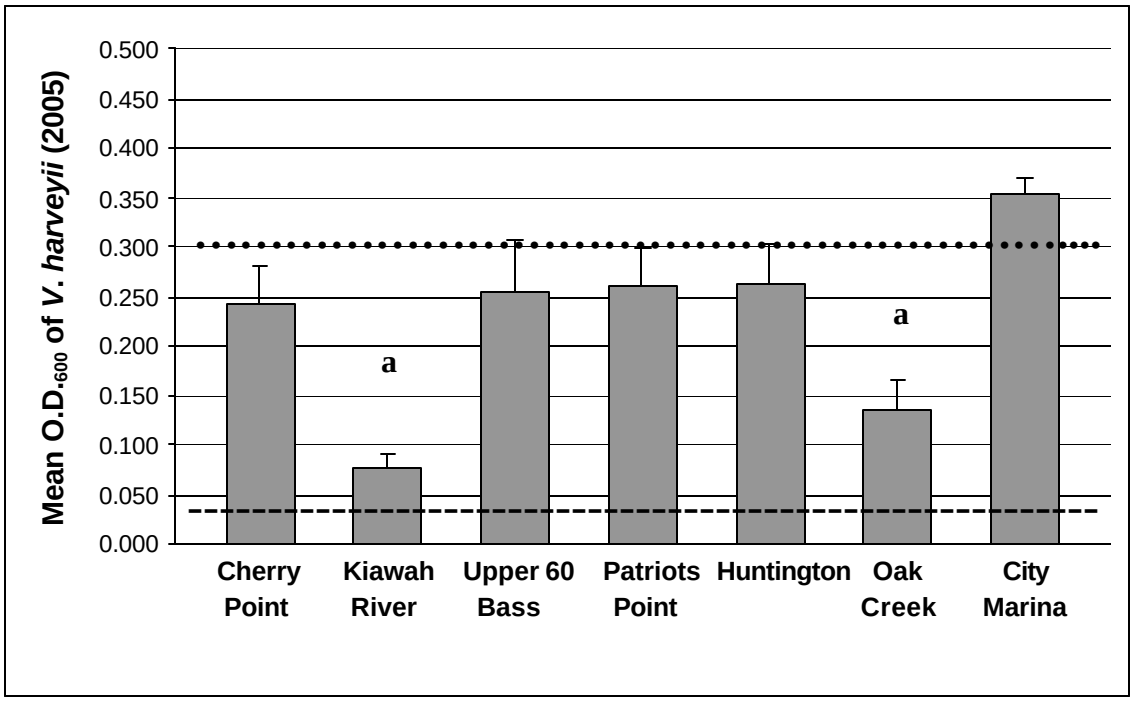

Mean O.D.600 of V. harveyii (2005)
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
a
a
Cherry Point
Kiawah River
Upper 60 Bass
Patriots Point
Huntington
Oak Creek
City Marina

Figure 9. **Variation in mean O.D. of *M. luteus* at each site**. Variation in the average antimicrobial activity (± SEM) against *M. luteus*, inferred by optical density, in crude methanol/water extracts of *L. virgulata* is presented for each field site between May and October 2005. Colonies of *L. virgulata* collected from Cherry Point Seafood Company, Kiawah River Bridge, and Oak Creek Bridge demonstrated strong antimicrobial activity against *M. luteus* at each of the 3 collection points between May and October 2005. Stippled, solid, and striped bars indicate the first (May-June), second (July-August), and third (September-October) points of collection at each site, respectively. The dotted line (·····) at 0.320 represents the negative control (90 µL of bacteria and 10 µL of methanol), i.e., microbial growth. The dashed line (-----) at 0.036 represents the positive control (90 µL of bacteria and 10 µL of 100 µM $Ala^{[8,13,18]}$ magainin-II-amide), i.e., inhibited growth. (a) Mean antimicrobial activity in *L. virgulata* sampled from Kiawah River Bridge differed significantly between August and October 2005 (pair-wise t-test, p= 0.01). (b) Mean antimicrobial activity in *L. virgulata* collected at Huntington Beach State Park–South Jetty in October 2005 was significantly different from June and August (pair-wise t-test, p= 0.0001).

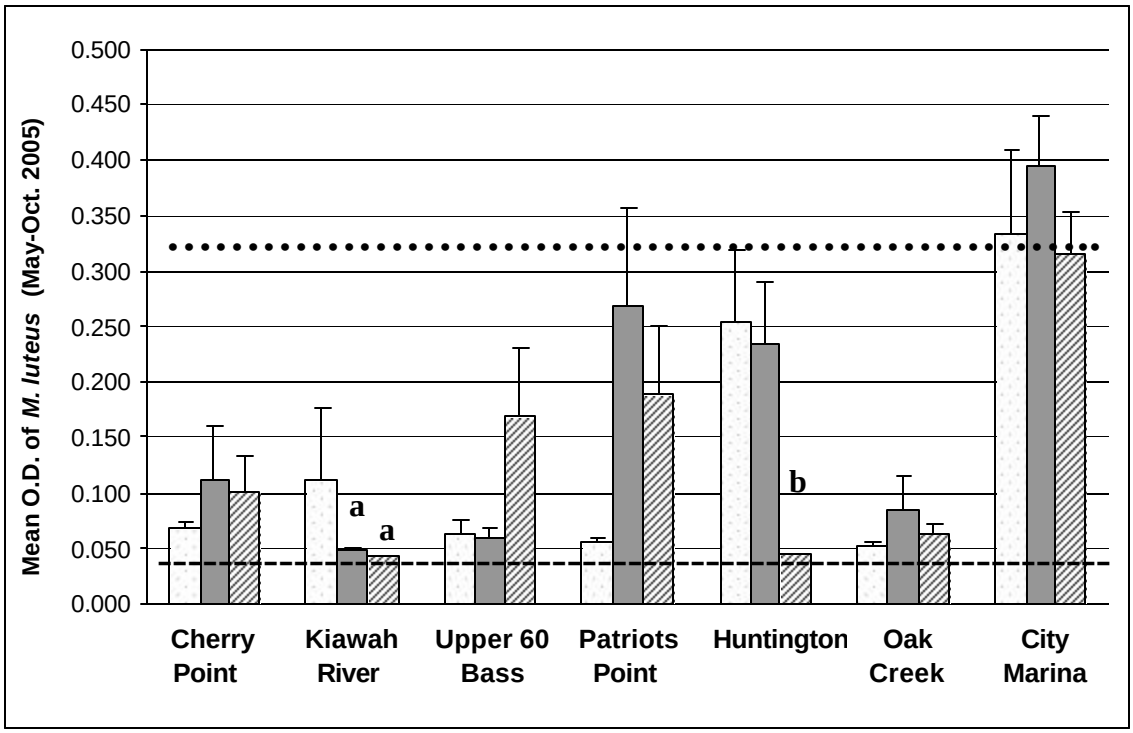

Mean O.D. of M. luteus (May-Oct. 2005)
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
a
a
b
Cherry Point
Kiawah River
Upper 60 Bass
Patriots Point
Huntington
Oak Creek
City Marina

Figure 10. **Variation in mean O.D. of *Bacillus* sp. at each site**. Variation in the average antimicrobial activity (± SEM) against *Bacillus* sp., inferred by optical density, in crude methanol/water extracts of *L. virgulata* is presented for each field site between May and October 2005. Stippled, solid, and striped bars indicate the first (May-June), second (July-August), and third (September-October) points of collection at each site, respectively. The dotted line (·····) at 0.338 represents the negative control (90 µL of bacteria and 10 µL of methanol), i.e., microbial growth. The dashed line (-----) at 0.037 represents the positive control (90 µL of bacteria and 10 µL of 100 µM Ala[8,13,18] magainin-II-amide), i.e., inhibited growth. Note the location of the dotted line (negative control) as compared to the height of the bars at Cherry Point Seafood Company, upper 60 Bass Creek–North Inlet, Patriots Point Marina, and the Charleston City Marina. Colonies of *L. virgulata* collected at these four sites between May and October 2005 did not inhibit *Bacillus* sp. growth. (a) Mean antimicrobial activity in *L. virgulata* collected at Huntington Beach State Park–South Jetty in October 2005 was significantly different from the mean activity recorded in June and August (pair-wise t-test, p= 0.0001).

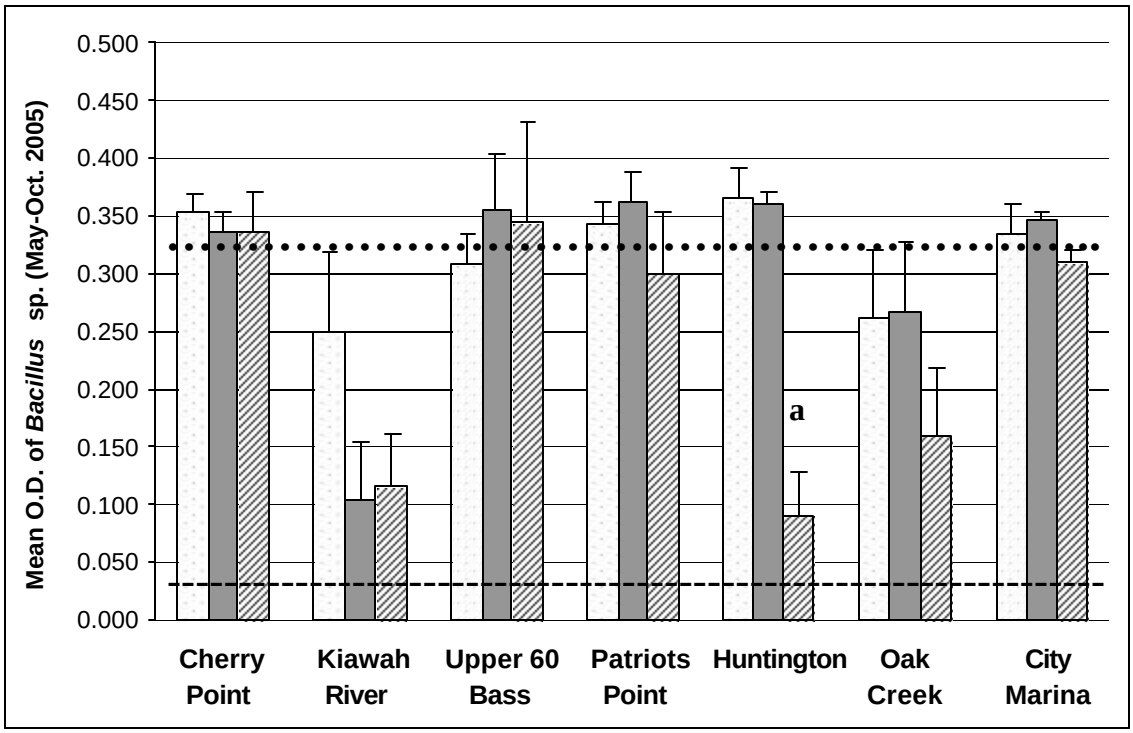

Mean O.D. of *Bacillus* sp. (May-Oct. 2005)
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
a
Cherry Point
Kiawah River
Upper 60 Bass
Patriots Point
Huntington
Oak Creek
City Marina

Figure 11. **Variation in mean O.D. of *E. coli* BL21 at each site**. Variation in the average antimicrobial activity (± SEM) against *E. coli* BL21, inferred by optical density, in crude methanol/water extracts of *L. virgulata* is presented for each field site between May and October 2005. Stippled, solid, and striped bars indicate the first (May-June), second (July-August), and third (September-October) points of collection at each site, respectively. The dotted line (·····) at 0.279 represents the negative control (90 µL of bacteria and 10 µL of methanol), i.e., microbial growth. The dashed line (-----) at 0.037 represents the positive control (90 µL of bacteria and 10 µL of 100 µM Ala[8,13,18] magainin-II-amide), i.e., inhibited growth. (a) Mean antimicrobial activity in *L. virgulata* at upper 60 Bass Creek in June 2005 differed significantly from the mean activity in August 2005 (pair-wise t-test, p= 0.05). (b) Mean antimicrobial activity in *L. virgulata* at Patriots Point differed significantly between July and September 2005 (pair-wise t-test, p= 0.05). (c) Average antimicrobial activity in *L. virgulata* samples from Huntington Beach State Park–South Jetty differed significantly among June, August, and October 2005 (pair-wise t-test, p= 0.05). (d) Antimicrobial activity was not observed in *L. virgulata* collected at the Charleston City Marina between May and October 2005. However, a slight depression in the O.D. of *E. coli* BL21 was identified in assays containing crude extracts of *L. virgulata* collected from the City Marina in October. Accordingly, the mean antimicrobial activity in *L. virgulata,* inferred by optical density, differed significantly between June and October 2005 (pair-wise t-test, p= 0.001).

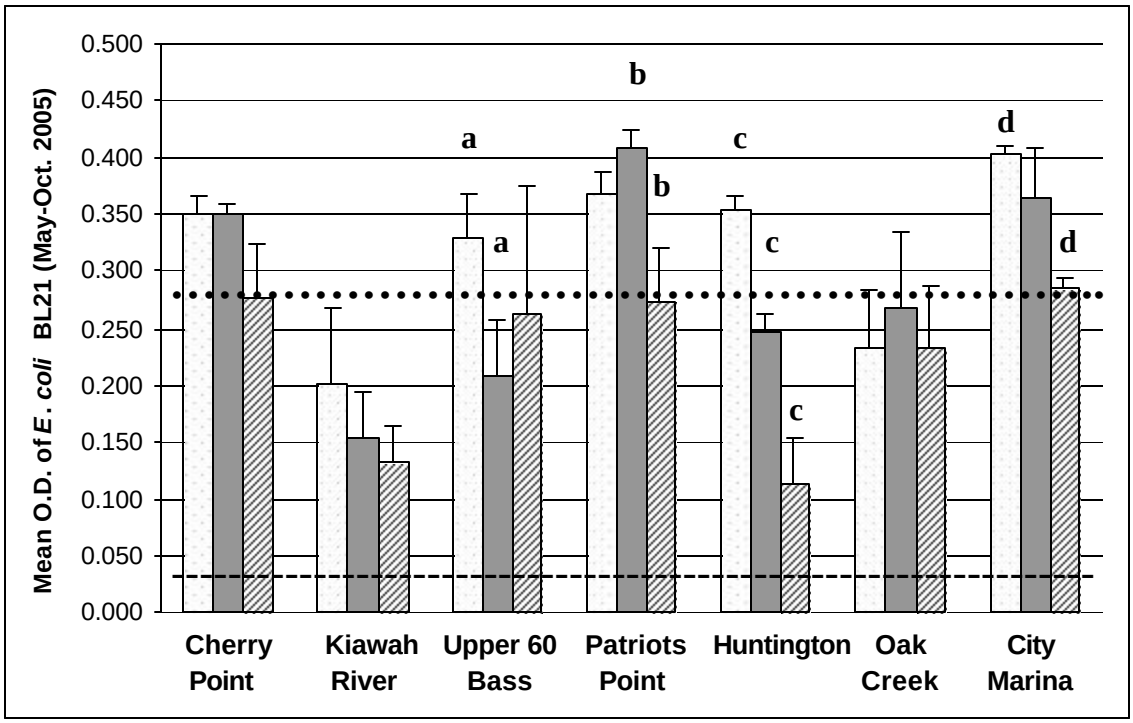

Mean O.D. of E. coli BL21 (May-Oct. 2005)
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
a
b
c
d
a
b
c
c
d
Cherry Point
Kiawah River
Upper 60 Bass
Patriots Point
Huntington
Oak Creek
City Marina

Figure 12. **Variation in mean O.D. of *V. harveyii* at each site**. Variation in the average antimicrobial activity (± SEM) against *V. harveyii*, inferred by optical density, in crude methanol/water extracts of *L. virgulata* is presented for each field site between May and October 2005. Stippled, solid, and striped bars indicate the first (May-June), second (July-August), and third (September-October) points of collection at each site, respectively. The dotted line (·····) at 0.302 represents the negative control (90 µL of bacteria and 10 µL of methanol), i.e., microbial growth. The dashed line (-----) at 0.036 represents the positive control (90 µL of bacteria and 10 µL of 100 µM Ala[8,13,18] magainin-II-amide), i.e., inhibited growth. (a) Mean antimicrobial activity in *L. virgulata* collected from Cherry Point Seafood Company differed significantly in May 2005 from July and September 2005 (pair-wise t-test, p= 0.05). (b) Average antimicrobial activity in *L. virgulata* at upper 60 Bass Creek differed significantly among June, August, and October 2005 (pair-wise t-test, p= 0.05). (c) Mean antimicrobial activity in the common seawhip collected from Patriots Point Marina was significantly different in May 2005 from July and September 2005 (pair-wise t-test, p= 0.0001). (d) Average antimicrobial activity in *L. virgulata* collected from Huntington Beach State Park–South Jetty in October 2005 was significantly different from the mean activity recorded in June and August (pair-wise t-test, p= 0.0001).

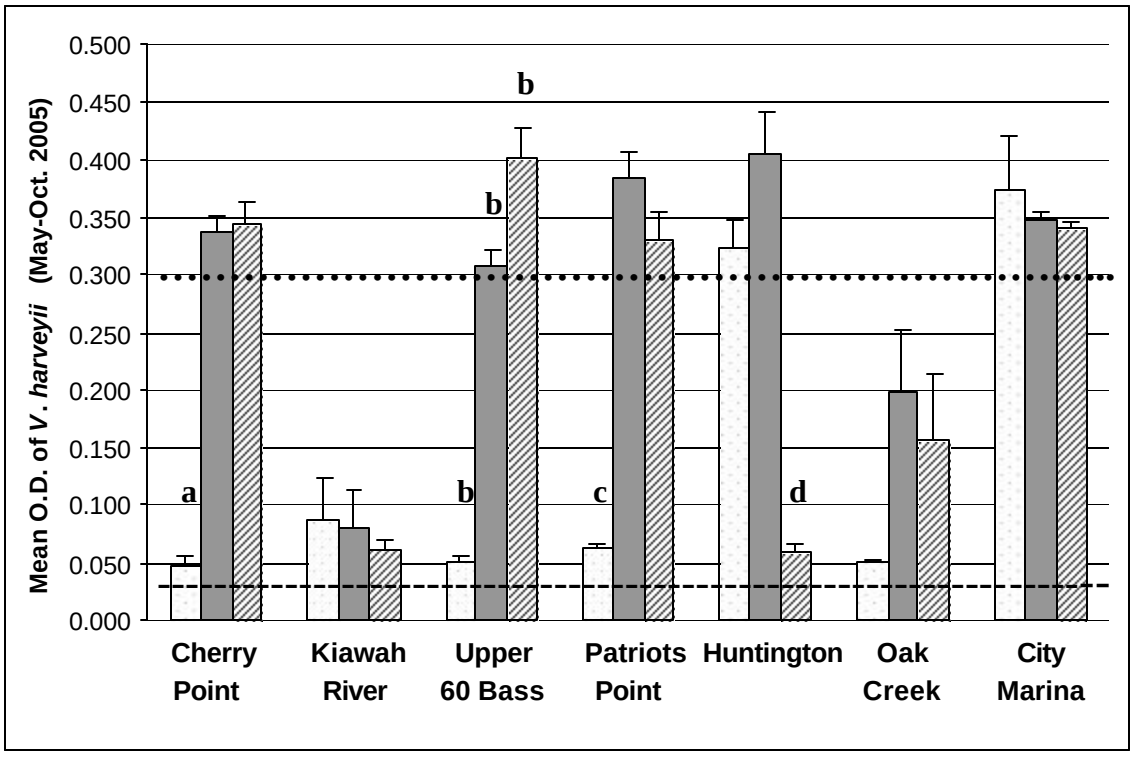
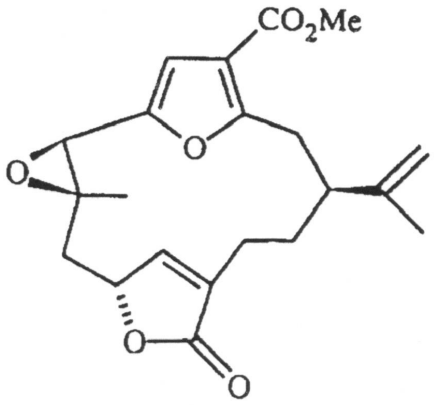
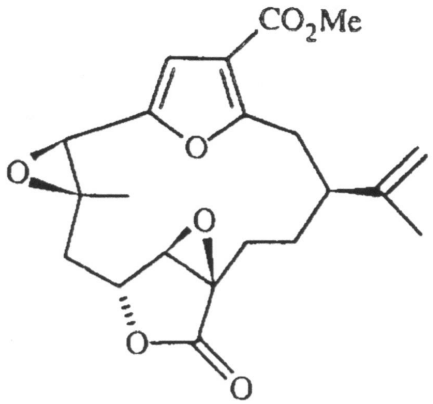

Mean O.D. of V. harveyii (May-Oct. 2005)
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
a
b
b
c
d
b
Cherry Point
Kiawah River
Upper 60 Bass
Patriots Point
Huntington
Oak Creek
City Marina

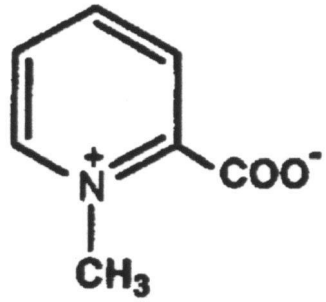
Figure 13. **Structures of pukalide, epoxypukalide, and homarine**. Images of pukalide and 11ß, 12ß-epoxypukalide are reproduced from Coll *et al*. (1989). The illustration of homarine is after Netherton and Gurin (1982). (a) The complex structure of pukalide ($C_{21}H_{24}O_6$), a diterpenoid, includes the 14-membered ring system typical to furanocembranolides. Associated functional groups include butenolide, an a, ß-unsaturated 5-membered ring lactone, epoxide, isopropenyl, and an a, a´-dialkyl methyl furan-ß-carboxylate (Missakian *et al*. 1975). (b) The structure of 11ß, 12ß-epoxypukalide ($C_{21}H_{24}O_7$), a furanocembranolide diterpene and pukalide derivative, is analogous to the structure of pukalide with the addition of a C_{11}-C_{12}-epoxide (Ksebati *et al*. 1984). (c) Homarine ($C_7H_7NO_2$), a.k.a. N-methyl picolinic acid or N-methyl-2-carboxypyridine, is a 5-membered betaine with N-methyl and carboxyl functional groups (Hoppe-Seyler 1933).



a) Pukalide



b) 11ß, 12ß - Epoxypukalide



c) Homarine

Figure 14. **FAB-MS analysis of analytically pure homarine**. Fast atom bombardment spectrometry (FAB-MS) of analytically pure homarine identified mass signatures corresponding to the pseudomolecular ion $(M + Na)^+$ at m/z = 160 and $(M + H)^+$ at m/z = 138. These signals were complemented by a prominent N-methylpyridinium fragment ion at m/z = 94, arising from the loss of CO_2 during FAB-MS fractionation (figure after Polychronopoulos *et al*. 2001, reproduced with permission from Prokopios Magiatis, Department of Pharmacy, University of Athens, Greece).

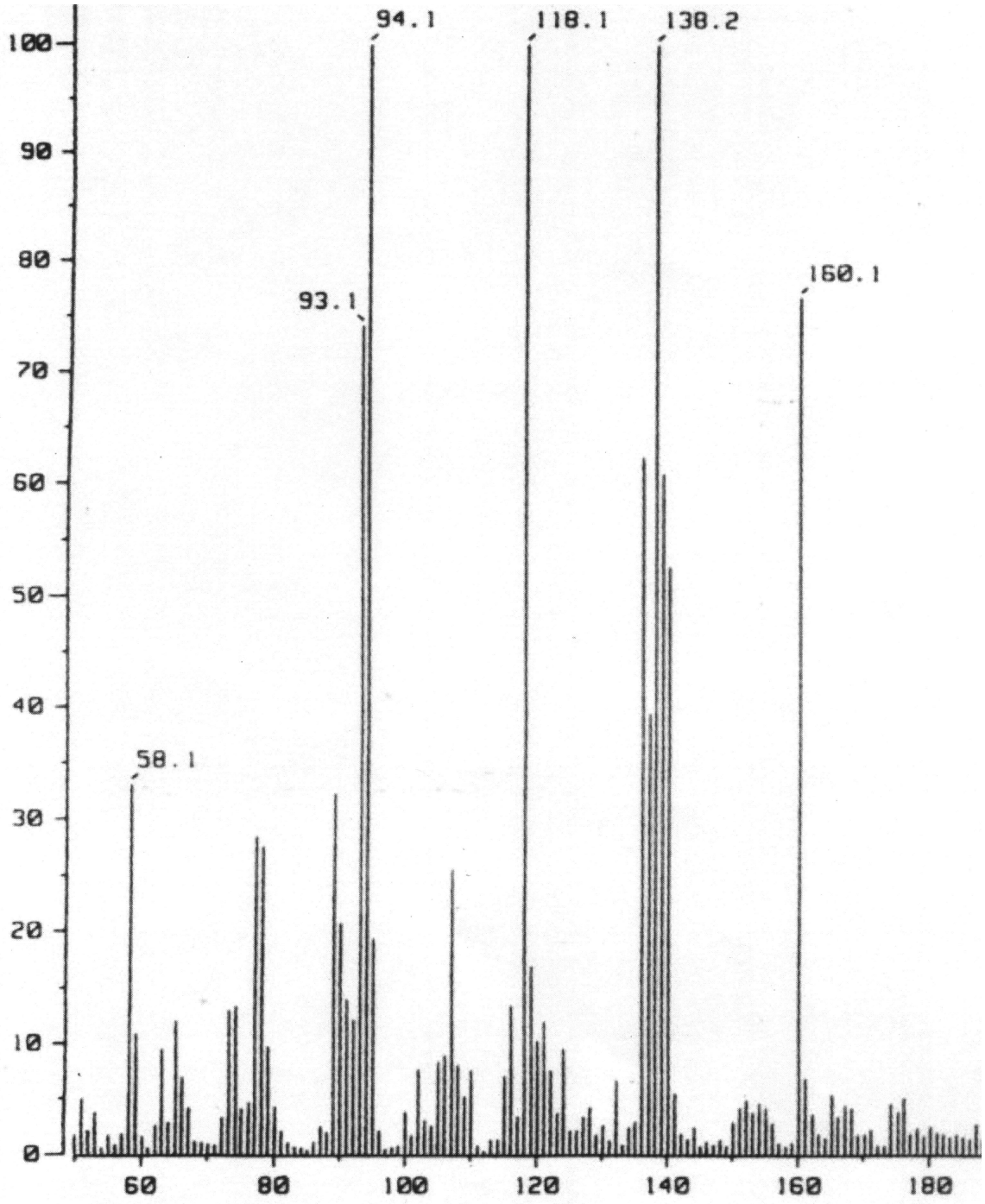

94.1
118.1
138.2
160.1
93.1
58.1
100
90
80
70
60
50
40
30
20
10
0
60
80
100
120
140
160
180

Figure 15. HPLC chromatograms of pooled active crude *L. virgulata* extracts. Crude aqueous methanol extracts of *L. virgulata* exhibiting antimicrobial activity were pooled into one sample, which was then separated into 50, 1-minute, sequential fractions by reverse-phase HPLC. Fractionation was monitored at 238, 248, and 272 nm, wavelengths representing the chromophore signatures for homarine (Polychronopoulos *et al*. 2001), pukalide (Missakian *et al*. 1975), and 11ß, 12ß-epoxypukalide (Ksebati *et al*. 1984), respectively. Strong chromophoric signals correlating to activity, designated by a black box on each of the three chromatograms, were recorded between minutes 10 and 49.

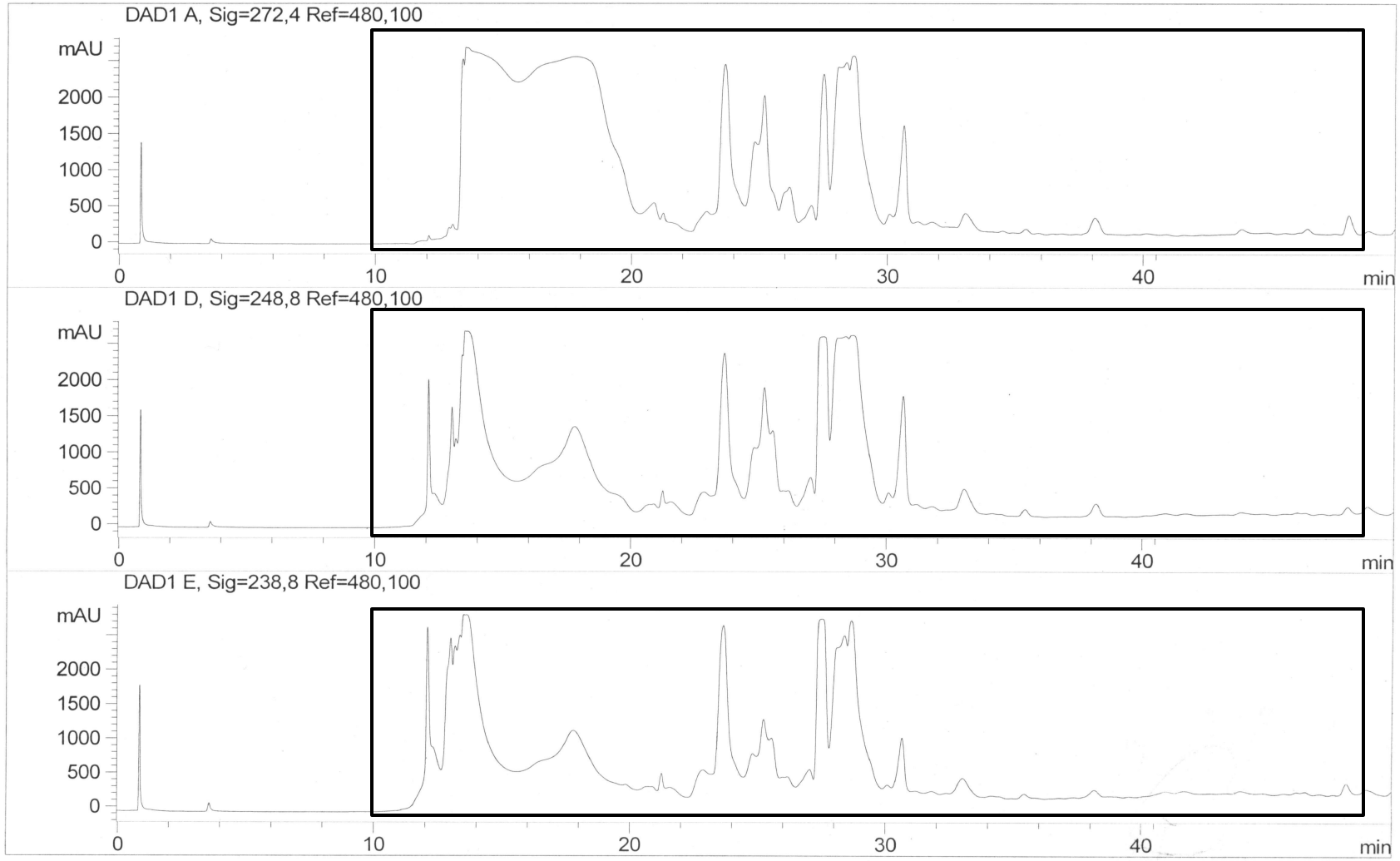

DAD1 A, Sig=272,4 Ref=480,100
mAU
2000
1500
1000
500
0
0
10
20
30
40
min
DAD1 D, Sig=248,8 Ref=480,100
mAU
2000
1500
1000
500
0
0
10
20
30
40
min
DAD1 E, Sig=238,8 Ref=480,100
mAU
2000
1500
1000
500
0
0
10
20
30
40
min

Figure 16. **^{1}H-NMR spectrum of analytically pure homarine** (courtesy of Prokopios Magiatis, Department of Pharmacy, University of Athens, Greece). The chemical shifts for isolated signals of pure homarine in the ^{1}H-NMR spectrum include (a) 4.42, s, N-CH_3; (b) 7.93, dd, 8, 6.5, C_5-H; (c) 8.07, d, 8, C_3-H; (d) 8.52, t, 8, C_4-H; and (e) 8.76, d, 6.5, C_6-H.

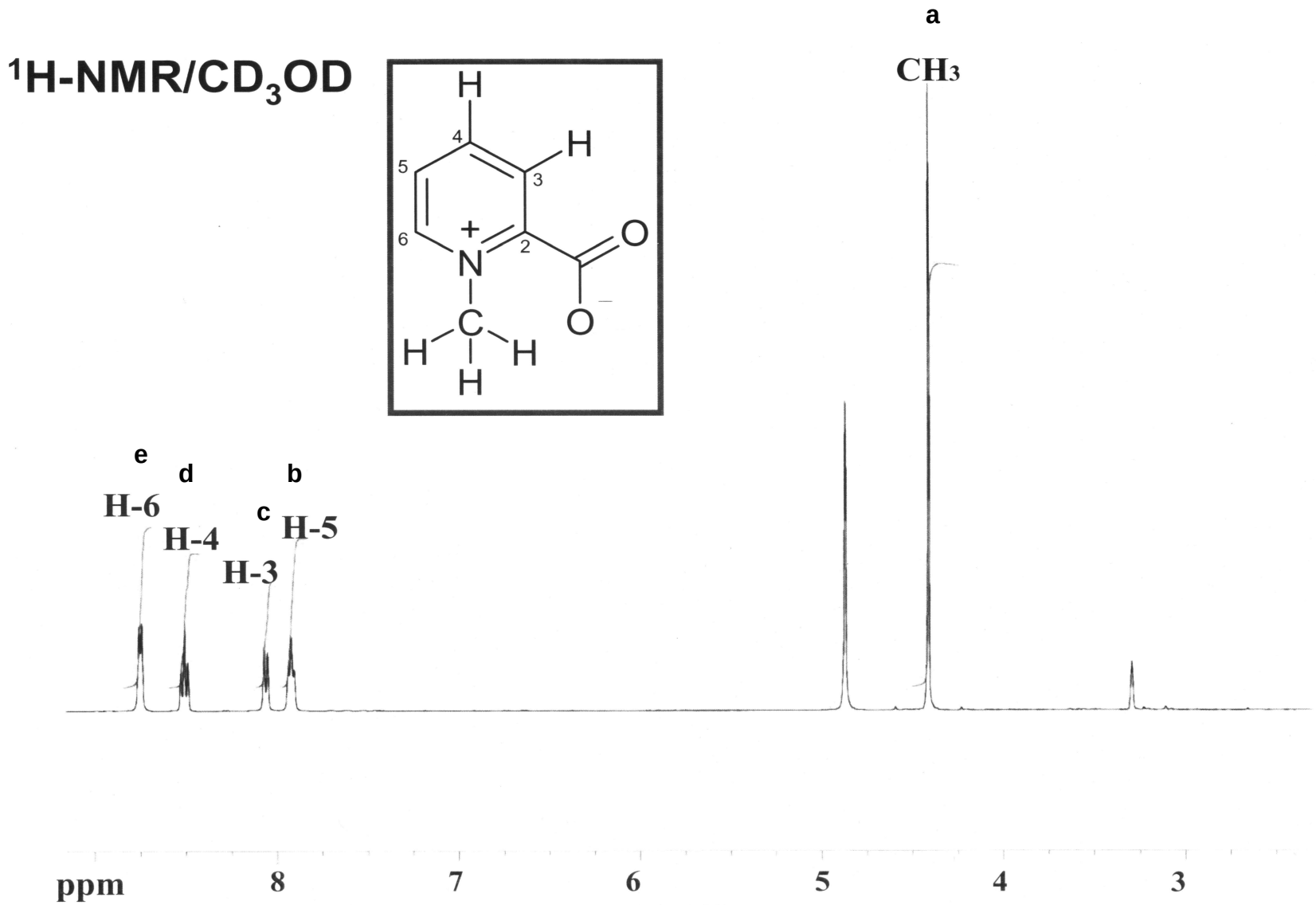

1H-NMR/CD3OD
a
CH3
e
d
b
c
H-6
H-4
H-3
H-5
ppm
8
7
6
5
4
3

Figure 17. **^{13}C-NMR spectrum of pure homarine** (courtesy of Prokopios Magiatis, Department of Pharmacy, University of Athens, Greece). The chemical shifts for isolated signals of pure homarine in the ^{13}C-NMR spectrum include (a) 47.5, N-CH_3; (b) 127.6, $C_{5,3}$; (c) 146.6, C_6; (d) 147.0, C_4; (e) 155.2, C_2; and (f) 165.2, CO.

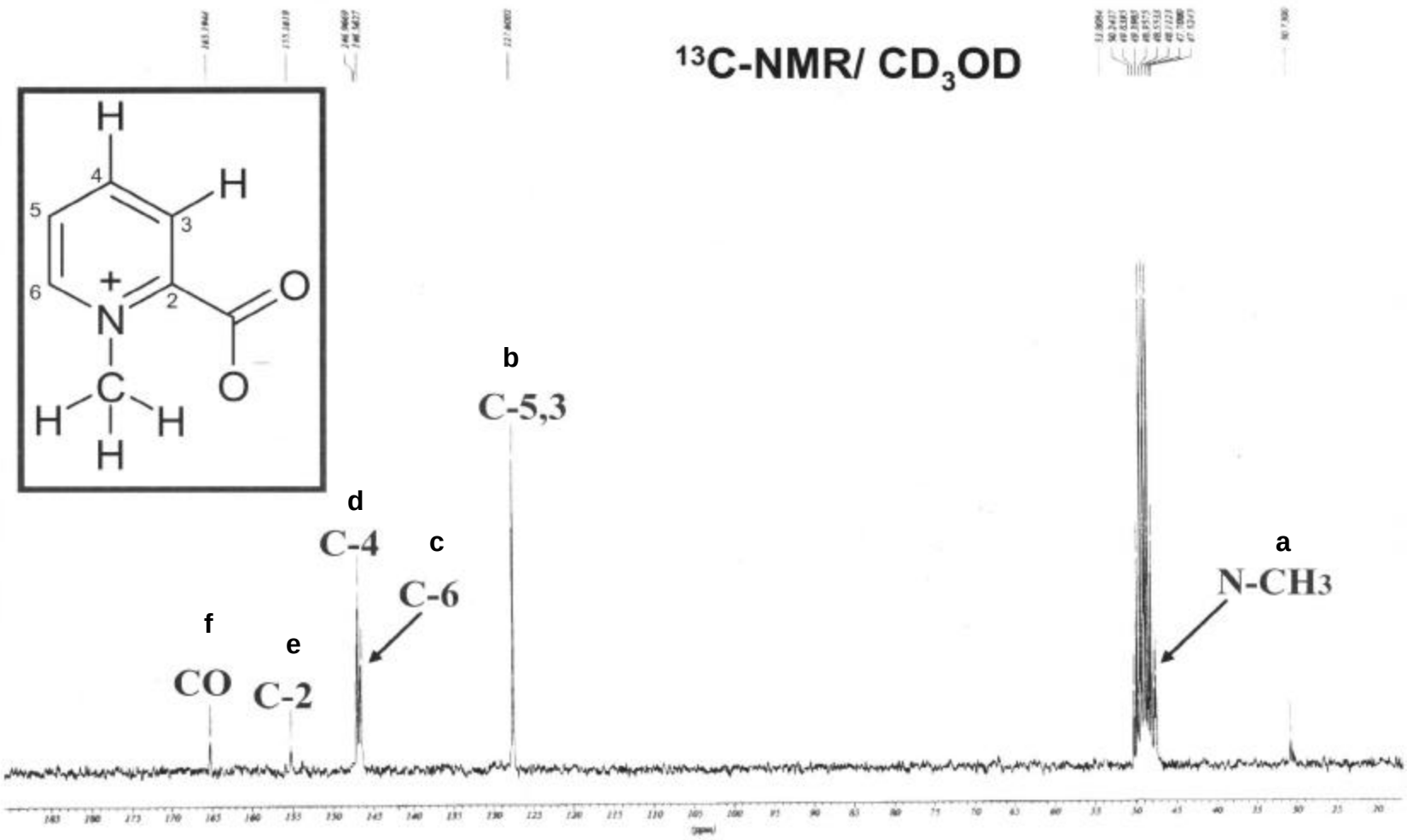

13C-NMR/ CD3OD
H
4
H
5
3
6
+
N
2
O
C
O
H
H
H
b
C-5,3
d
C-4
c
C-6
a
N-CH3
f
CO
e
C-2

Figure 18. **^{1}H-NMR spectrum of pure pukalide** (courtesy of Paul Keifer, Eppley Institute for Research in Cancer, University of Nebraska Medical Center). The structure of pukalide is displayed as an inset. Atom numbers (1-21) correspond to individual carbons within the molecule. Chemical shifts (Missakian *et al*. 1975) for isolated signals of pure pukalide in the ^{1}H-NMR spectrum include (a) C_{21}, 3.75; (b) C_5, 6.33; (c) C_7, 4.04; (d) C_{19}, 1.00; (e) C_9, 2.20, 2.50; (f) C_{10}, 5.20; (g) C_{11}, 7.06; (h) C_{13}, 2.35; (i) C_1, 3.55; (j) C_2, 2.90, 3.00; (k) C_{17}, 4.91, 5.20; and (l) C_{16}, 1.75.

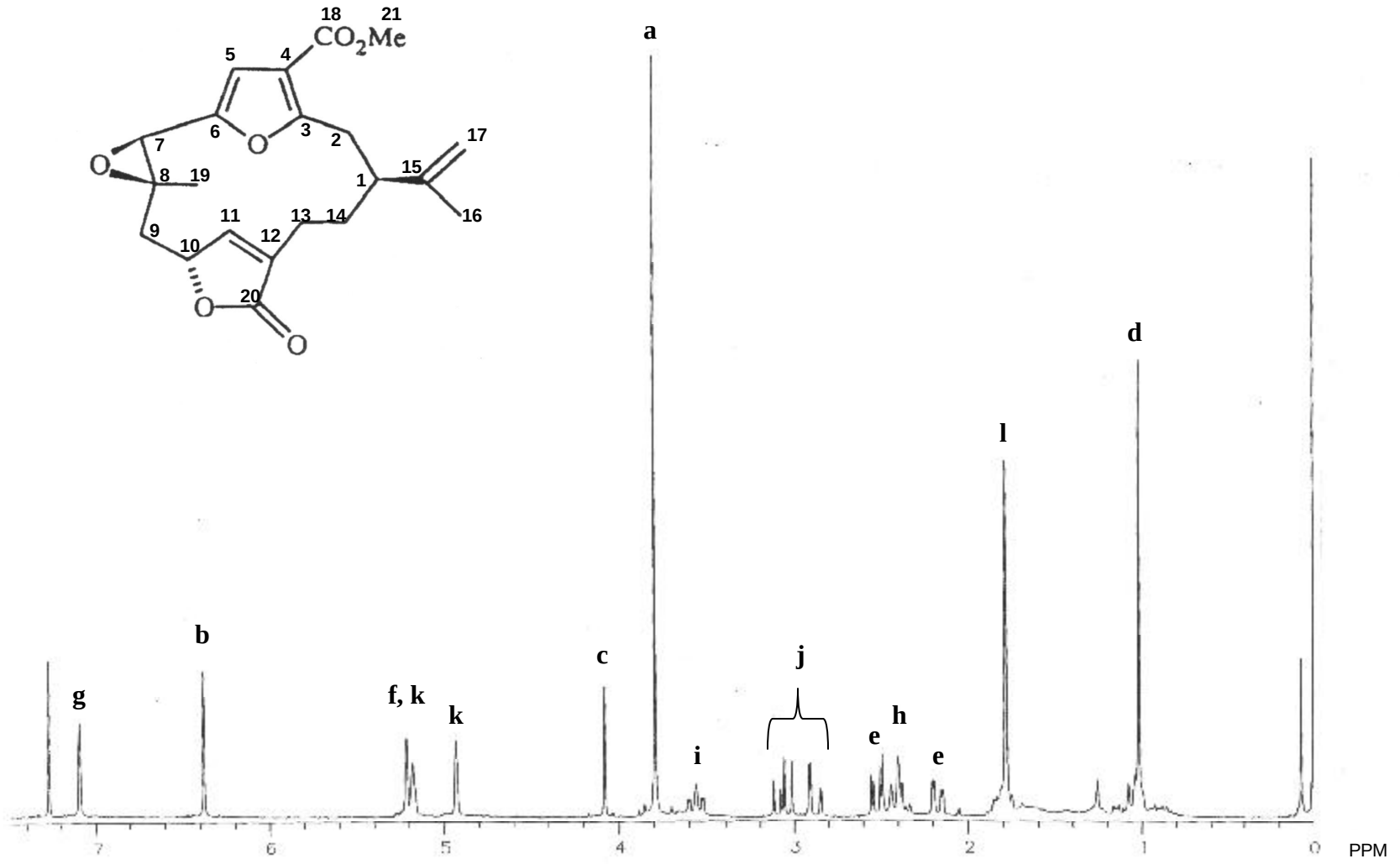

18
21
CO_2Me
5
4
6
O
3
2
17
7
O
8
19
1
15
16
11
13
14
9
12
10
O
20
O
a
b
g
f, k
k
c
i
j
e
h
e
l
d
7
6
5
4
3
2
1
0
PPM

Figure 19. **^{1}H-NMR spectrum of pure 11ß, 12ß-epoxypukalide** (courtesy of Paul Keifer, Eppley Institute for Research in Cancer, University of Nebraska Medical Center). The structure of 11ß, 12ß-epoxypukalide is displayed as an inset. Atom numbers (1-21) correspond to individual carbons within the molecule. Chemical shifts (Ksebati *et al*. 1984) for isolated signals of pure 11ß, 12ß-epoxypukalide in the ^{1}H-NMR spectrum include (a) C_{21}, 3.79; (b) C_5, 6.38; (c) C_7, 4.07; (d) C_{19}, 1.01; (e) C_9, 2.18, 2.51; (f) C_{10}, 5.18; (g) C_{11}, 7.08; (h) C_{13}, 2.38; (i) C_{14}, 1.7-; (j) C_1, 3.55; (k) C_2, 2.88, 3.05; (l) C_{17}, 4.93, 5.20; and (m) C_{16}, 1.78.

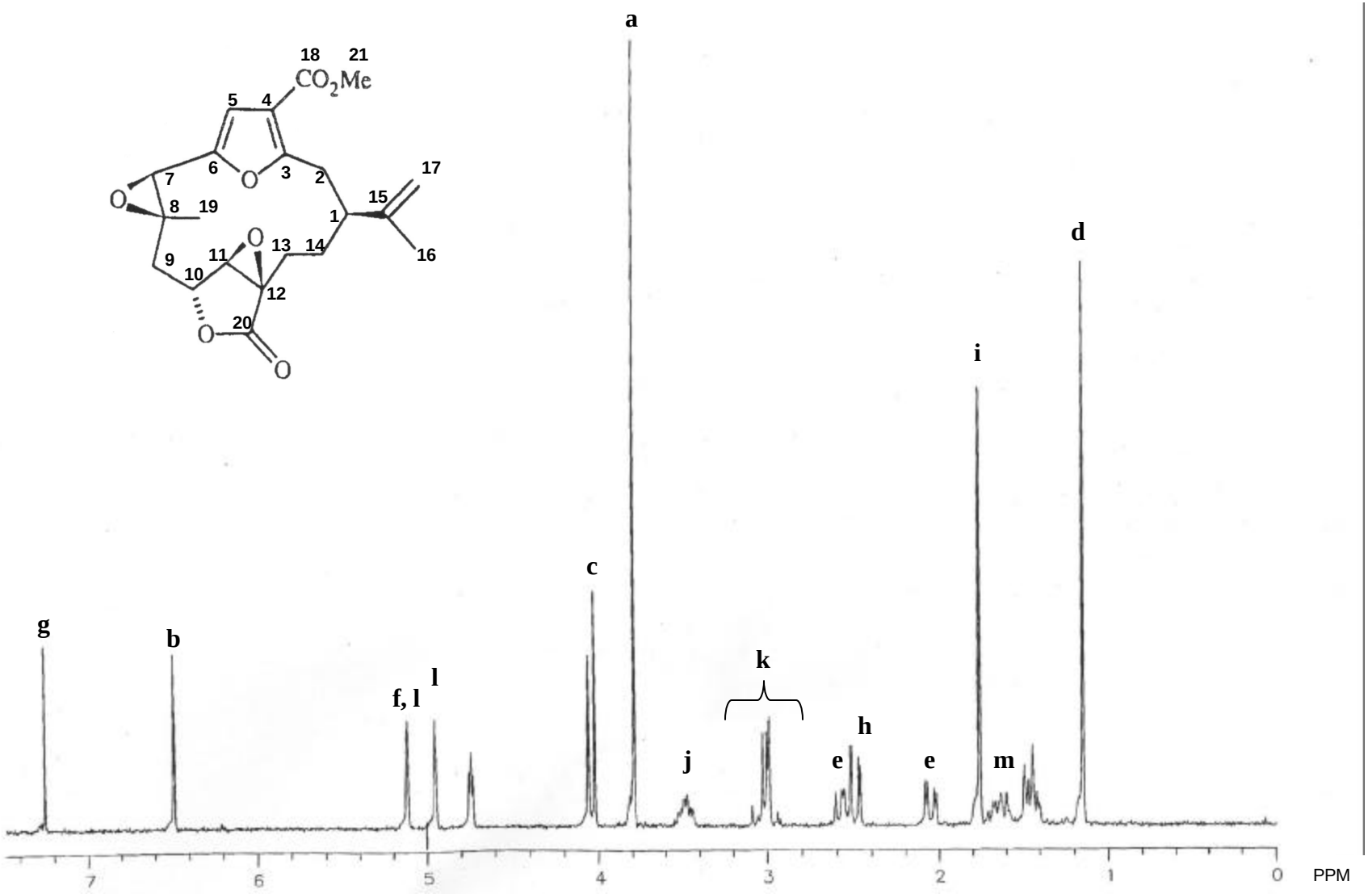

18
21
CO2Me
5
4
6
O
3
2
7
17
O
8
19
15
1
16
O
13
14
9
11
10
12
O
20
O
a
d
i
c
g
b
f, l
l
k
j
h
e
e
m
7
6
5
4
3
2
1
0
PPM

Figure 20. **LC-MS and UV spectra corresponding to homarine**. MS peaks in the mass spectrum (b) of crude extracts of *L. virgulata* revealed signals corresponding to the molecular weight of homarine at m/z = 94.3 and 138.3 ($C_7H_7NO_2$, M^+, 94.1, 138.2, 160.1, Polychronopoulos *et al*. 2001). These MS peaks were observed at retention times between 0.9-3.5 minutes. Of note is a signal in the UV chromatogram (254 nm) (a), indicated by a black box centered at 2.6 minutes. This signal (UV peak) is associated with the MS peaks of interest, depicted by a * in the total ion chromatogram (TIC) (a) and in the MS spectrum (b). It suggests the presence of a chromophore associated with homarine in the uncharacterized compounds in *L. virgulata.*

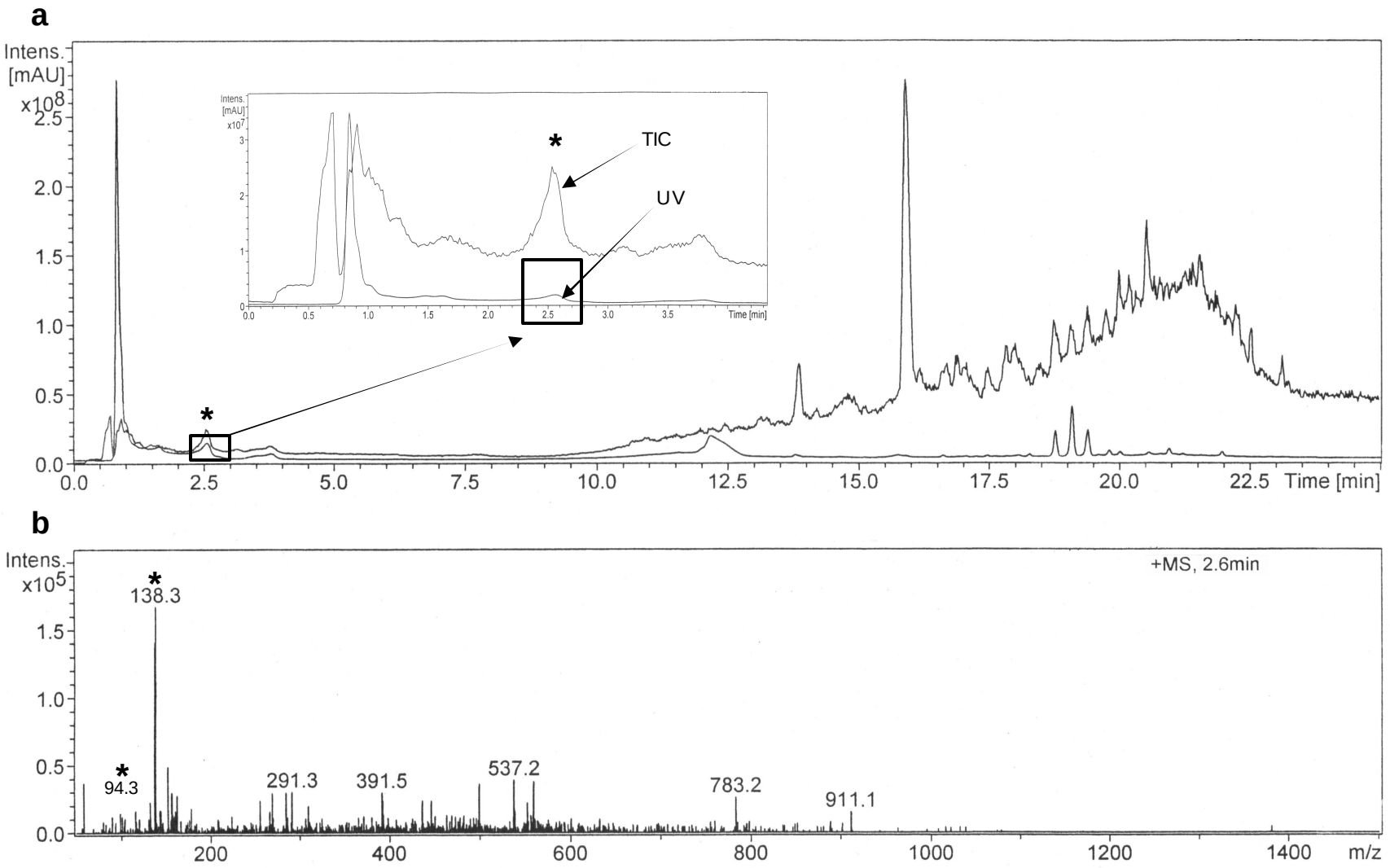

a
Intens.
[mAU]
x10^8
2.5
2.0
1.5
1.0
0.5
0.0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
Time [min]
*
Intens.
[mAU]
x10^7
3
2
1
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Time [min]
*
TIC
UV
b
Intens.
x10^5
1.5
1.0
0.5
0.0
+MS, 2.6min
*
138.3
*
94.3
291.3
391.5
537.2
783.2
911.1
200
400
600
800
1000
1200
1400
m/z

Figure 21. **LC-MS and UV spectra corresponding to pukalide**. An MS peak in the mass spectrum (b) of crude extracts of *L. virgulata* revealed a signal corresponding to the molecular weight of pukalide at m/z = 372.6 ($C_{21}H_{24}O_6$, M^+, 372.1564, Missakian *et al*. 1975). This MS peak was observed at retention times between 18.5-19.0 minutes. Of note is a signal in the UV chromatogram (254 nm) (a), indicated by a black box centered at 18.8 minutes. This signal (UV peak) is associated with the MS peak of interest, depicted by a * in the total ion chromatogram (TIC) (a) and in the MS spectrum (b). It suggests the presence of a chromophore associated with pukalide in the uncharacterized compounds in *L. virgulata.*

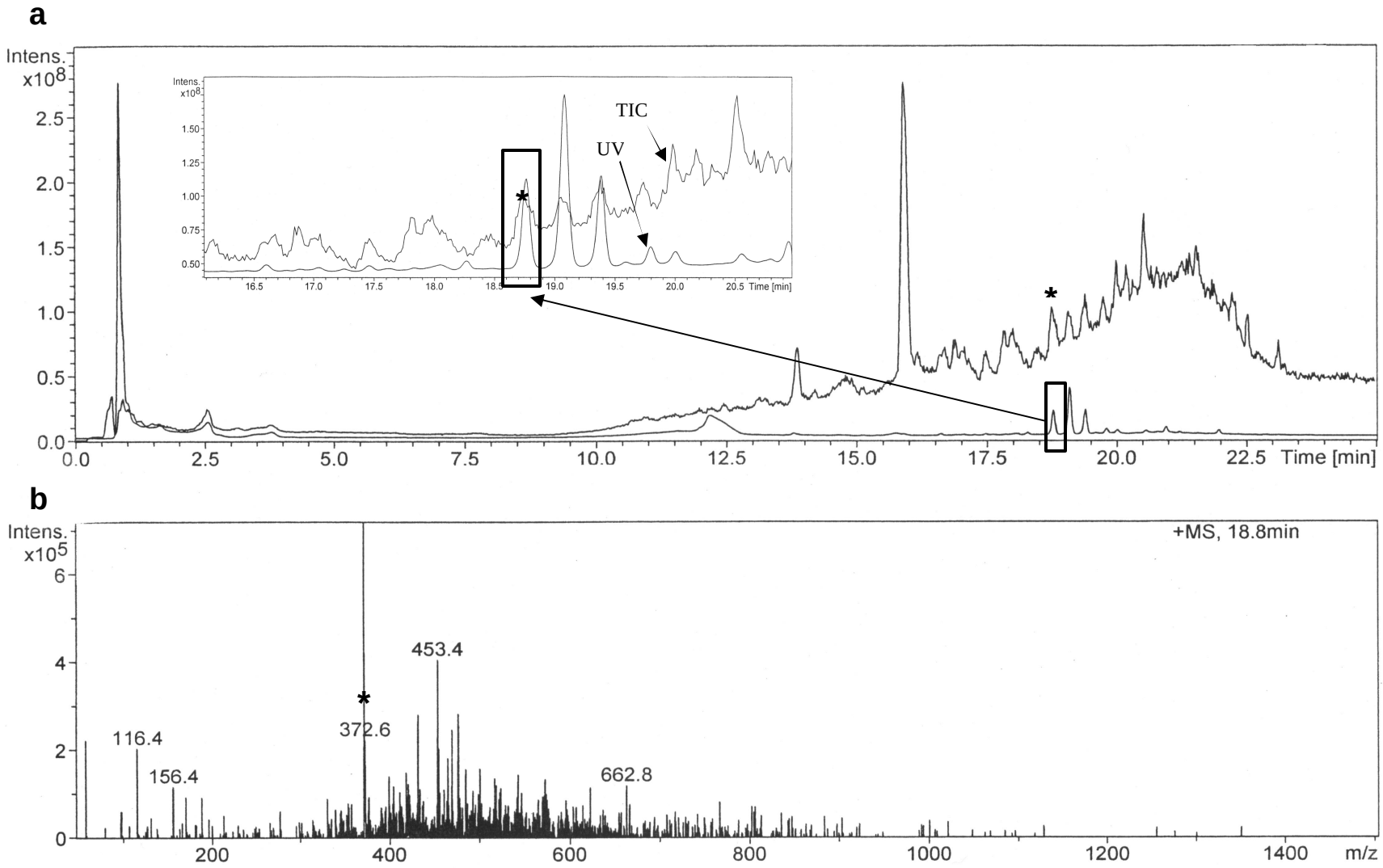

a
Intens.
x10^8
2.5
2.0
1.5
1.0
0.5
0.0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
Time [min]
TIC
UV
*
b
Intens.
x10^5
+MS, 18.8min
6
4
2
0
453.4
372.6
116.4
156.4
662.8
200
400
600
800
1000
1200
1400
m/z

Figure 22. **LC-MS and UV spectra corresponding to epoxypukalide**. An MS peak in the mass spectrum (b) of crude extracts of *L*. *virgulata* revealed a signal corresponding to the molecular weight of 11ß, 12ß-epoxypukalide at m/z = 389.9 ($C_{21}H_{24}O_7$, M^+, 388.15221, Ksebati *et al*. 1984). This MS peak was observed at retention times between 19.0-19.5 minutes. Of note is a signal in the UV chromatogram (254 nm) (a), indicated by a black box centered at 19.3 minutes. This signal (UV peak) is associated with the MS peak of interest, depicted by a * in the total ion chromatogram (TIC) (a) and in the MS spectrum (b). It suggests the presence of a chromophore associated with 11ß, 12ß-epoxypukalide in the uncharacterized compounds in *L*. *virgulata.*

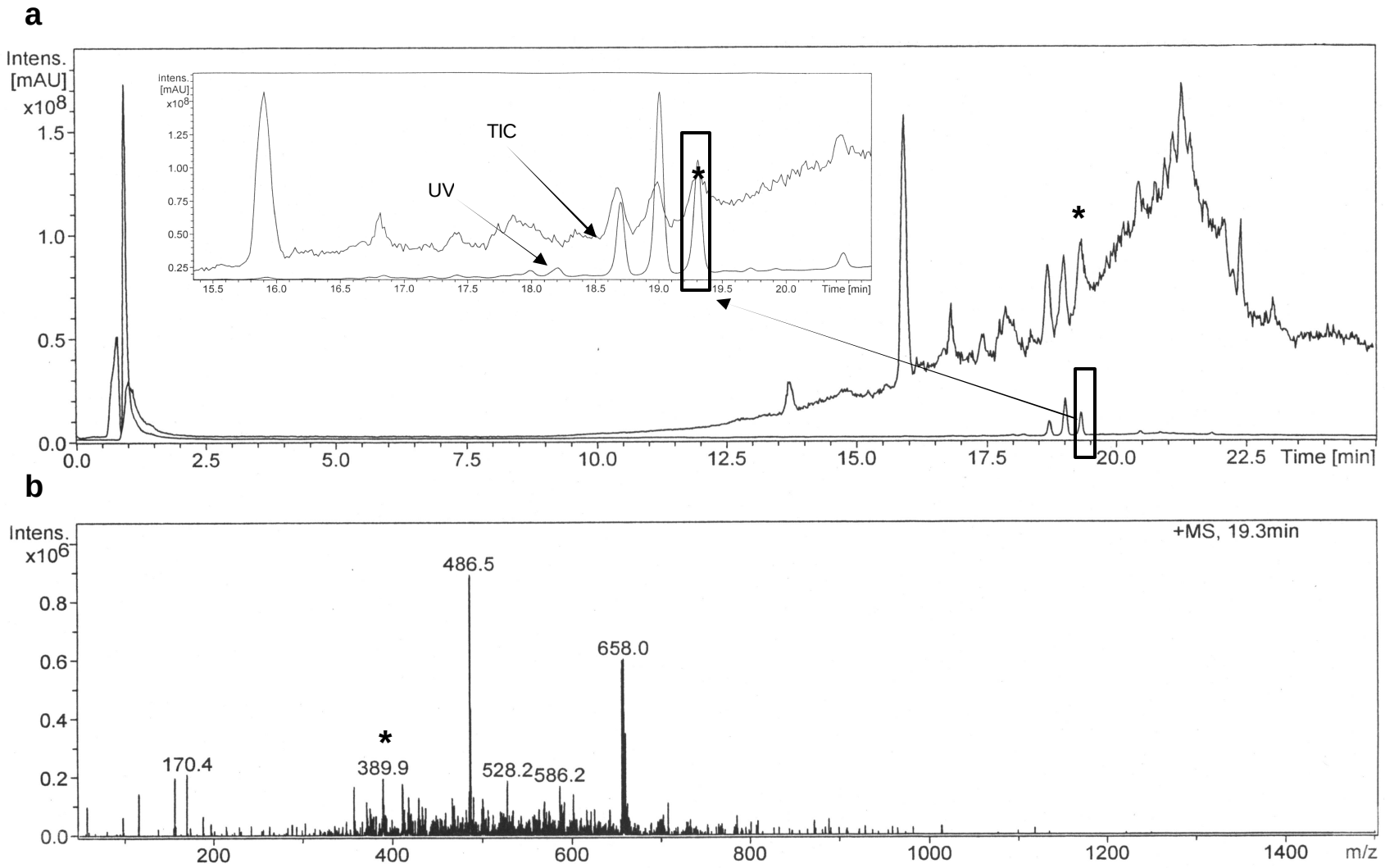

a
Intens.
[mAU]
x10^8
1.5
1.0
0.5
0.0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
Time [min]
TIC
UV
*
*
b
Intens.
x10^6
+MS, 19.3min
486.5
658.0
*
170.4
389.9
528.2
586.2
0.8
0.6
0.4
0.2
0.0
200
400
600
800
1000
1200
1400
m/z

Figure 23. **Time-averaged MS spectra analogous to pukalide and epoxypukalide**. The LC-MS spectra of one crude extract of *L. virgulata* with antimicrobial activity, taken from a set of 8 samples, is shown. Identical molecular weights to the signals of pukalide (m/z = 372.6) and 11ß, 12ß-epoxypukalide (m/z = 389.9) were undetectable when the UV chromatograms and MS spectra of each of the 8 uncharacterized crude extracts of *L. virgulata* were time-averaged in five-minute increments. Dotted arrows in the MS spectrum (b) at m/z 372.6 and 389.9 indicate the absence of diagnostic mass signatures associated with pukalide and epoxypukalide. The black box centered at 15-20 minutes in the time-averaged total ion (TIC) and UV chromatograms (a) includes the retention times analogous to pukalide and 11ß, 12ß-epoxypukalide at 18.8 and 19.3 minutes, respectively.

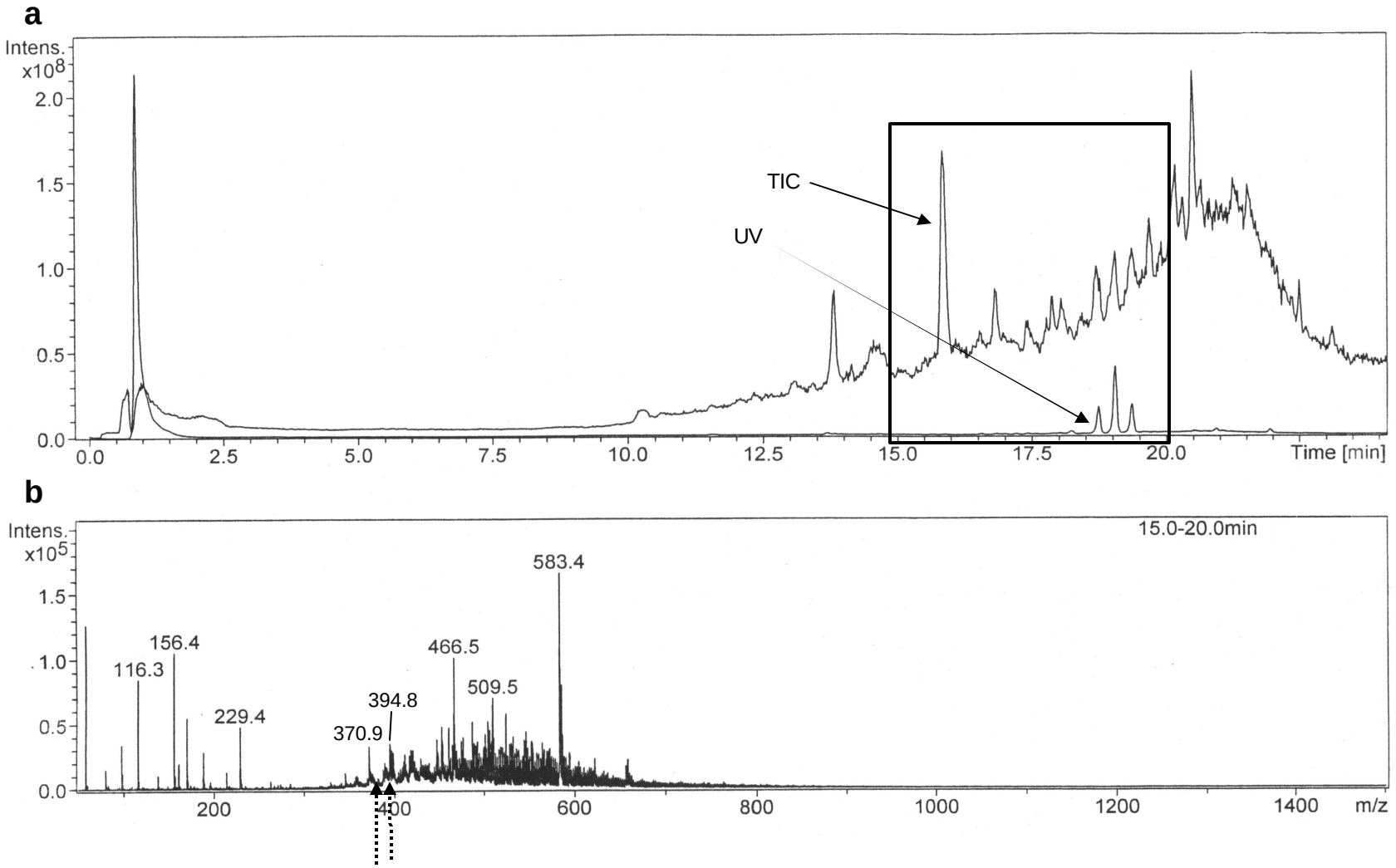

a
Intens.
x10^8
2.0
1.5
1.0
0.5
0.0
TIC
UV
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Time [min]
b
Intens.
x10^5
15.0-20.0min
583.4
156.4
116.3
466.5
509.5
394.8
229.4
370.9
1.5
1.0
0.5
0.0
200
400
600
800
1000
1200
1400
m/z

Figure 24. **Time-averaged MS spectra analogous to homarine**. The LC-MS spectra of one active (a/b) and one biologically inactive (c/d) crude extract, taken from a set of 8 samples, is shown. Mass peaks corresponding to the molecular weights of homarine (indicated by a * at m/z = 94.3, 138.3), identified in the MS spectra (b, d) of the active and non-active crude extracts of *L. virgulata,* were time-averaged in five-minute increments. The two LC-MS spectra depicted (a/b and c/d), time-averaged between 0.0-5.0 minutes, as indicated by the dotted black box on the total ion (TIC) and UV chromatograms (a, c), include the retention times analogous to homarine at 0.9-3.5 minutes. Note that the relative intensity, designated by a solid black box, of MS spectral peaks associated with homarine is greater in magnitude in the biologically active crude extract (a/b) than in the non-active extract (c/d), although the wet tissue weight of *L. virgulata* extracted was identical in both samples.

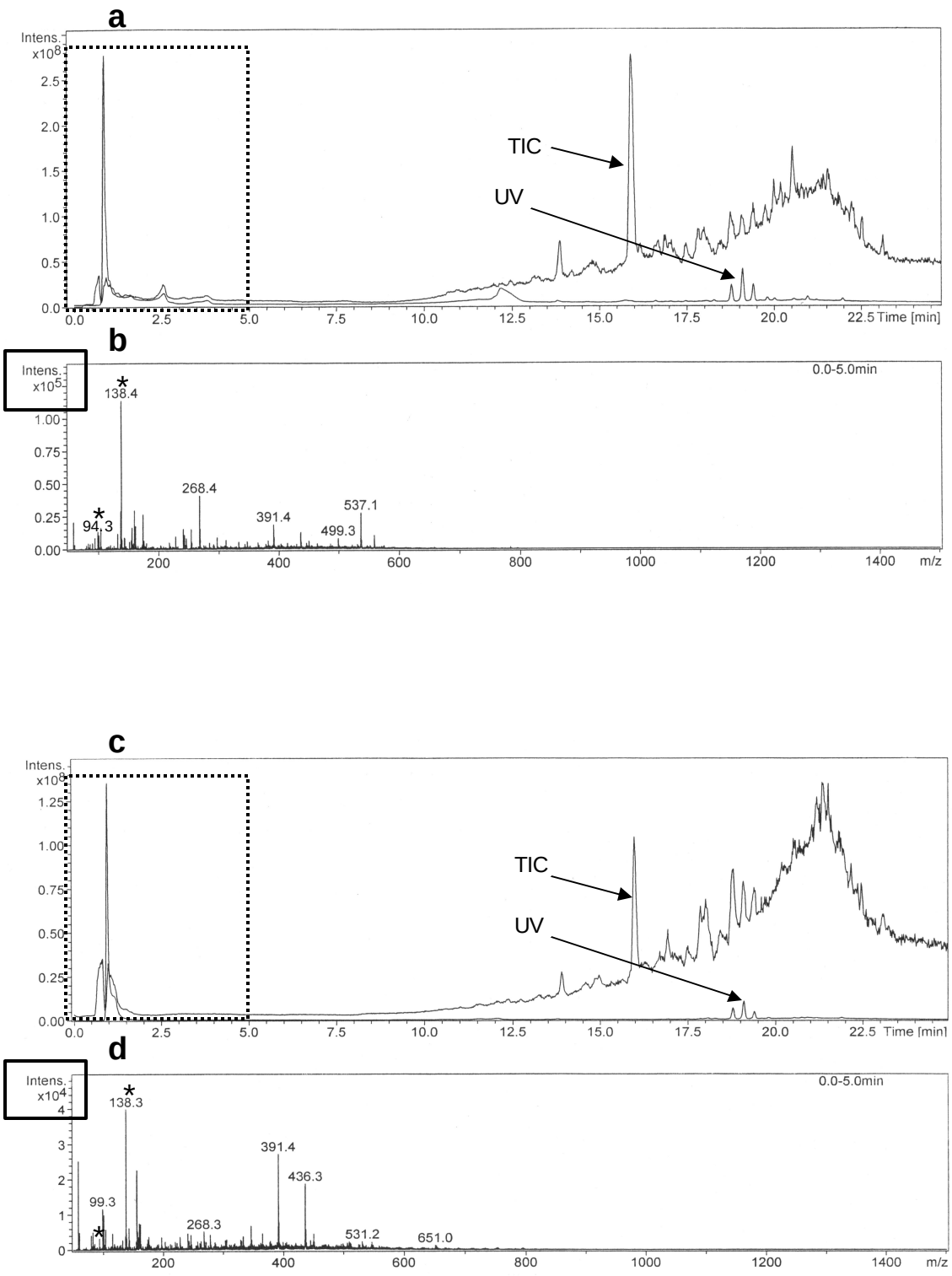

a
Intens.
x10^8
2.5
2.0
1.5
1.0
0.5
0.0
TIC
UV
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5 Time [min]
b
Intens.
x10^5
1.00
0.75
0.50
0.25
0.00
0.0-5.0min
138.4
268.4
94.3
391.4
499.3
537.1
200
400
600
800
1000
1200
1400
m/z
c
Intens.
x10^8
1.25
1.00
0.75
0.50
0.25
0.00
TIC
UV
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5 Time [min]
d
Intens.
x10^4
4
3
2
1
0
0.0-5.0min
138.3
391.4
436.3
99.3
268.3
531.2
651.0
200
400
600
800
1000
1200
1400
m/z

Figure 25. **LC-MS analysis of HPLC fraction 17**. Illustrated are the time-averaged (0.0-5.0 minutes), expanded UV chromatogram (a, denoted by an arrow) and mass spectrum (b) of HPLC fraction 17 from crude extracts of *L. virgulata* with documented antimicrobial activity. Note the peaks in the MS spectrum, indicated by a *, corresponding to the diagnostic mass signatures of homarine [m/z 94.3, 138.3].

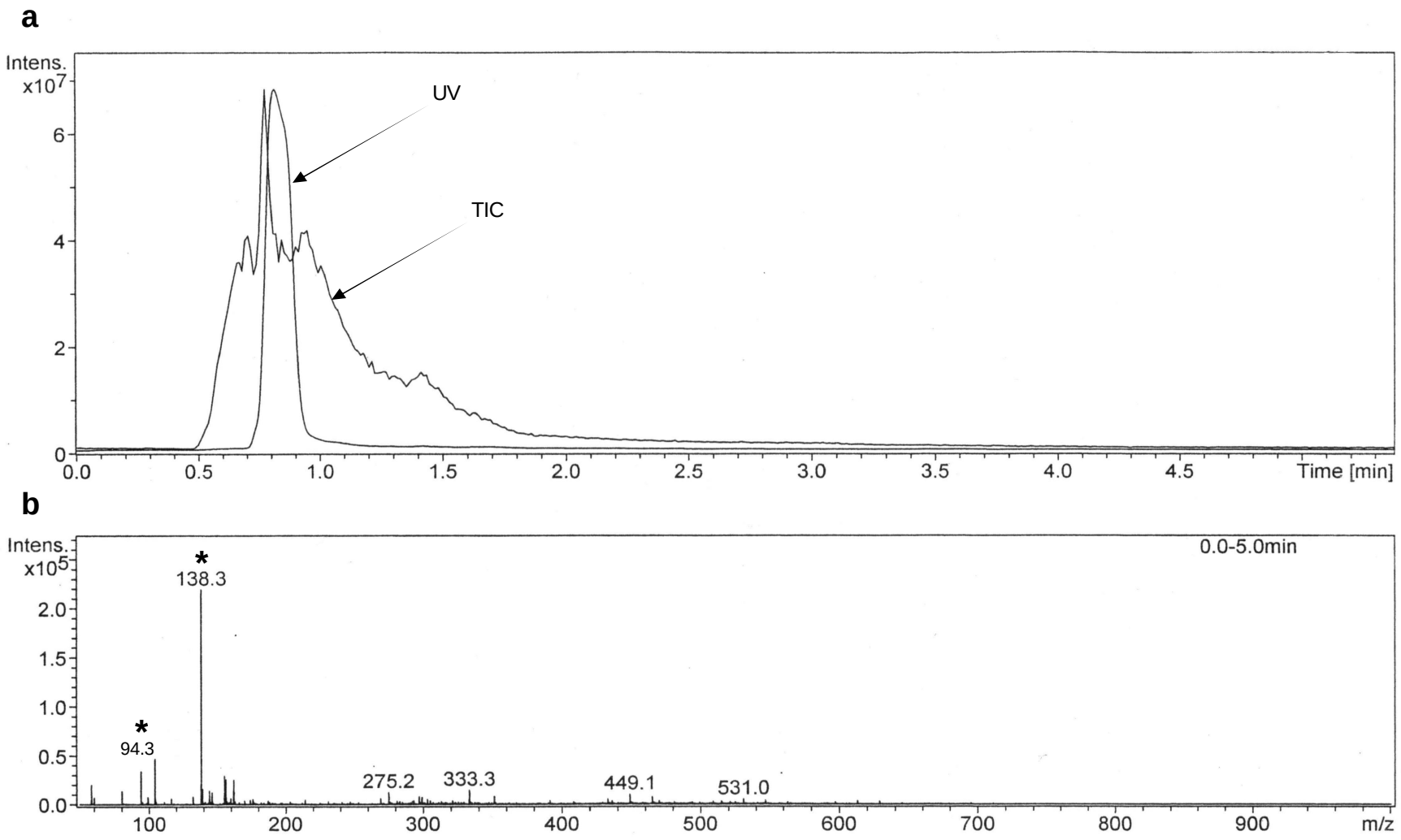

a
Intens.
x10^7
6
4
2
0
UV
TIC
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time [min]
b
Intens.
x10^5
2.0
1.5
1.0
0.5
0.0
0.0-5.0min
*
138.3
*
94.3
275.2
333.3
449.1
531.0
100
200
300
400
500
600
700
800
900
m/z

Figure 26. **LC-MS analysis of fraction 18**. Illustrated are the time-averaged (0.0-5.0 minutes), expanded UV chromatogram (a, denoted by an arrow) and mass spectrum (b) of HPLC fraction 18 from crude extracts of *L. virgulata* with documented antimicrobial activity. Note the peaks in the MS spectrum, indicated by a *, corresponding to the diagnostic mass signatures of homarine [m/z 94.3, 138.2].

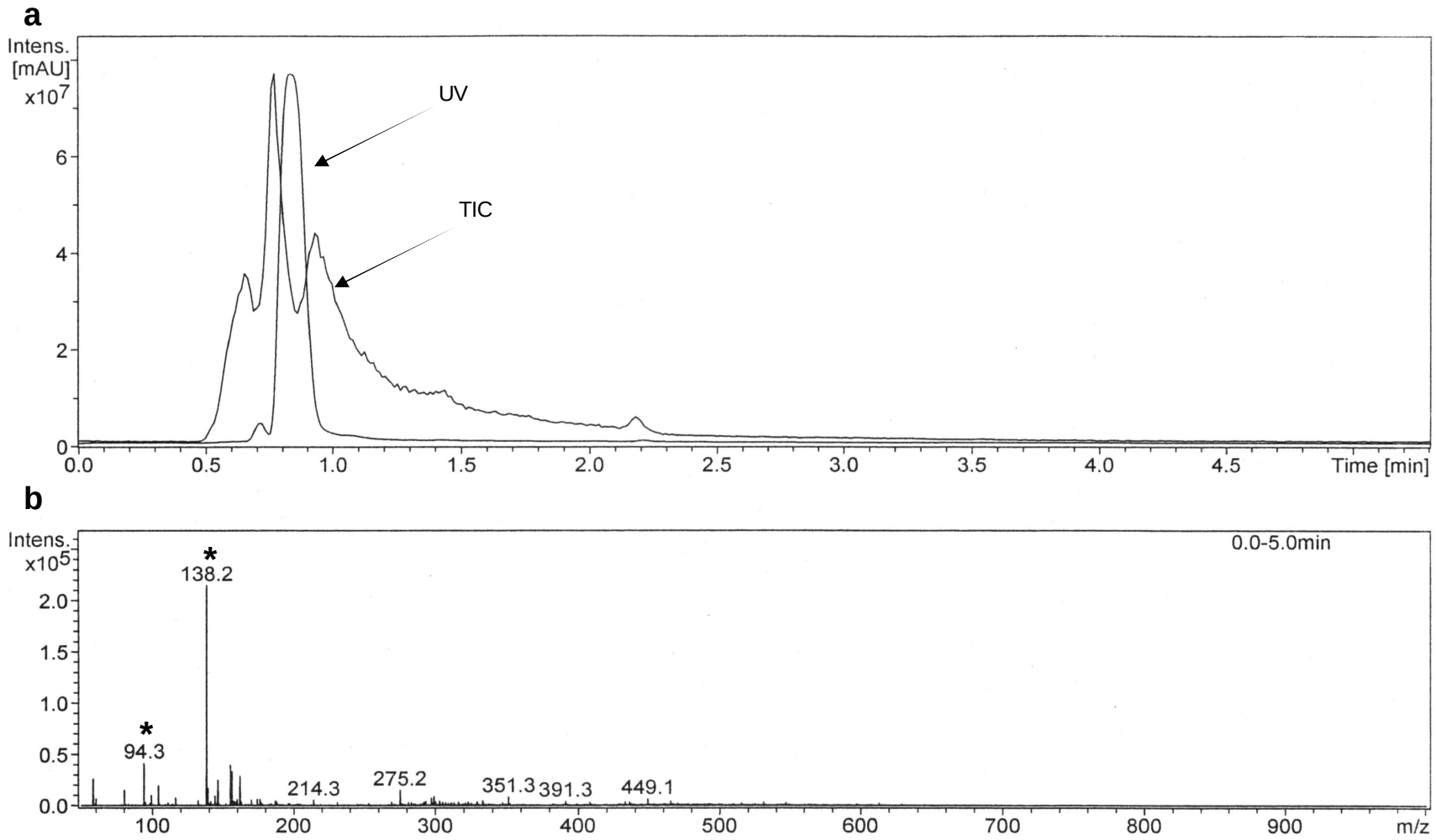

a
Intens.
[mAU]
x10^7
6
4
2
0
UV
TIC
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time [min]
b
Intens.
x10^5
2.0
1.5
1.0
0.5
0.0
0.0-5.0min
*
138.2
*
94.3
214.3
275.2
351.3
391.3
449.1
100
200
300
400
500
600
700
800
900
m/z

Figure 27. **LC-MS analysis of fraction 19**. Illustrated are the time-averaged (0.0-5.0 minutes), expanded UV chromatogram (a, denoted by an arrow) and mass spectrum (b) of HPLC fraction 19 from crude extracts of *L. virgulata* with documented antimicrobial activity. Note the peaks in the MS spectrum, indicated by a *, corresponding to the diagnostic mass signatures of homarine [m/z 94.4, 138.3].

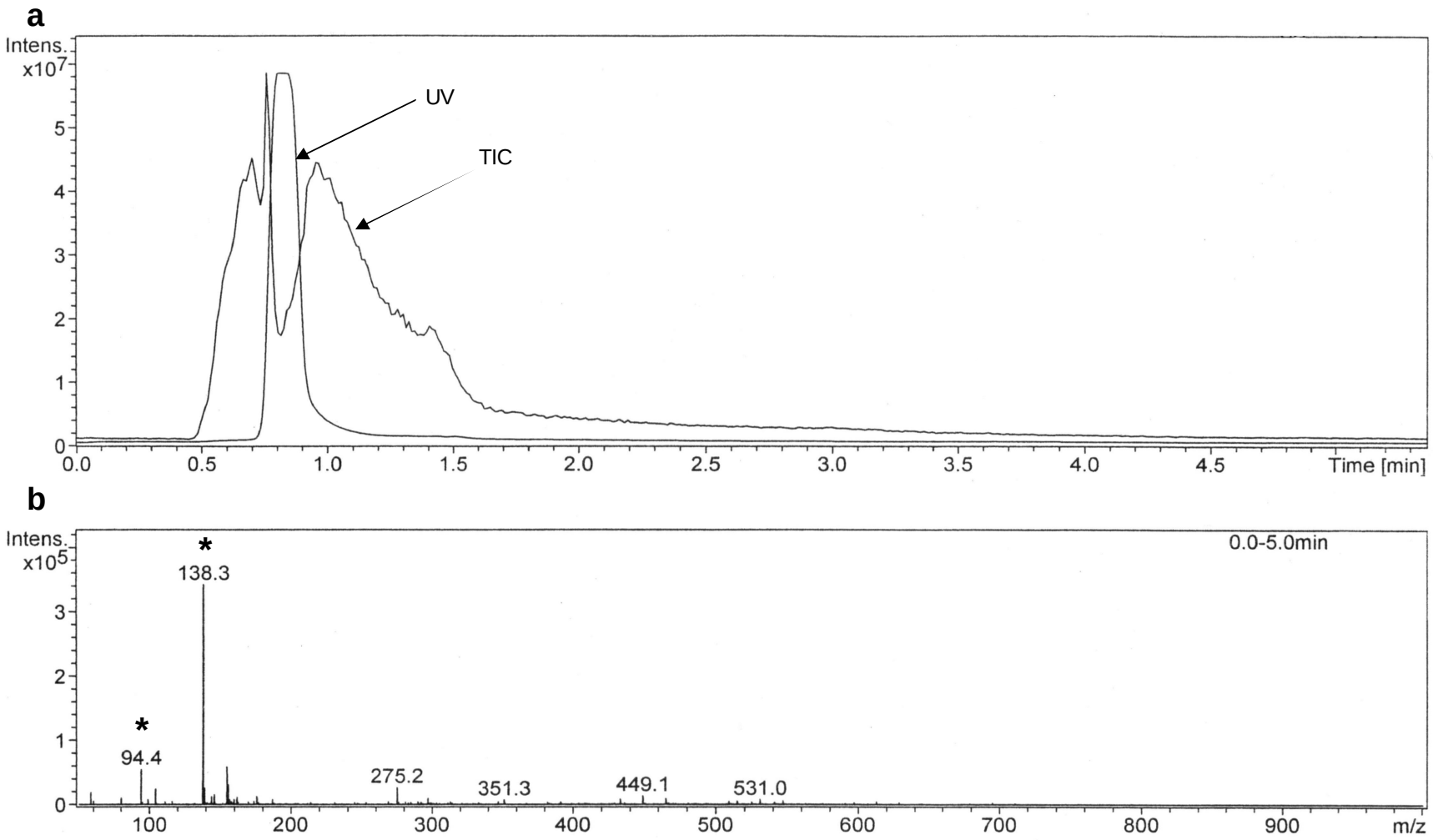

a
Intens.
x10^7
5
4
3
2
1
0
UV
TIC
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time [min]
b
Intens.
x10^5
3
2
1
0
0.0-5.0min
*
138.3
*
94.4
275.2
351.3
449.1
531.0
100
200
300
400
500
600
700
800
900
m/z

Figure 28. **^{1}H-NMR spectrum of fractions 14-21**. The chemical shifts (in ppm, d_4-methanol) for isolated signals of pooled fractions 14-21 in the ^{1}H-NMR spectrum include (a) 4.43, (b) 7.96, (c) 8.10, (d) 8.54, and (e) 8.79. These chemical shifts were identical to those reported by ^{1}H-NMR for analytically pure homarine (Figure 16, Table 3) and indicate the presence of homarine in fractions 14-21.

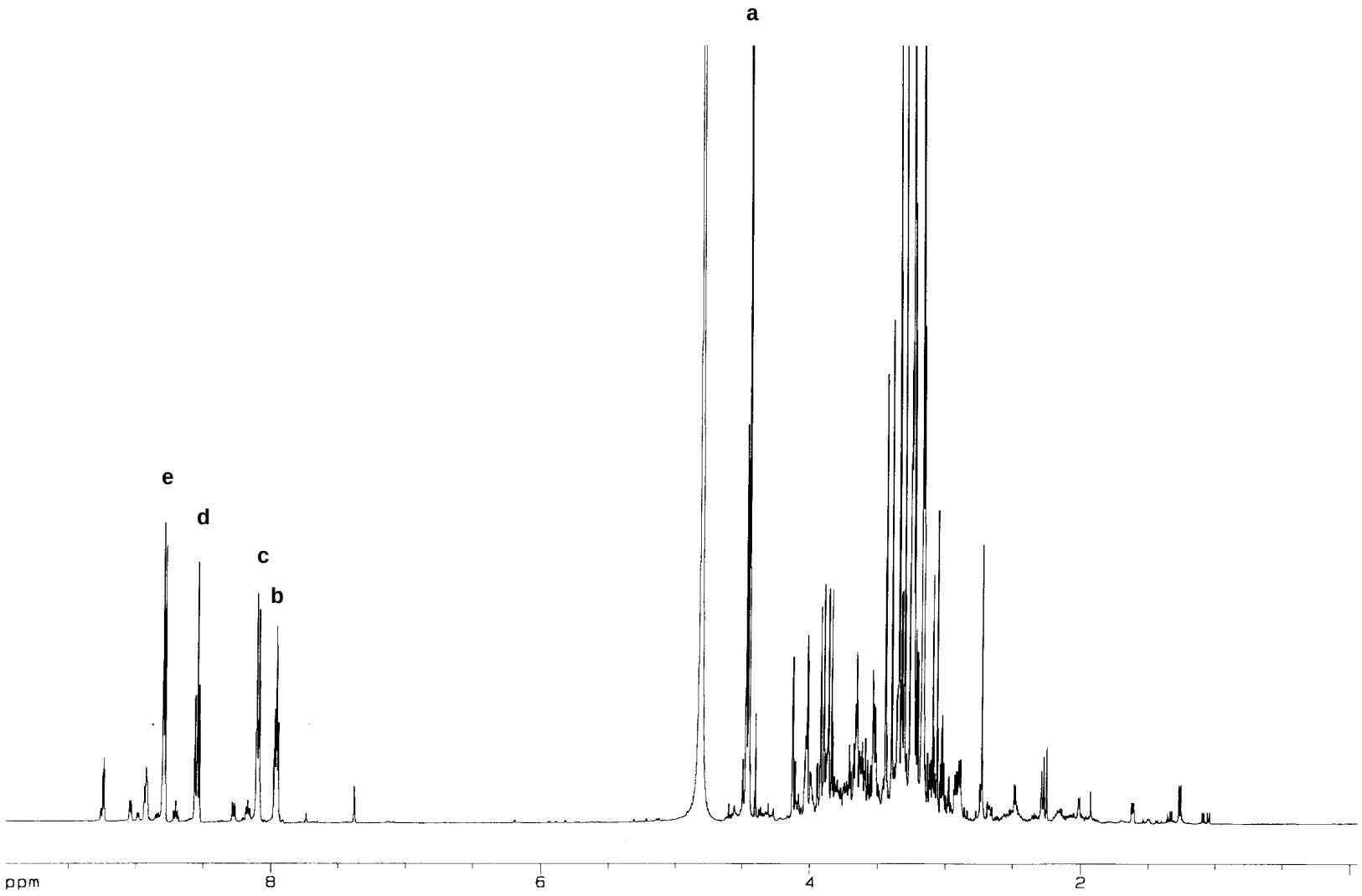

a
e
d
c
b
ppm
8
6
4
2

Figure 29. **Excised portion of ^{1}H-NMR spectrum of fractions 14-21**. Expansion of the 7.0-9.5 ppm region of the ^{1}H-NMR spectrum of pooled fractions 14-21 (Figure 28) indicated the presence of signals identical to the chemical shifts reported for homarine (a-d) (Figure 16, Table 3) and a homarine analog (e-h). The chemical shifts (in ppm, d_4-methanol) for isolated signals of fractions 14-21 representative of homarine and a homarine analog include (a) 7.96, C_5-H; (b) 8.10, C_3-H; (c) 8.54, C_4-H; and (d) 8.79, C_6-H; as well as a triplet at (e) 8.17, C_5-H; doublet at (f) 8.28, C_3-H; triplet at (g) 8.70, C_4-H; and a doublet at (h) 8.84, C_6-H.

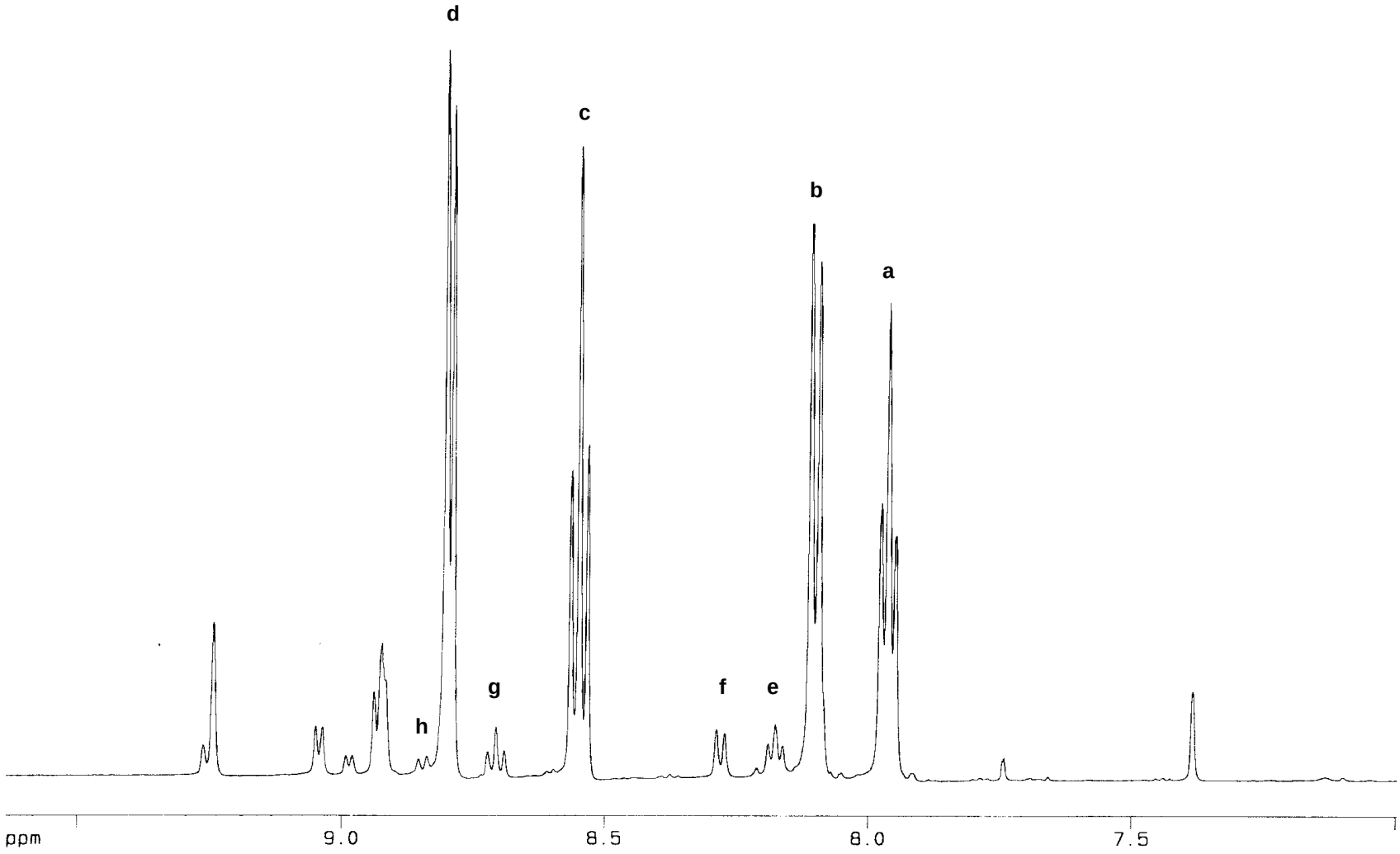

d
c
b
a
h
g
f
e
ppm
9.0
8.5
8.0
7.5

Figure 30. **^{13}C-NMR spectrum of fractions 14-21**. The chemical shifts for isolated signals of pooled fractions 14-21 in the ^{13}C-NMR spectrum include (a) 46.8, (b) 126.14, (c) 145.05, (d) 145.60, (e) 154.2, and (f) 163.95. These chemical shifts were identical to those reported by ^{13}C-NMR for analytically pure homarine (Figure 17, Table 3) and indicate the presence of homarine in fractions 14-21.

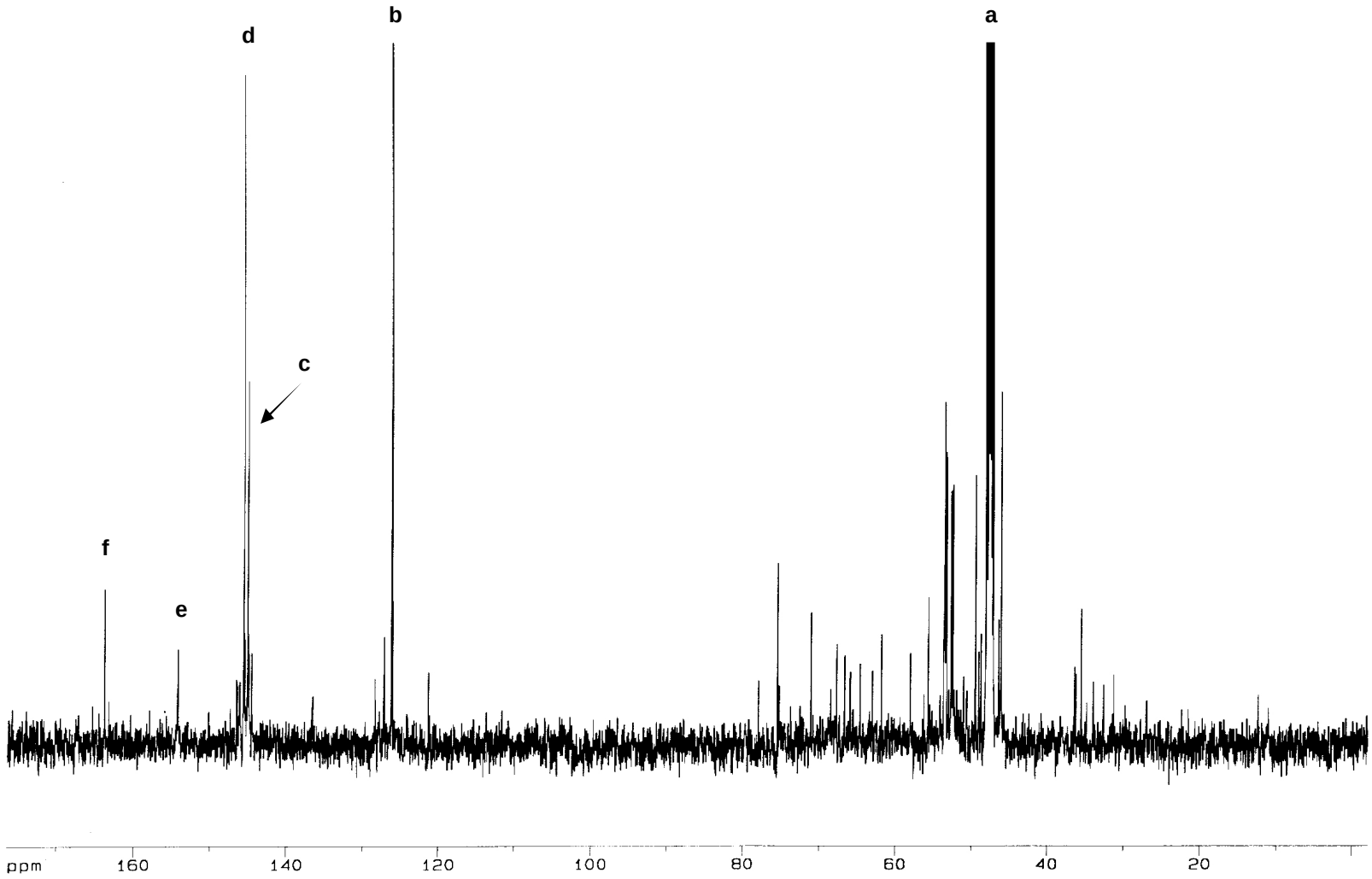

a
b
d
c
f
e
ppm
160
140
120
100
80
60
40
20

Figure 31. **Excised portion of ^{13}C-NMR spectrum of fractions 14-21**. Expansion of the 120-170 ppm region of the ^{13}C-NMR spectrum of pooled fractions 14-21 (Figure 30) indicated the presence of signals identical to the chemical shifts reported for homarine (Figure 17, Table 3). The chemical shifts (in ppm, d_4-methanol) for isolated signals of fractions 14-21 include (a) 126.14, $C_{5,3}$; (b) 145.05, C_6; (c) 145.60, C_4; (d) 154.2, C_2; and (e) 163.95, CO and indicate the presence of homarine in these pooled fractions.

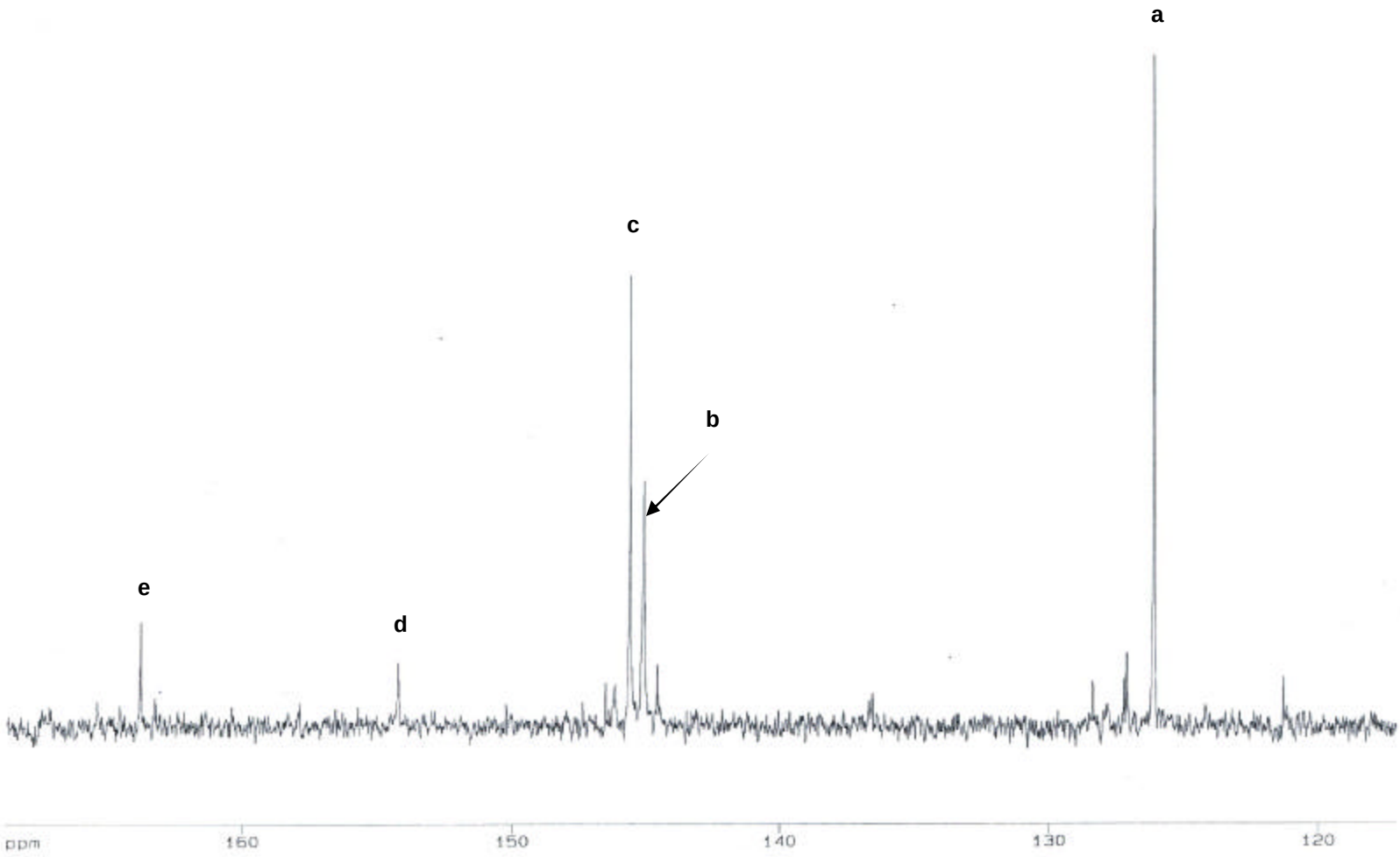

a
c
b
e
d
ppm
160
150
140
130
120

Figure 32. **^{1}H-NMR spectrum of fractions 22-24**. The chemical shifts (in ppm, d_4-methanol) for isolated signals of pooled fractions 22-24 in the ^{1}H-NMR spectrum include (a) 4.44, (b) 7.94, (c) 8.09, (d) 8.53, and (e) 8.76. These chemical shifts were identical to those reported by ^{1}H-NMR for analytically pure homarine (Figure 16, Table 3) and indicate the presence of homarine in fractions 22-24.

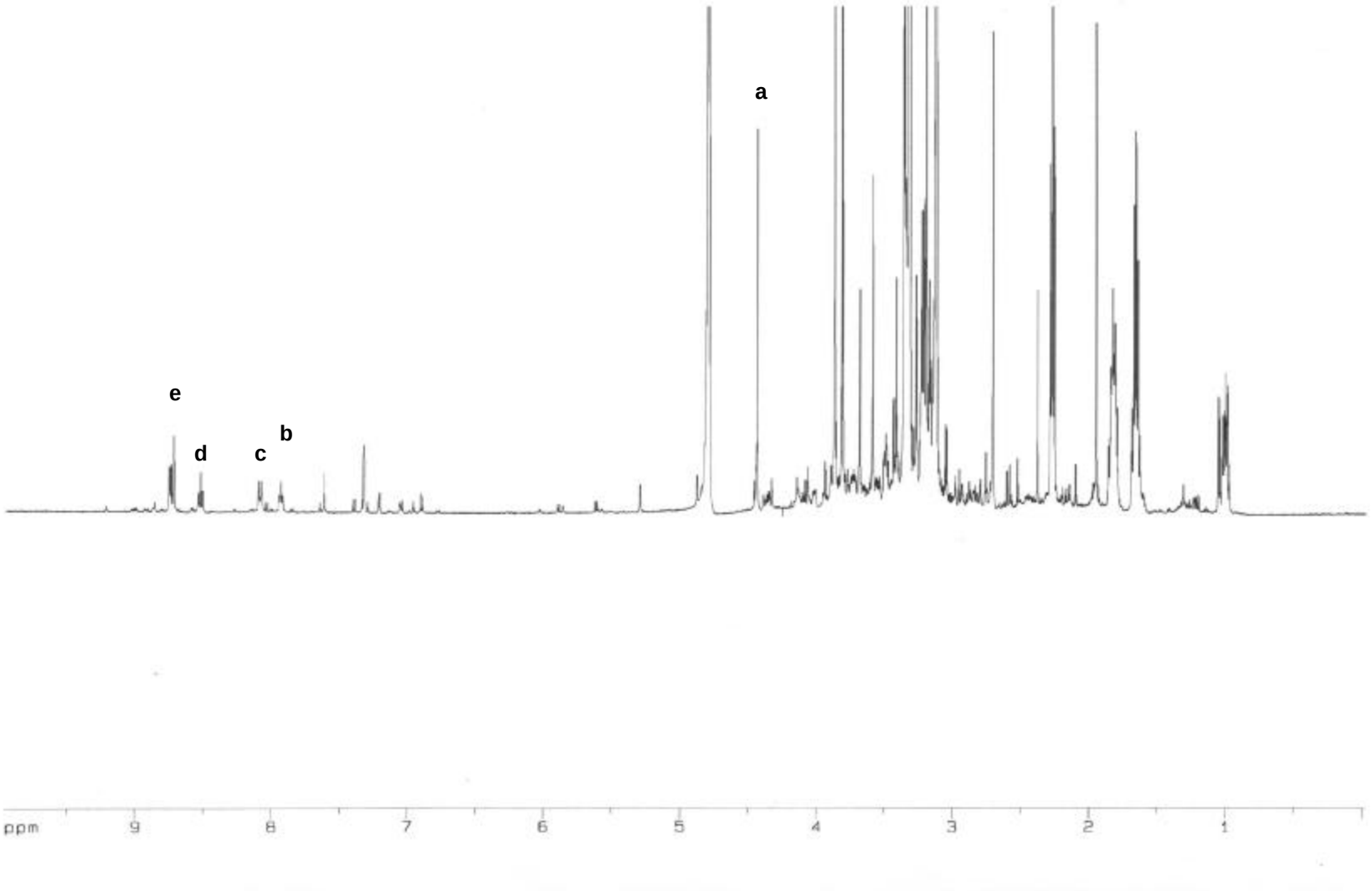
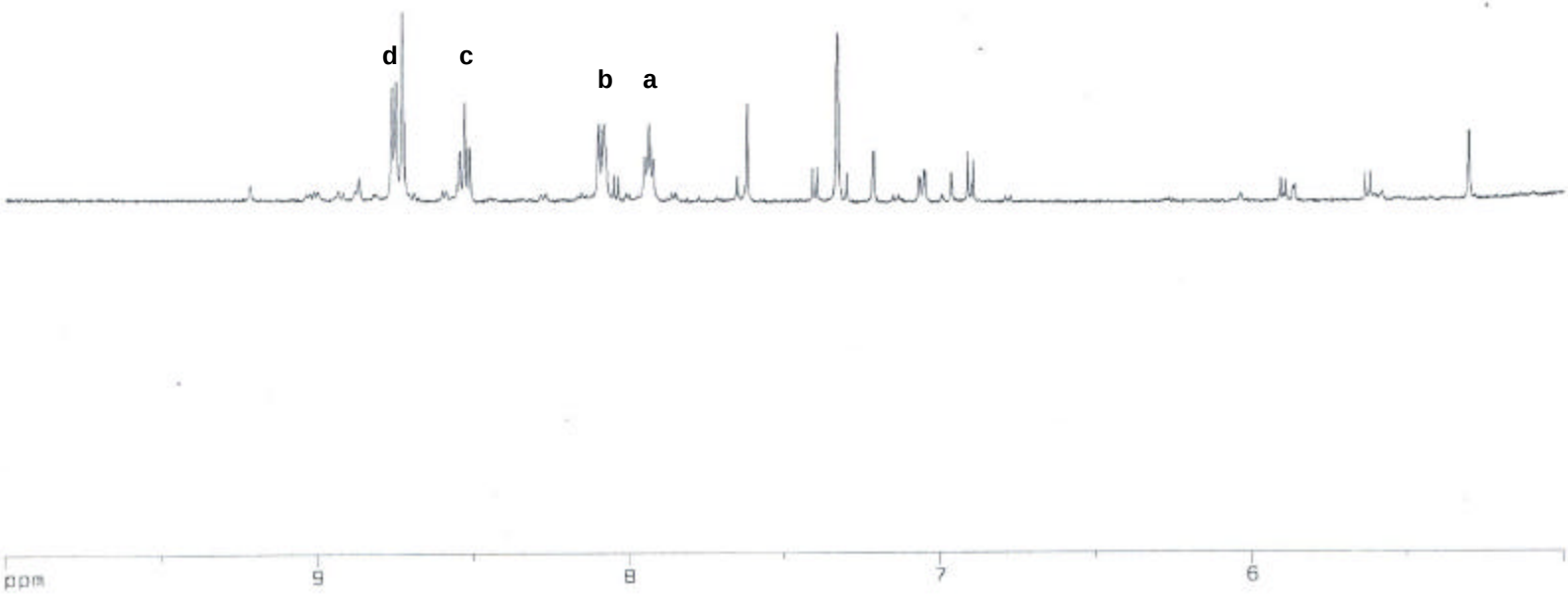

a
e
d
c
b
ppm
9
8
7
6
5
4
3
2
1

Figure 33. **Excised portion of ^{1}H-NMR spectrum of fractions 22-24**. Expansion of the 5.0-10.0 ppm region of the ^{1}H-NMR spectrum of pooled fractions 22-24 (Figure 32) indicated the presence of signals identical to the chemical shifts reported for homarine (Figure 16, Table 3). The chemical shifts (in ppm, d_4-methanol) for isolated signals of fractions 22-24 include (a) 7.94, C_5-H; (b) 8.09, C_3-H; (c) 8.53, C_4-H; and (d) 8.76, C_6-H and indicate the presence of homarine in these pooled fractions.


d
c
b
a
ppm
9
8
7
6