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Marine natural products chemistry: Investigations in marine ecology and structure determination

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University of Hawaii, 1993

MARINE NATURAL PRODUCTS CHEMISTRY: INVESTIGATIONS IN MARINE ECOLOGY AND STRUCTURE DETERMINATION

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
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IN

CHEMISTRY

DECEMBER 1993

By

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For my wife, Gail.

I love you.

ACKNOWLEDGMENTS

Five years ago, this month, Gail and I landed in these islands and embarked on an adventure that ends with this dissertation. The path that we walked was smoothed by many people that we now know as friends. These people are varied and many but we owe them all a debt of gratitude that a mere mention in an acknowledgment can not begin to cover. I know that Gail and I will never forget the aloha that was shown us during our stay.

I would like to start by thanking Dr. Scheuer and my research committee for their guidance, support and patience over the past five years. The Scheuer research group, including all the post-docs and visiting faculty over the years, deserve a thanks also. Drs. Brian Popp and Fred Dobbs, whom I had the honor of collaborating with, showed me kindness and guidance that I will never forget. A big mahalo is due to my fellow graduate students for their friendship and support.

The support staff here at the Chemistry Department are a fantastic bunch - always willing to go the extra mile, especially if there's beer at the end. Faith Caplan and Kay Larsen have provided test results and a shoulder to cry on. Wes Yoshida is like a brother to me. Dr. Walt Niemczura and Mike Burger have always come through for me when it comes to data or broken electronics. Bill Cooper is a friend, an artist and a craftsman. Whenever things got hairy the glass shop was a place of refuge for me.

ABSTRACT

Chemical investigation of five marine invertebrates has led to the isolation and identification of a variety of new compounds and insights into the ecology of the organisms. A majority of the compounds exhibit some type of biological activity, including antiviral, cytotoxic and immunomodulatory properties. A new approach to the study of marine ecology utilizing carbon isotope ratio mass spectrometry as a tracer technique showed promising preliminary results.

Ulapualide B (1) is a macrolide that was first isolated from the eggmasses of the nudibranch *Hexabranchus sanguineus* but which presumably is sequestered by the mollusk from a dietary source as a defensive chemical. The compound is then passed on to its eggmasses which have a known predator, the aeolid nudibranch *Favorinus japonicus*. This hypothesis was tested by using isotope ratio mass spectrometry to "fingerprint" ulapualide and follow the compound from the nudibranch to its eggmasses.

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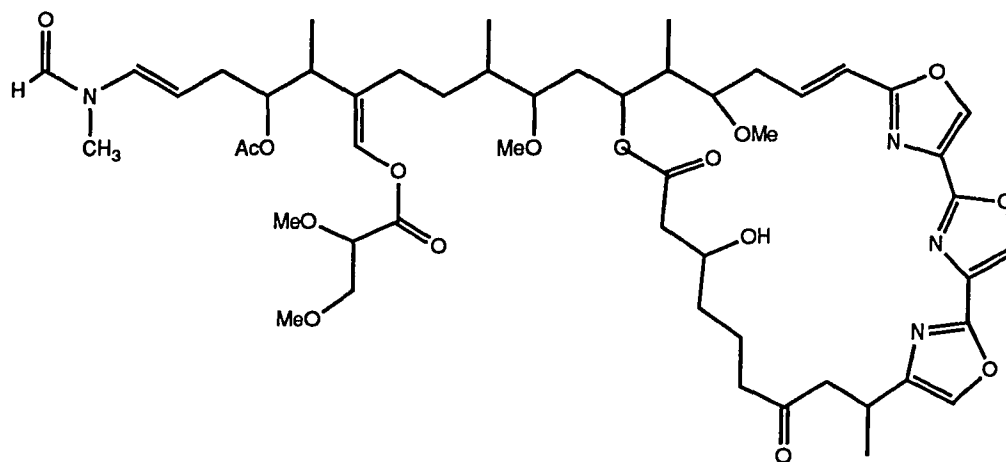
Preliminary results indicate that ulapualide is not simply passed from the nudibranch to the eggmasses. It was also shown that ulapualide is present in *Favorinus japonicus*.

A sponge of the genus *Strongylophora*, collected at Puako, Hawaii, has yielded three C-19 oxidized pregnanes (**2, 3** and **4**) from the lipophilic extract. One of the compounds showed marginal cytotoxicity against two tumor cell lines. Some pregnanes are human sex hormones which suggests their potential therapeutic use. The structure of **2** was confirmed by x-ray diffraction crystallography.

| **2** | **3** | **4** |
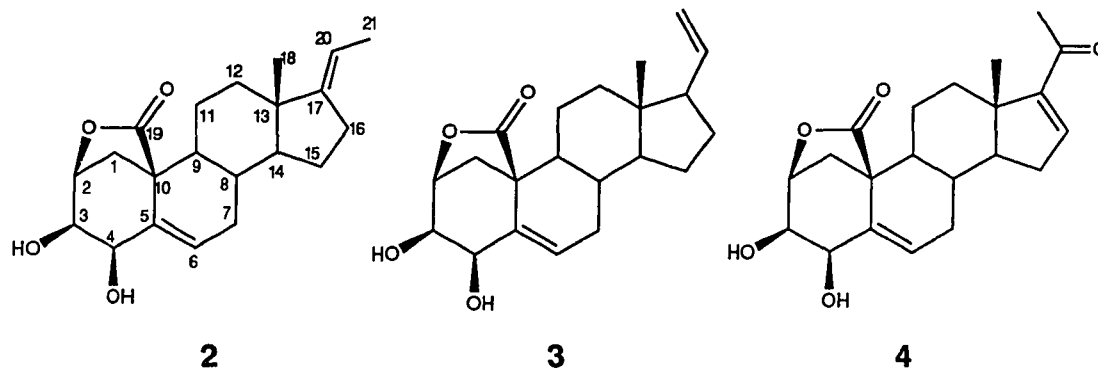
The volatile halogenated constituents of the acorn worm, *Balanoglossus aurantiacus* were determined by gas chromotography-mass spectrometry. These compounds were under investigation for their possible role as mediators of the microbiology in the animals' habitat. Twenty compounds containing

halogens were identified in the extracts of the animal. The compounds consisted of one chlorinated aromatic, twelve brominated aromatics, five aromatic compounds containing both chlorine and bromine, and two nitrogenous compounds containing chlorine. None of the chlorinated compounds had previously been reported from acorn worms.

A sponge of the genus *Rhabdastrella*, collected in Manado, Sulawesi, Indonesia, yielded stelliferinoside (5), a new isomalabaricane triterpenoid containing a ribopyranose glycoside, and the previously described stelliferin A (6). Both compounds show marginal cytotoxicity against two tumor cell lines.
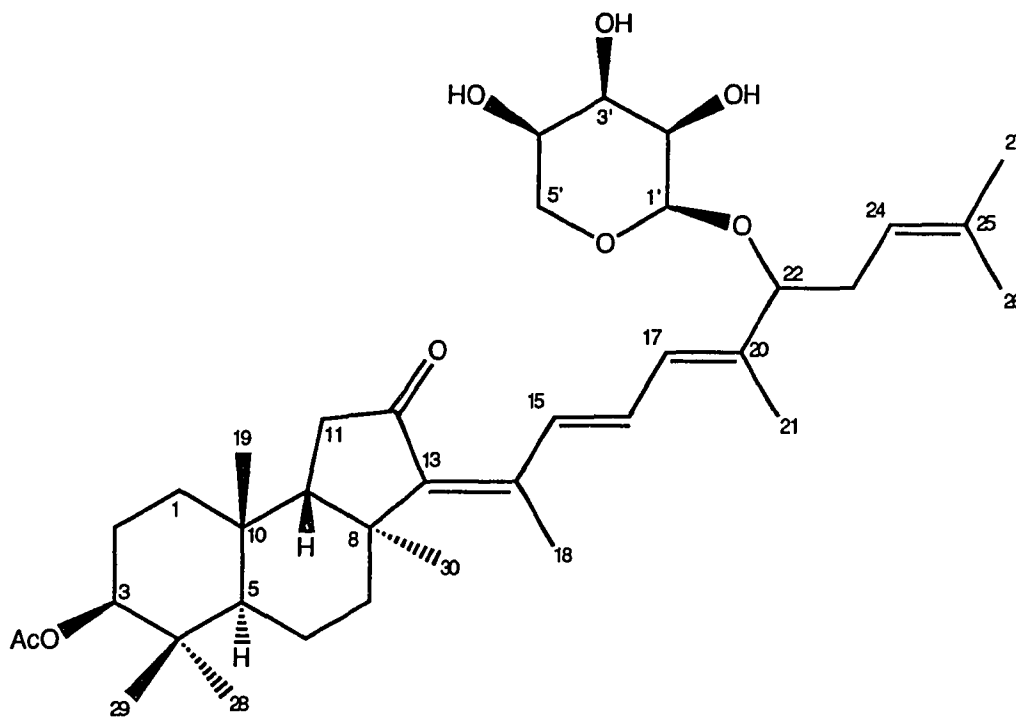
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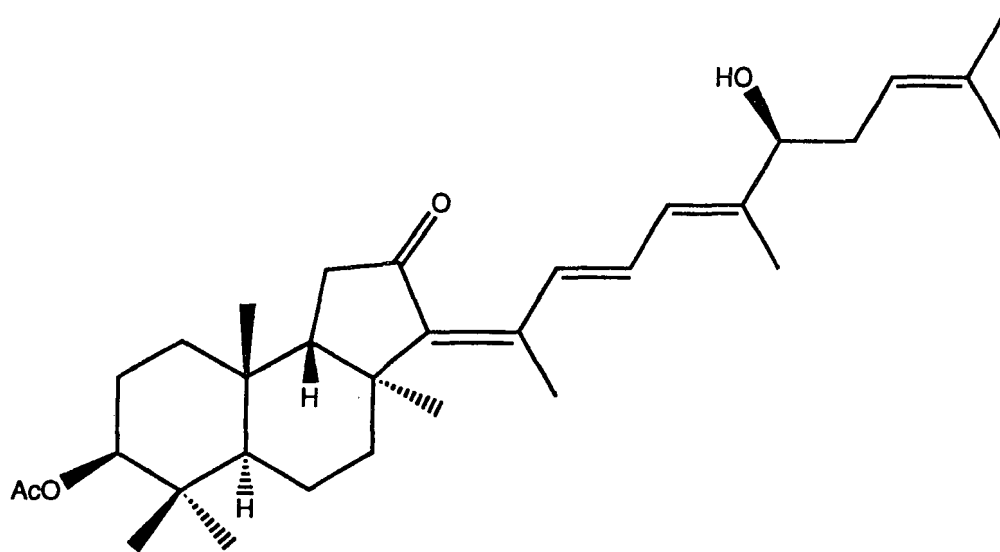
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A phenolic compound **7** isolated from a sponge collected in Manado, Indonesia, contains three stereocenters but has zero optical rotation at the sodium D line. This compound is structurally related to compounds shown to inhibit H, K-ATPase activity.

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LIST OF ABBREVIATIONS

A-549	= Human lung carcinoma cell cultures
br	= broad signal
COSY	= ^{1}H-^{1}H Correlation spectroscopy
CV-1	= African green monkey kidney cell cultures
d	= doublet
DHFR	= Dihydrofolate reductase
DMAP	= Dimethylaminopyridine
DNA	= Deoxyribonucleic acid
EIMS	= Electron impact mass spectrometry
FABMS	= Fast atom bombardment mass spectrometry
HIV-1	= Human acquired immunodeficiency virus
HIV-RT	= Human acquired immunodeficiency virus reverse transcriptase
HMQC	= ^{1}H-^{13}C (1 bond) Heteronuclear multiple quantum coherence spectroscopy
HMBC	= ^{1}H-^{13}C (2 and 3 bond) Heteronuclear multiple bond correlation spectroscopy
HPLC	= High pressure liquid chromatography
HSV- I, II	= Herpes simplex virus I, II
HT-29	= Human colorectal adenocarcinoma cell cultures
IC$_{50}$	= Inhibitory concentration 50%
IR	= Infrared spectroscopy
IRMS	= Isotope ratio mass spectrometry

LIST OF ABBREVIATIONS (CONTINUED)

KB	= Human epidermoid carcinoma cell cultures
L1210	= Mouse lymphocytic leukemia cell cultures
LoVo	= Human colorectal adenocarcinoma cell cultures
m	= Multiplet
MHz	= Megahertz
M. I. C.	= Minimum inhibitory concentration
NOE	= Nuclear Overhauser enhancement
o. d.	= Outside diameter
p	= Pentet
P-388	= Mouse lymphocytic leukemia cell cultures
PI	= Phosphatase inhibition
q	= Quartet
RNase H	= Ribonuclease H
ROESY	= Rotating frame nuclear Overhauser spectroscopy
s	= singlet
t	= triplet
TS	= Thymidylate synthetase
TLC	= Thin layer chromatography
UV	= Ultraviolet spectroscopy

CHAPTER 1. INTRODUCTION

A. General Remarks

Although the majority of plant and invertebrate phyla are either exclusively or predominately marine, natural products of marine origin had been generally neglected by chemists before the 1950's because of the difficulties associated with the collection of marine organisms. Since the advent of scuba, organic chemists have looked to the marine environment for organisms containing secondary metabolites which possess properties valuable to man. These properties include compounds possessing biological activity including, but not limited to, anticancer, antiviral, and antiparasitic. An analysis of the phyletic distribution of compounds isolated from the marine environment in the decade 1977-1987 shows that 93% originate from four groups: algae (35%), sponges (29%), coelenterates (22%) and echinoderms (7%).[1] This distribution reflects the ease of collecting large quantities of the organism rather than predominance of bioactive compounds. In fact, ascidians, while not greatly studied due to the difficulty in collecting them, are responsible for some of the most interesting and exciting marine natural products being studied. Chemists have only just begun to harvest the huge resource of bioactive compounds that are produced in the marine environment.

Natural product chemists have historically investigated the origin of conspicuous properties. The rich purple color of Tyrian purple, isolated from a marine mollusc for use as a dye, inspired chemical investigation of the structure

of the compound.[2] Palytoxin, the toxic component of the zooanthid *Palythoa toxica*, was investigated because of a legend involving ancient Hawaiians dipping their spears in the organism before battle.[3] The observation of lack of predation of large brightly colored eggmasses of the nudibranch *Hexabranchus sanguineus* led chemists to the isolation of the macrolide ulapualide.[4] Recently, because of advances in microbiology, isolation has also been guided by assays related to specific bioactivity. Both these approaches have led to the discovery of new and useful compounds. To quote A. V. Hill, the distinguished British physiologist; "By the method of comparative physiology, or of experimental biology, by the choice of suitable organ, tissue or process, in some animal far removed in evolution, we may often throw light upon some function or process in higher animals or in man."[5]

B. Antitumor and Cytotoxic Metabolites

The primary focus of many researchers working in the field of marine natural products chemistry has been the isolation of compounds displaying antitumor or cytotoxic properties. These compounds are typically isolated by a bioassay-guided isolation scheme. These bioassays consist of measuring toxicity of extracts against cultured cancer cell lines. The cancer cell lines most commonly used *in vitro* include human epidermoid carcinoma cell cultures (KB), human colorectal adenocarcinoma cell cultures (LoVo) and mouse lymphocytic leukemia cell cultures (P388 and L1210). Simpler assays which correlate to cytotoxicity include the brine shrimp assay and inhibition of development of fertilized sea urchin or starfish eggs.[6]

The National Cancer Institute defines cytotoxicity as toxicity to cultured tumor cells, an active cytotoxic extract as having an $IC_{50} < 20$ µg / mL and an active cytotoxic compound as having an $IC_{50} < 4$ µg / mL. The terms antitumor and antineoplastic are reserved for *in vivo* actitivity in experimental models while anticancer applies to describing result of clinical trials in humans.[6]

A review of compounds displaying cytotoxic activity from the marine environment reported prior to 1987 listed approximately 185 compounds.[7] A similar review covering the literature from 1987 to 1991 showed 404 new compounds.[7] This reflects both the increase in the number of researchers doing marine natural product research and the success of bioassay-guided isolation schemes.

1. Spongothymidine

Spongothymidine (**1**, arabinosyl thymine, ara-T) and spongouridine (arabinosyl uracil; ara-U) were isolated from the sponge *Cryptotethya crypta* collected in the Carribbean in the early 1950s[8] and, after many years and numerous serendipitous events, became the prototypes of important drugs. An analog of arabinosyl cytosine (**2**, ara-C, cytarabine) displayed *in vivo* antileukemic activity. This activity is due to the conversion of arabinosyl cytosine to arabinosyl cytosine triphosphate, incorporation into cellular DNA and subsequent inhibition of DNA polymerase.[9] Arabinosyl cytosine is now in clinical use for the treatment of acute myelocytic leukemia[10] and non-Hodgkin's

lymphoma.[11] Another analog, ara-A (arabinosyl cytosine, vidabarine) is indicated for the treatment of herpes infections of the eye.

1   2
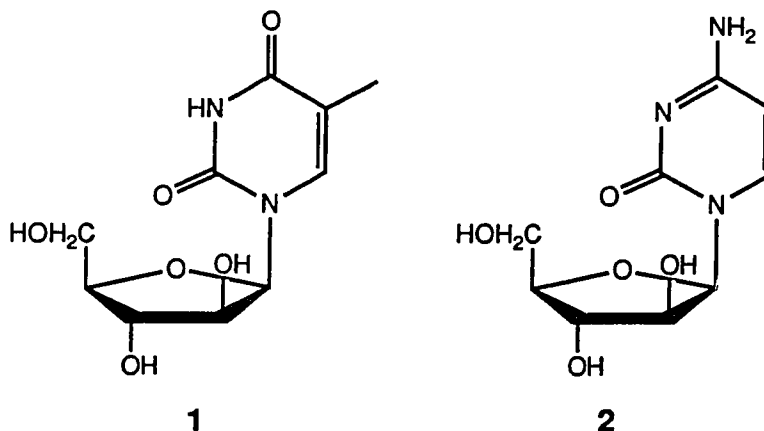
2. The Didemnins

The didemnins are cyclic depsipetides isolated from *Trididemnum solidum*, a gray-green tunicate that covers rocks or coral at depths to 120 feet in the waters off Belize, Honduras, Mexico, Colombia and Panama. The structures of didemnins A, B (3) and C were elucidated using NMR spectroscopy and various mass spectrometry techniques including the then new technique of fast atom bombardment. In all, nine different didemnins have been reported of which didemnin B has received the most attention from the pharmaceutical industry.[12]
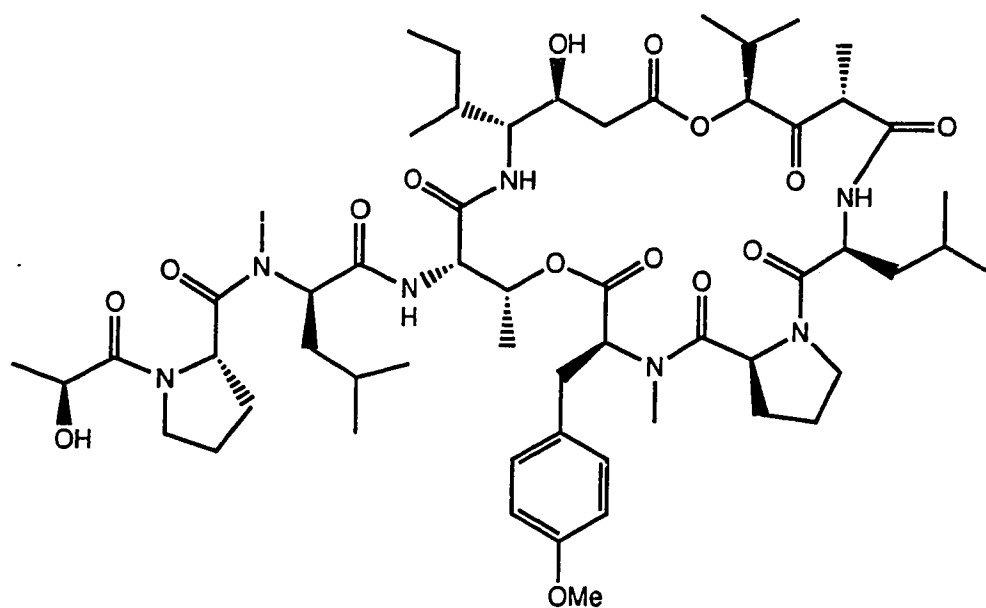
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These compounds display an incredible array of bioactivity. In addition to antitumor activity, this family of compounds has displayed antiviral and immunosuppressive activities. The antitumor activity results from inhibition of lymphokine-induced protein and DNA synthesis.[13] Didemnin B advanced to a phase II clinical trial against advanced renal carcinoma for which it proved not to be efficacious.[14] Preliminary results of phase II clinical trials against colorectal cancer are also unfavorable.[15]

The ultimate fate of the didemnins as clinically useful compounds is yet undecided. The recent discovery of dehydrodidemnin B from *Aplidium*

albicans, a Mediterranean tunicate of a different family from that of *Trididemnum solidum*, has regenerated excitement in this family of compounds. Dehydrodidemnin B has proven to be three to five times more potent against P388 leukemia, B16 melanoma and lung tumors than didemnin B in *in vivo* testing.[16] *In vivo* testing also showed significant differences in antiviral bioactivities between didemnin A and B although they differ only in their side chain. This suggests that chemical modification of this family of compounds may result in improved therapeutic potential.

3. The Bryostatins

The bryostatins are highly oxygenated macrolides isolated primarily from the bryozoan *Bugula neritina* collected in waters off California, in the Gulf of California, the Gulf of Mexico and the Gulf of Sagami.[17] Bryostatin 1 (4) was found to activate DNA synthesis in a fashion similar to phorbol esters. While bryostatin 1 induces only a subset of the biological responses induced by the phorbol esters and competes with phorbol esters for binding to protein kinase C, It inhibits tumor promotion and blocks the effects of phorbol esters.[18] These compounds show potential for inhibiting protein kinase C tumor promotion and are under continuing investigation as antitumor agents.
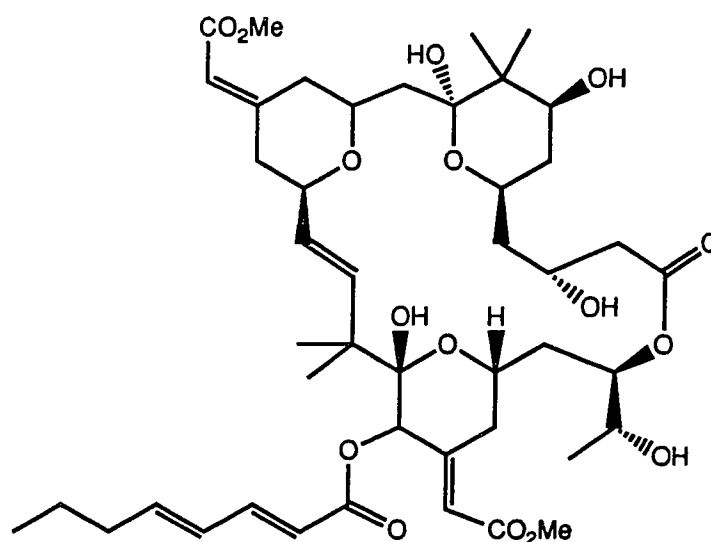
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The bryostatins reflect one of the more critical aspects of marine natural products, low yield. These compounds are isolated in low and variable yields and although there is a synthetic route, it is not considered commercially feasible.[19] Bryostatins have also been isolated from the sponge *Lissodendoryx isodictyalis*[20] and the ascidian *Aplydium californicum*.[21] Although some of the bryostatins isolated from these organisms were unique, samples collected for taxonomy were found to be contaminated with *Bugula neritina*.

C. Antiviral Metabolites

With the current AIDS epidemic, interest in antiviral compounds has increased dramatically. Antiviral drugs are rare and toxicity is a serious problem. Many classes of compounds, including peptides, heterocycles and terpenes, have shown antiviral properties. The modes of action of many of these varied compounds are unstudied and will provide an active area of research in the coming years.

Antiviral compounds are typically isolated by bioassay-guided separation schemes. In addition, compounds that display antimitotic properties are candidates for antiviral testing because they interfere with cell division rather than nucleic acid or protein synthesis.[22] Common antiviral bioassays test against *Herpes simplex* virus type 1 and 2 (HSV 1 and 2) and human immunodefiency virus type 1 (HIV-1). Activity has been arbitrarily defined as very active ($IC_{50} < 1$ μg / mL or well), active ($IC_{50} = 1 - 10$ μg / mL or well) and modestly active ($IC_{50} > 10$ μg / mL or well) in *in vitro* assays.[23]

1. Avarol

Avarol (**5**) and avarone (**6**) are examples of a class of compounds containing sesquiterpenes attached to a quinone or hydroquinone unit.[24] This class of compounds are typically isolated from sponges of the genus *Dysidea* and have shown a diverse array of bioactivity including antileukemic activity, both *in vitro* and *in vivo*,[25] T-lymphotropic cytostatic activity *in vitro*[26] and anti-HIV activity *in*

vitro in the H-9 cell system.[27] Recently, avarol and avarone derivatives containing a 6'-hydroxyl group showed inhanced ability to inhibit both the DNA polymerase and ribonuclease H (RNase H) activities of HIV1-reverse transcriptase over the parent compounds. A related compound, ilimaquinone (7), primarily inhibits the RNase H function. The inhibitory site for ilimaquinone was determined to be a hydroxyl moiety at the ortho position to the carbonyl group of the quinone ring. Determination of the inhibitory site of these compounds is an important step towards synthetic drug design.[28] Avarol and avarone inhibit HIV at doses of 0.1 - 1 µg / mL *in vitro*, are able to cross the blood brain barrier and thus are gaining attention as potential weapons in the battle against AIDS.[27]
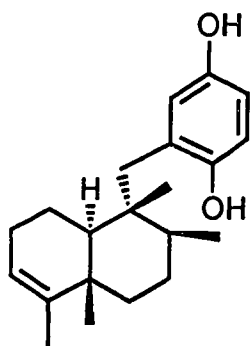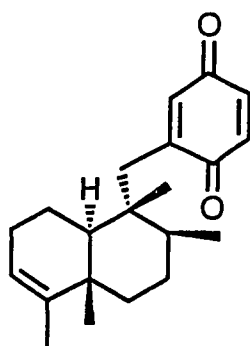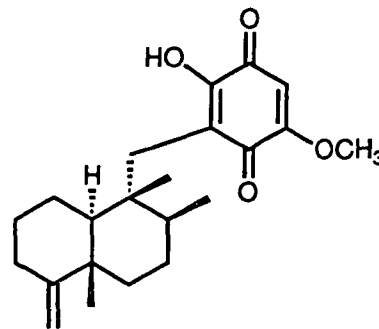
5   6   7

2. Sulfonic Glycolipids

A class of sulfonic acid-containing glycolipids (sulfoquinovosyl diacylglycerols, e.g. **8**) isolated from blue-green algae were found to inhibit HIV-1 at noncytotoxic concentrations *in vitro*. These glycolipids are structural components of chloroplast membranes occurring widely in higher plants, algae and photosynthetic microorganisms and had not been previously associated with HIV-1 inhibitory activity.[29] A total synthesis of the sulfolipids has been accomplished mitigating the difficulties associated with isolating these compounds for *in vivo* testing. The mechanism of action of the glycolipids has been hypothesized to involve the incorporation of the sulfolipids into the cellular membrane interfering with viral recognition of cell surface receptors.[30] Related compounds, sulfated polysaccharides, of the λ-carrageenan family were also found to selectively inhibit HIV-RT *in vitro*. These polysaccharides may inhibit HIV infection by interfering with virus adsorption as well as inhibiting HIV RT.[31]
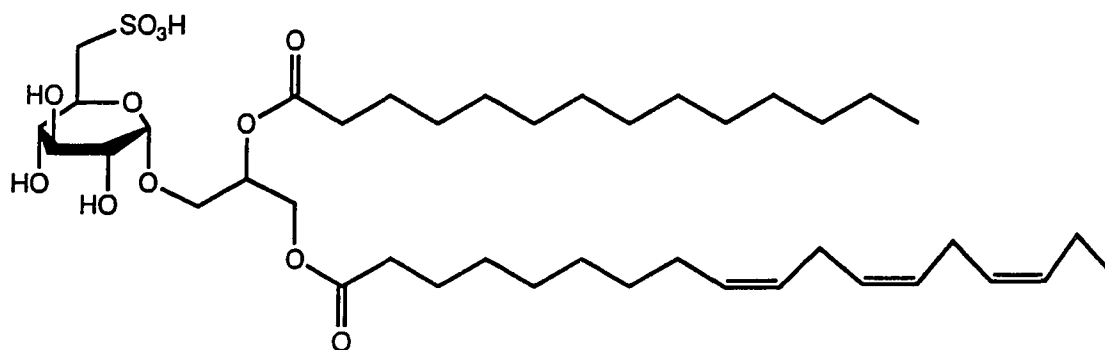
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3. Eudistomins

The eudistomins are a family of seventeen compounds characterized by a β -
carboline ring system (e.g. **9** and **10**). These compounds were originally
isolated from the tunicate *Eudistoma olivaceum* collected among mangrove
roots in Mexico, Belize and Florida[32] and have since been isolated from an
Okinawan tunicate, *Eudistoma glaucus*[33] and a New Zealand ascidian,
Ritterella sigillinoides.[34] The eudistomins have shown *in vitro* antiviral potency
ranging from 5 to 500 ng/well against HSV-1. Synthetic efforts have resulted in
obtaining the most active eudistomins in amounts sufficient for extensive *in vivo*
testing.[35] Structure-activity relationship studies for (-)-debromoeudistomin K
(**11**) indicate that the natural stereochemistry at both C-1 and C-13b and the
presence of the C-1 NH_2 and a C-10 OMe substituent are required for high
potency in both antiviral and antitumor models. The similarity in structure-
activity relationships for antiviral and antitumor activities suggests that the
eudistomins act on a biochemical process that is essential to both virus and
tumor cell growth.[36]

9 = H   **11**
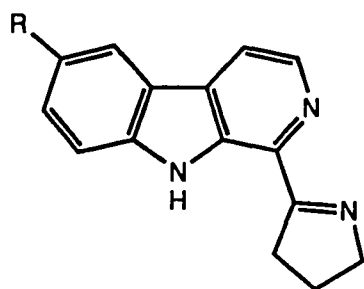
10 = Br
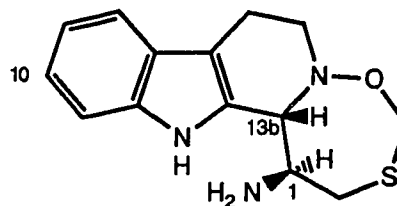
D. Antiparasitic and Antiprotozoal Metabolites

Infectious diseases caused by parasites are common throughout the world; the helminth *Necator americanus* causes hookworm; improperly prepared pork can contain the helminth *Trichinella spiralis* which causes trichinosis; and malarial infections are due to *Plasmodium* parasites. These diseases can typically be controlled by chemotherapy although drug resistant strains, especially of the malarial causative, *Plasmodium falciparum*,[37] are a problem. Marine natural product chemists, pharmaceutical concerns and funding agencies have focused their funding and efforts on "glamour" diseases like cancer and AIDS that promise rich rewards for their investments. The major market for antiparasitic drugs are the peoples of developing countries who cannot afford to pay for new expensive medications.[38] As a result only a fraction of the compounds isolated from the marine environment are tested in antiparasitic disease models.

Anthelmintic and antiprotozoal agents are typically isolated by bioassay-guided separation schemes. For anthelmintic agents, the *in vitro* bioassay involves a screen against helminth targets such as the intestinal roundworm *Nippostrongylus brasiliensis*. Activity is measured by observing viability, mobility and the ability of the larvae to molt to the adult (cast) after exposure to either an extract or a pure compound at a concentration of 50 μg/mL. An active extract is defined as showing > 60% activity against casts. Pure compounds that show > 95% activity against casts are classified as very active *In vivo* assays utilize a mouse model with a mixed helminth infection of the roundworm *Nematospirodes dubius* and the tapeworm *Hymenolepis nana*. The infected

mouse is fed test material in feed pellets at 200-1000 ppm with activity being defined as reduction of one or both worms by > 60%. Bioassays, both *in vitro* and *in vivo*, against *Trypanosoma*, *Giardia* and *Plasmodium* constitute coverage of the protozoans. Activity is generally reported as IC_{50} values. Unfortunately, the highly specialized life cycles of both parasitic helminths and protozoans limit the applicability of the *in vitro* results to clinical use.[39]

1. Kainic Acid

Kainic acid (12) is the only marine natural product used clinically as an antiparasitic agent. Kainic acid was first isolated in the 1950's from the red alga *Digenea simplex*. This alga was used by the Japanese as a folk medicine against intestinal parasites. A single 5 - 10 mg dose of the compound affords up to a 70% reduction of parasitic intestinal nematodes.[40]

12
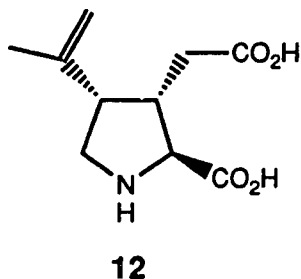
2. Bengamides

The bengamides (e.g. **13**) are a family of heterocycles characterized by a seven-membered lactam ring.[41] The compounds were isolated from the sponge *Jaspis* sp. collected in Fiji and the Solomon Islands. Many of these compounds were "powerfully active" *in vitro* against the intestinal roundworm *Nippostrongylus brasiliensis*, but only bengamides E and F were screened *in vivo*. Bengamide E was inactive, while bengamide F (**13**) showed a 75% reduction in population of the tapeworm *Hymenolepis nana* at a concentration of 225 ppm.[39]
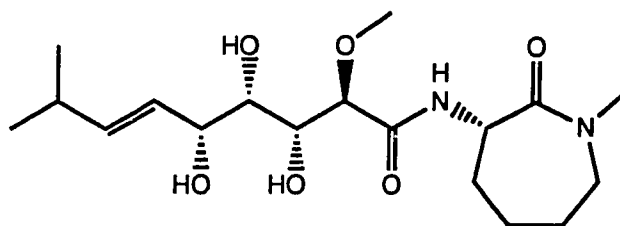
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3. Axisonitrile-3

Axisonitrile-3 (**14**) is a sesquiterpene containing an isonitrile moiety which was originally isolated from the sponge *Axinella cannabina*.[42] This compound showed *in vitro* anthelmintic activity, but no *in vivo* activity.[39] It is also one of the

few marine natural products to be tested for antiprotozoal properties. Two strains of *Plasmodium falciparum*, a chloroquine-sensitive West African clone (D-6) and a chloroquine-resistant Indochina clone (W-2), were tested *in vitro*. Axisonitrile-3 proved to be active against strain D-6 with an IC_{50} = 32 ng/mL (chloroquine had an IC_{50} = 3 ng/mL) and inactive against the W-2 clone. No other marine natural product in the literature displays comparable activity against *Plasmodium*.[43]
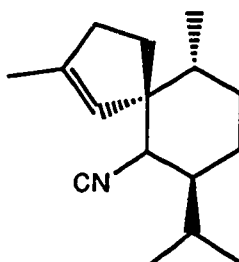
14

E. Research Objectives

The search for drugs from the sea has added another dimension to the discovery of therapeutic agents. The diseases tested against, and the examples of compounds from, the marine environment given in this chapter are representative of the approach marine natural product chemists take towards drug discovery. Marine natural products are being explored for other medicinal properties including anti-inflammatory, antibiotic and antiulcer therapeutics.

Compounds, especially toxins, from marine sources have given scientists new insights into biological processes. It has been hypothesized that secondary metabolites must, by definition, have biological activity (natural product / receptor interactions), or the organism would not synthesize the compound.[44]

Isolation and structure determination is often considered the first step towards identifing useful marine natural products. In fact, the first step is marine ecology. Understanding the interactions of an organism with its environment can provide leads, what type of compounds may be present and what type of activity these compounds may possess. The observation that sessile, slow-growing marine organisms must contain some form of chemical defense to prevent predation is responsible for a major research effort involving these organisms. One of the primary objectives of marine natural product research is a better understanding of the complex interactions of the organisms that inhabit the marine environment.

CHAPTER 2. RESULTS AND DISCUSSION

A. Project IRMS: A Carbon Isotope Study of Ulapualide B

The ulapualides, the kabiramides, the halichondramides and the jaspisamides are macrolides characterized by three contiguous oxazole rings. Ulapualide B (**15**) is the most abundant of the ulapualides, which are isolated from *Hexabranchus sanguineus* and its eggmasses in Hawaiian waters.[4] The halichondramides were isolated from a sponge of the genus *Halichondria* and from the nudibranch, *Hexabranchus sanguineus*, which feeds on *Halichondria*, collected in Micronesia.[45] The kabiramides were originally isolated from unidentified eggmasses collected in the Ryukyu islands[46] and have since been found in *Halichondria* and in *Hexabranchus sanguineus* in Micronesia.[47] The mycalolides were isolated from a sponge of the genus *Mycale* collected at Gokasho Bay, Japan.[48] Recently, the jaspisamides have been isolated from a sponge of the genus *Jaspis*.[49]

15
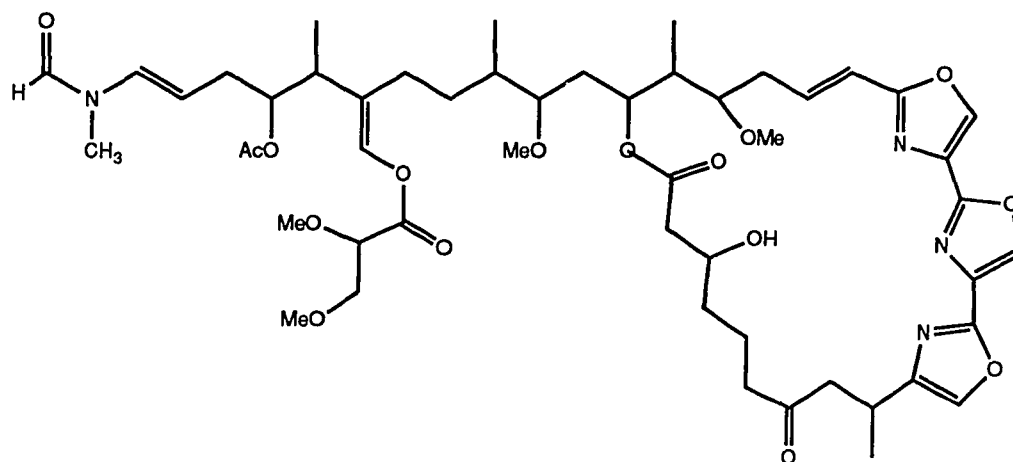
Figure 1. Photograph of *Hexabranchus sanguineus* eggmass

(north shore of Oahu, ~ 10 m).

Figure 2. Photograph of *Hexabranchus sanguineus*

(north shore of Oahu, ~ 10 m).

The halichondramides and the kabiramides were shown to be defensive agents of the sponge *Halichondria*, and a large, slow-moving brightly colored nudibranch, *Hexabranchus sanguineus* and its conspicous eggmasses.[47] The nudibranch, which feeds on the sponge, apparently sequesters these compounds and passes the compound to its eggmasses. It is not known whether the sponge or a symbiont produces the macrolide. The macrolides that provide the chemical defense against predation are related to the scytophycins, a group of macrolides isolated from cultures of terrestrial blue-green algae.[50] The possibility that the sponge macrolides were produced by symbiotic cyanobacteria was ruled out by the absence of chlorophyll a and b in the extracts of *Halichondria* sp.[47] The presence of heterotrophic bacterial symbionts or blue-green algae living heterotrophically in the tissues of *Halichondria*, and their involvement in the production of the macrolides, could not be excluded.

The goal of this study was to discover the source of the ulapualides in the sponge or in a symbiont of the sponge by comparing the carbon isotopic composition of ulapualide from the sponge that contains ulapualide, from the nudibranch that feeds on the sponge, and from the eggmasses of the nudibranch. If ulapualide is produced by the sponge and the compound is sequestered by the nudibranch, the carbon isotopic composition of ulapualide will be identical, as changes in the carbon isotopic composition occur only during biosynthesis with addition or loss of carbon.[51]

Furthermore, the eggmasses of *Hexabranchus sanguineus* have an aeolid predator, *Favorinus japonicus*, which lays its eggmasses on the *Hexabranchus sanguineus* eggmasses. Many aeolids store nematocysts (stinging cells), which they sequester from coelenterates (e.g. hydroids, anemones, jellyfish), in a specialized organ called a cnidosac.[52] It has been proposed that if an aeolid does not sequester nematocysts, as is the case with *Favorinus japonicus*, the cnidosacs contain a defensive agent derived from a food source.[53] A second goal of this study was to determine whether ulapualide is present in *Favorinus japonicus* and its eggmasses and, if so, to use carbon isotopic composition to test the hypothesis that *Favorinus japonicus* sequesters ulapualide from the eggmasses of *Hexabranchus sanguineus*.
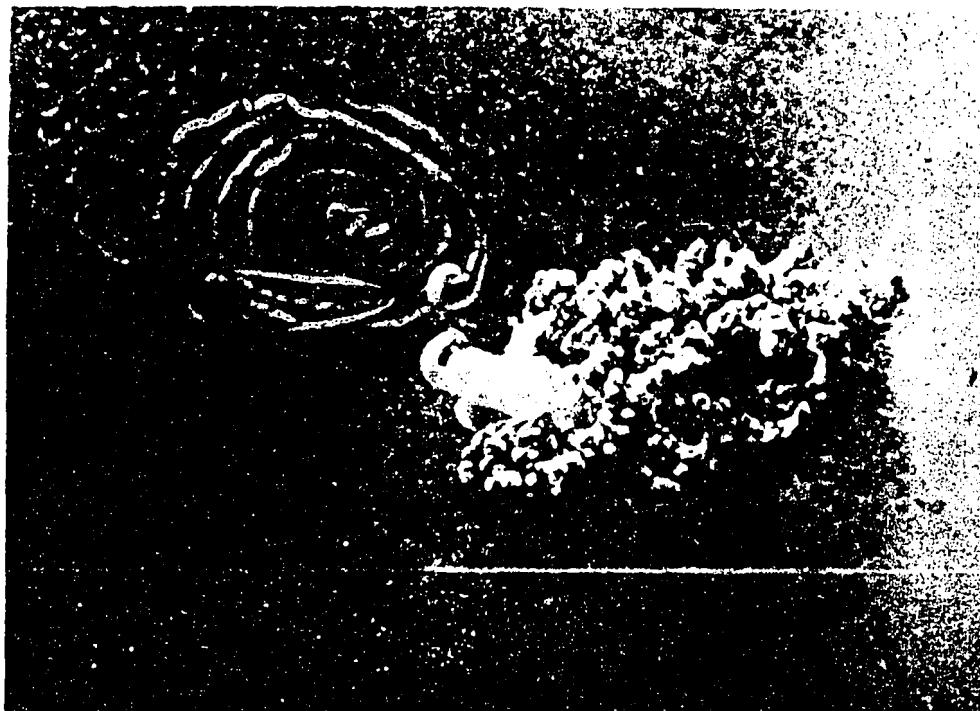
Figure 3. Photograph of *Favorinus japonicus* (north shore of Oahu, ~ 15 m).

All nudibranchs and eggmasses were collected at Pupukea on the north shore of Oahu to eliminate environmental influences on the carbon isotopic composition due to differences in food sources since spongivory by *Hexabranchus sanguineus* is nonselective.[54] The nudibranchs were dissected and the gut and its contents removed, thereby preventing contamination of ulapualide from the nudibranch with ulapualide from any sponge that may be present in the digestive tract.

Ulapualide B was isolated from the nudibranchs and eggmasses by a modification of the method used by Roesener and Scheuer (Fig. 4).[4] All samples for carbon isotopic analysis were placed in quartz tubes and freeze-dried overnight. An oxidizing agent was added; the quartz tubes were evacuated and subjected to combustion temperature. The generated gases were cryogenically distilled and then sent for analysis to Indiana University.[55]

Unfortunately, no sponge containing ulapualide was found, although I observed *Hexabranchus sanguineus* feeding on many different sponges. A literature search revealed that two *Halichondria* spp were reported from a fouling community on Coconut Island in Kaneohe Bay.[56] The sponges were collected but did not contain ulapualide. Examination of the gut contents of a nudibranch revealed the presence of both calcareous and siliceous sponge spicules evidencing the

Hexabranchus sanguineus

Dissected

Digestive glands and reproductive organs

Mantle and excudate

extracted with MeOH
partitioned with hexane

Hexane

Aqueous

Partitioned with CCl₄

CCl₄

Aqueous

HPLC RP C-18 Phenomenex
Semi-prep.
MeOH/H₂O 80:20

Ula A

Ula B
R.T. = 42 min
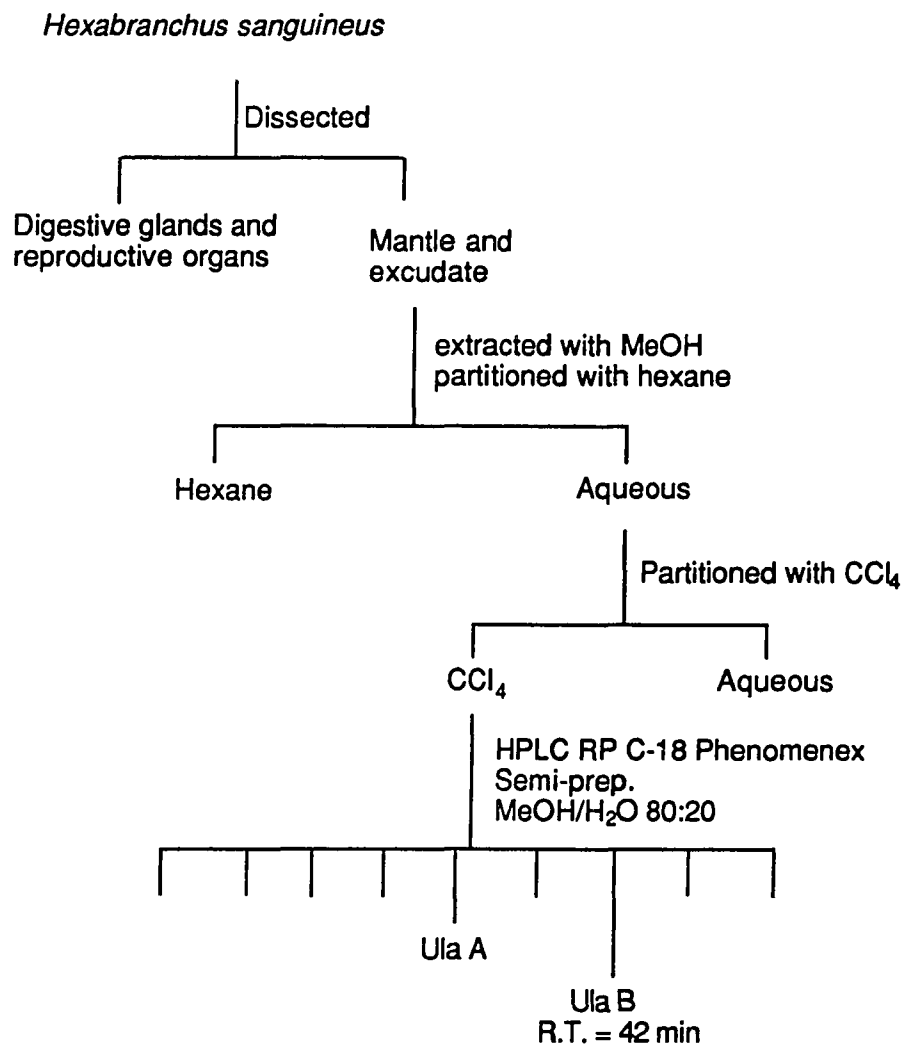
Figure 4. Separation scheme of ulapualide from the nudibranch

Hexabranchus sanguineus.

varied spongivory of the nudibranch. Extraction and HPLC of the gut contents showed only trace amounts of ulapualide, which may have originated from the mucous exudate that surrounds the animal and contaminated the gut contents during dissection.

Ulapualide B isolated from the eggmasses had δ values which ranged from -19.83 to -19.68 ‰. Three samples of ulapualide were isolated from 60 g of eggmasses (Table 1). The amount of gas generated from combustion of ulapualide B in these samples was large enough to offset the analytical blanks present.

Table 1. The Carbon Isotopic Composition of Ulapualide B
from the Eggmasses of *Hexabranchus sanguineus*

Source	Amount	Sample	Amount CO_2 (μmol)	δ Value vs. PDB	Determined δ Value
Eggmass	60 g	EM-1	>100	-19.83 ‰	
		EM-2	>100	-19.68 ‰	
		EM-3	>100	-19.71 ‰	-19.8 ± 0.2 ‰[*]

[*]indicated uncertainties are at the 95% confidence level

Ulapualide B from a single specimen of *Hexabranchus sanguineus* yielded 4.0 μmol of CO_2 with an isotopic composition of -21.56 ‰, whereas a second specimen of *Hexabranchus sanguineus* yielded 5.0 μmol of CO_2 with an isotopic composition of -20.68 ‰. Seven more specimens of *Hexabranchus sanguineus* were collected and ulapualide B isolated to determine if the difference in the carbon isotopic composition of ulapualide B from the two animals was due to experimental error (Table 2).

Table 2. The Carbon Isotopic Composition of Ulapualide B

from *Hexabranchus sanguineus*

Source	Amount	Sample	Amount CO_2 (μmol)	δ Value vs. PDB	Detemined δ Value
Hexabranchus sanguineus	1 specimen				
	10 g	SD-3	11.3	-20.36 ‰	
	6 specimens				
	52 g	6SD-1	18.0	-19.81 ‰	
		6SD-2	32.0	-19.34 ‰	
		6SD-3	47.0	-19.03 ‰	-18.7 ± 0.3 ‰ [*]

[*]δ value solved for analytical blank, indicated uncertainties are at the 95% confidence level

If the size and isotopic composition of the analytical blank is constant in all samples, these multiple analysis of ulapualide B can be used to correct for the analytical blank by plotting δ vs. $1/n$.[57] Because the last four samples of

ulapualide B were isolated at the same time under identical conditions and provided the largest samples for analysis, they were used to determine the blank-corrected carbon isotopic value of ulapualide B. A blank-corrected δ value of -18.7 $\pm$ 0.3‰ is thus obtained for the isotopic composition of ulapualide B from *Hexabranchus sanguineus* (Fig. 5).
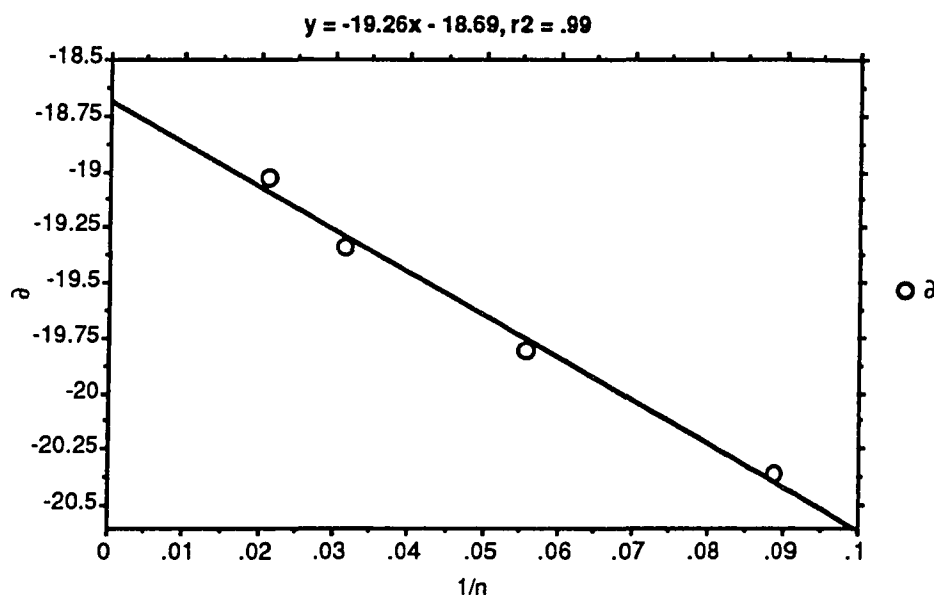
Figure 5. Plot of isotopic composition vs. the inverse molar amount of ulapualide B isolated from *Hexabranchus sanguineus*.

On the basis of the limited samples evaluated, the δ values for the individual and the group of nudibranchs precludes the possibility that ulapualide B catabolism accounts for the 1 ‰ difference in isotopic composition between the eggmasses and the nudibranch.

Isotope effects are expressed when the flow of carbon is divided at a branch point of an enzymatic reaction pathway.[51] An example of a branch point in an enzymatic reaction pathway is shown in Figure 6. Acetate can be shunted into the citric acid cycle, where it produces amino acids, or it can be used for fatty acid synthesis. Isotopic fractionation occurs at this branch point.[58] If the same scheme is used for ulapualide, there

Acetate

TCA cycle   Fatty acids

Amino acids
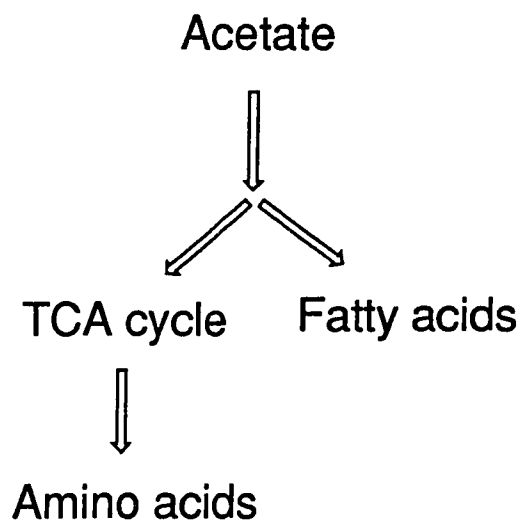
Figure 6. The branch point for isotopic fractionation of acetate.

are two possible places where fractionation can occur (Fig. 7). If catabolism occurs prior to storage, the eggmasses and the nudibranch should have identical isotopic compositions, since the compound is derived from the same pool of ulapualide. If the nudibranch catabolizes ulapualide after it has laid its eggs and catabolysis of ulapualide does not occur in the eggmasses, the δ

value of the eggmasses would be a "snapshot" of the δ value at the time at which the nudibranch laid its eggs. Ulapualide in the nudibranch would slowly become enriched in ^{13}C because ^{12}C metabolizes (reacts) faster. If this were the case, a scatter of δ values would be expected among individuals. Instead, the results show that the δ value of an individual is identical with those of a group of nudibranchs (after correcting for the analytical blank). If catabolism is causing fractionation, the chances are exceedingly small that a group of nudibranchs has the same isotopic composition as an individual.

These preliminary results of carbon isotope analysis of ulapualide B from *Hexabranchus sanguineus* and its eggmasses suggest that ulapualide B is not directly passed from the nudibranch to its eggmass. A 1‰ discrepency between the carbon isotopic composition of ulapualide B from the nudibranch and from its eggmasses in our sample set suggests that ulapualide is not simply sequestered from a dietary source and transferred intact to the eggmasses. This conclusion must be tempered by the limited size of our sample set and the fact that the eggmasses almost certainly did not come from the same animals that were collected. Due to a collecting season limited by the large surf that occurs on the North shore during the winter months and the difficulties associated with finding the organism, we were not able to gather enough data to make these findings statistically meaningful.

Ulapualide

catabolism   storage

nudibranch   eggmass

catabolism   nudibranch
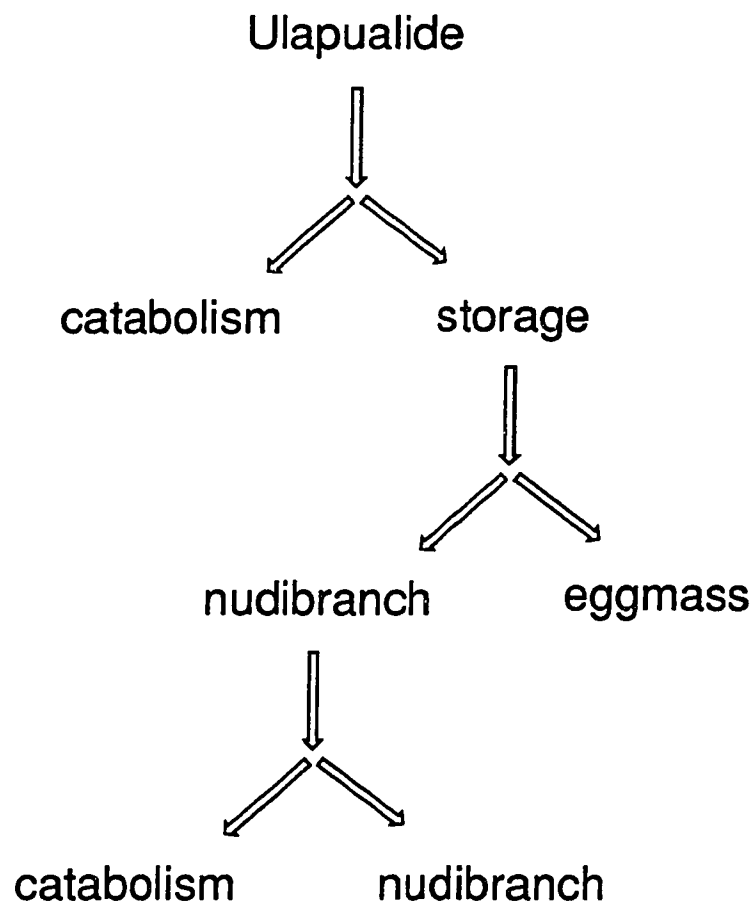
Figure 7. Possible branch points that would isotopically fractionate ulapualide B.

If the 1‰ discrepancy is significant, one possible explanation is that ulapualide is produced by a heterotrophic bacterium, which lives in or on *Hexabranchus sanguineus*. As an example, bacteria which provide pufferfish or newts with a toxic metabolite are responsible for the biosynthesis of tetrodotoxin.[59] Such a bacterium may be deposited with the eggmasses and, in a different environment with a different feedstock, produces ulapualide with a different isotopic composition. It is well established that the carbon isotopic composition of heterotrophic bacteria reflects that of their food source.[51] It is interesting to note that structurally related macrolides have been found among sponges of the genera *Halichondria*, *Mycale* and *Jaspis*, which belong to different orders. This strengthens the hypothesis that these macrolides are synthesized by epi- or symbiotic microorganisms.

Ulapualide A and B were present in *Favorinus japonicus*, but neither was detected in the eggmasses of the aeolid. The small amount of eggmass collected (~5 mg) may have yielded concentrations of ulapualide too small to detect by UV spectrophotometry. Interestingly, the relative amounts of ulapualide A and B in *Favorinus japonicus* differed from the relative amounts in the eggmasses of *Hexabranchus sanguineus*. In *Favorinus japonicus*, ulapualide A was the major constituent. This may imply that *Favorinus japonicus* is either selectivly sequestering ulapualide A or chemically altering ulapualide B. The results of carbon isotope ratio mass spectrometry were inconclusive because of the small amounts of ulapualide isolated.

B. Project BIP 6: Three Pregnane-10,2-Carbolactones from a Sponge, *Strongylophora* sp.

Pregnane steroids are important mediators of many functions in mammals, which suggests their potential therapeutic use. For example, pregnanes and their congeners exhibit varied hormonal activity, including development of male secondary sex characteristics (testosterone), regulation of blood pressure (aldosterone),[60] and depression of the central nervous system (alphaxalone).[61]

16   **17**   **18**
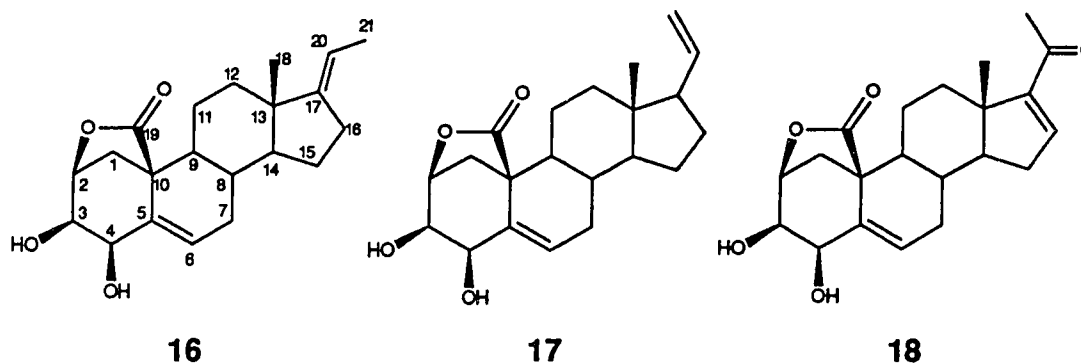
Three C-19 oxygenated pregnane steroids (**16**, **17** and **18**) were isolated from a sponge, *Strongylophora* sp. (family Pterosiidae, order Nepheliospongida, Fig. 8). Although sponges of the order Nepheliospongiida have proven to be a rich source of marine sterols,[62] this is the first report of pregnanes isolated from this order. The few pregnanes that have been isolated from the
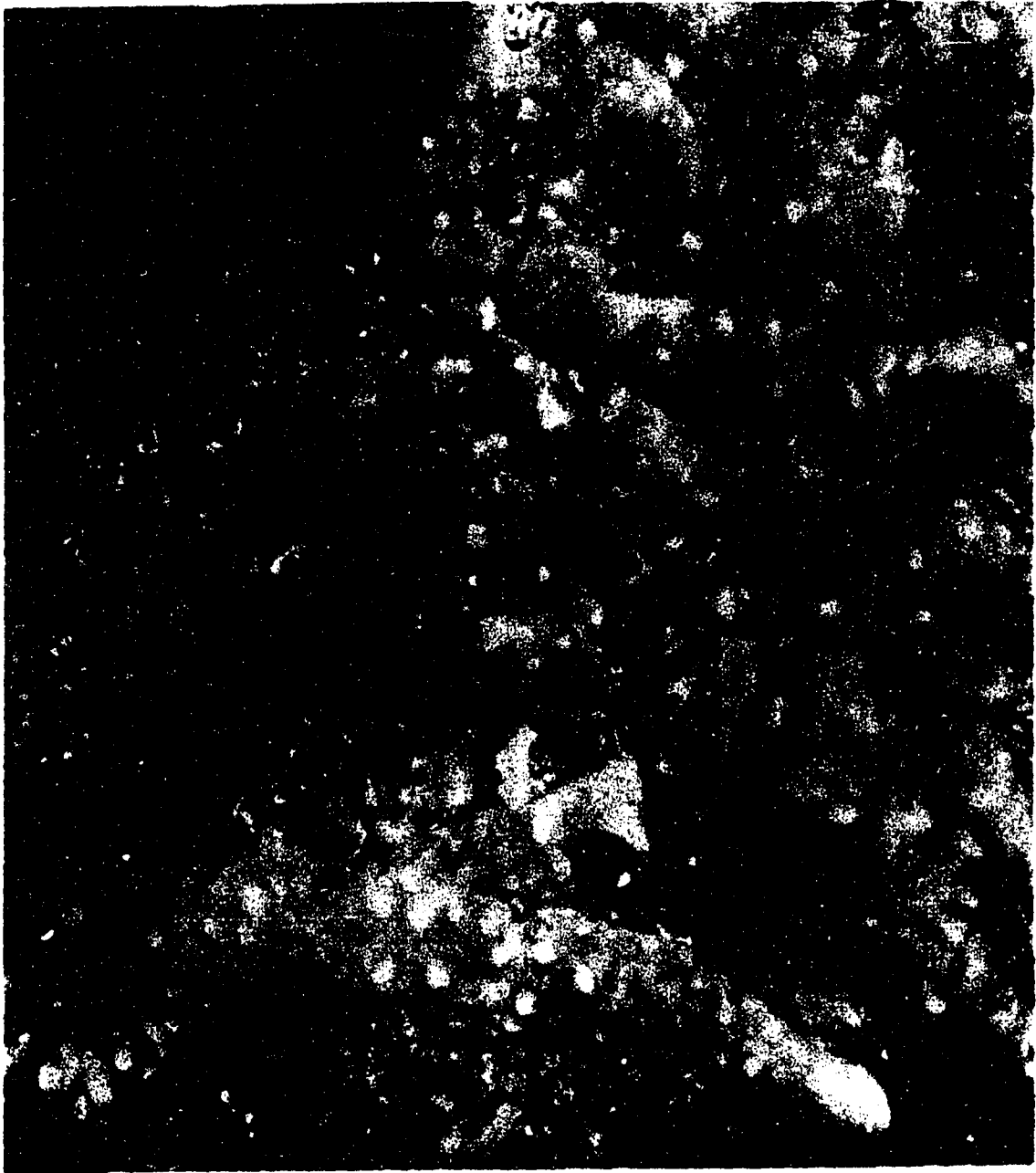
Figure 8. Photograph of *Strongylophora* sp. (Puako, Hawaii, ~ 15 m).

marine environment come from a diverse array of organisms. Four sponges (*Haliclona rubens*,[63] *Damiriana hawaiiana*,[64] *Stylopus australis*,[65] and *Axinella agnata*[66]), an octocoral,[67] a clam,[68] a gorgonian[69] and four alcyonacean corals[70,71,72] have yielded free pregnanes while several sea stars[68,73] and two gorgonians[74,75] elaborate pregnane glycosides.

A sponge, identified as a *Strongylophora* sp.,[76] was collected by SCUBA at Puako, Hawaii. A concentrated EtOH extract of the sponge was partitioned with CH_2Cl_2. Upon concentration of the CH_2Cl_2 extract, two layers formed. High pressure liquid chromatography of the top layer on silica gel yielded a white powder (**16**, Fig. 9). The sponge was recollected and, upon re-isolation, a new pregnane (**17**) co-eluted with **16** during silica gel HPLC. Pregnane **17** was separated from **16** by reverse phase HPLC. Pregnane **18** was isolated from the combined CH_2Cl_2 percipitates along with additional **16** and **17** using reverse phase high pressure liquid chromatography (Fig. 10).

3,4-Dihydroxy-5,17-pregnadiene-10,2-carbolactone (**16**) was initially isolated as a white powder of composition $C_{21}H_{28}O_4$ (U = 8) determined by HREIMS. One carbonyl (176 ppm) and four olefinic carbons (151.0, 136.0, 132.0 110.6 ppm) in the ^{13}C NMR spectrum (Fig. 13) of **16** pointed to a pentacyclic compound. The carbonyl frequency was compatible with an ester and an IR band at 1775 cm^{-1} pointed to a 5-membered lactone.[77]

BIP 6 Sponge
Strongylophora sp.
1 kg wet weight
11/19/89

extract with
EtOH
(3 X 200 mL)

1)concentrate
2)partition with
   CH_2Cl_2 (3 X 100 mL)

CH_2Cl_2 Aqueous

concentrate

Top Layer   Bottom Layer

silica HPLC
2.5% iPrOH in CH2Cl2

pregnane
16
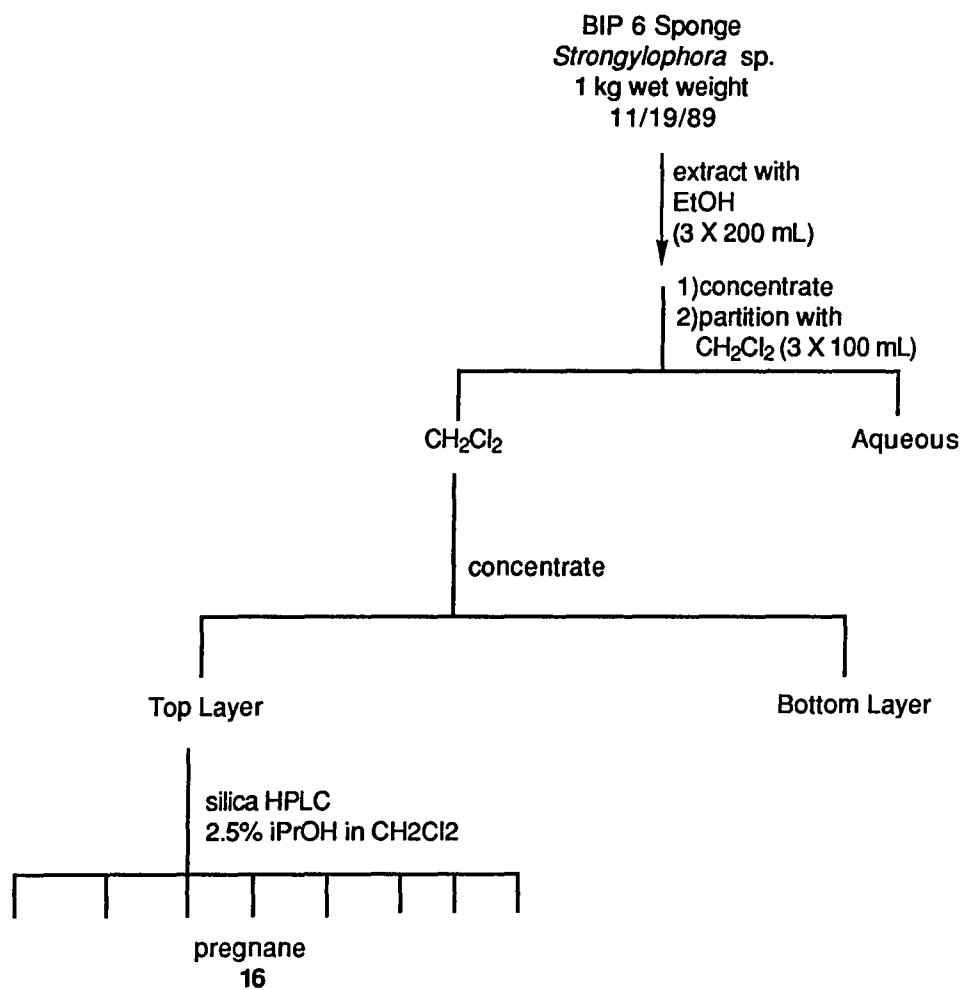
Figure 9. Separation scheme for the first collection of *Strongylophora* sp.

2BIP 6 sponge
Strongylophora sp.
2 kg wet weight
4/9/91

extract with
EtOH
(3 X 300 mL)

1)concentrate
2)partition with
CH_2Cl_2(3 X 200 mL)

CH_2Cl_2 Aqueous

concentrate

Top Layer   Bottom layer

silica HPLC
2.5% IPrOH in CH2Cl2

RP C-18 HPLC
MeCN / H_2O (10:1)

pregnane
17

pregnane
16

RP C-18 HPLC
MeOH/H_2O (50:3)

repeated
RP C-18 HPLC
MeCN / H_2O (1:1)

pregnane
16

pregnane
17

pregnane
18
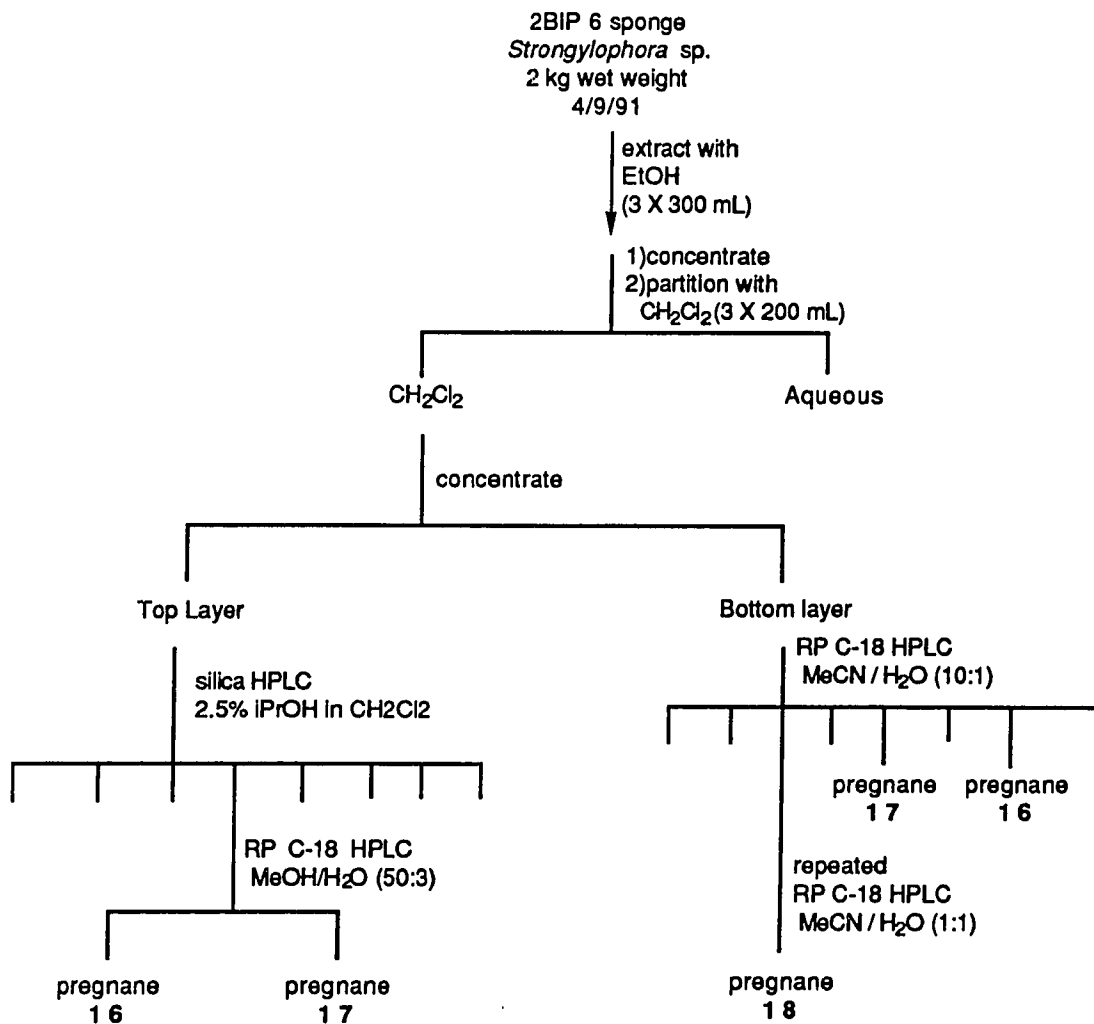
Figure 10. Separation scheme for the second collection of
Strongylophora sp.

^{1}H NMR data (Fig. 12) defined the ring A spin system (H$_2$-1, H-2, H-3, H-4) of the tetracyclic steroid nucleus. A second system, defined by a COSY[78] NMR experiment, encompassed the remaining protons (H-6, H$_2$-7, H-8, H-9, H$_2$-11, H$_2$-12, H-14, H$_2$-15, H$_2$-16) except those of the C-18 methyl and the side chain. An HMBC[78] NMR experiment located the lactone by correlating the carbonyl carbon (176 ppm) with H-2 (4.73 ppm) and H-9 (1.25 ppm). HMBC also established the olefin in ring B by correlating H-1a (2.70 ppm) and H-1b (1.86 ppm) to C-5 (136.0 ppm), and H-4 (4.48 ppm) to C-6 (132.0 ppm). The C-17 double bond was located by long range COSY of H-20 (5.06 ppm) with H-16a (2.35 ppm) and H-16b (2.26 ppm), and by HMBC correlations of H-20 with C-16 and C-13 (26.3, 43.7 ppm). Relative stereochemistry was determined by NOE experiments and by ^{1}H coupling constants (Fig. 11).

A   B
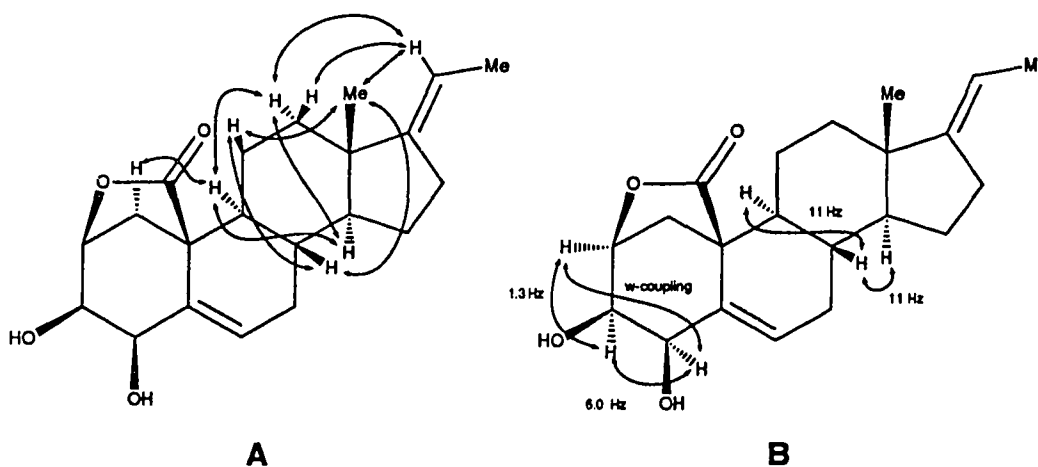
Figure 11. Important NOE correlations (A) and ^{1}H-^{1}H coupling constants (B) for 3,4-dihydroxypregna-5,17-diene-10,2- carbolactone (16).
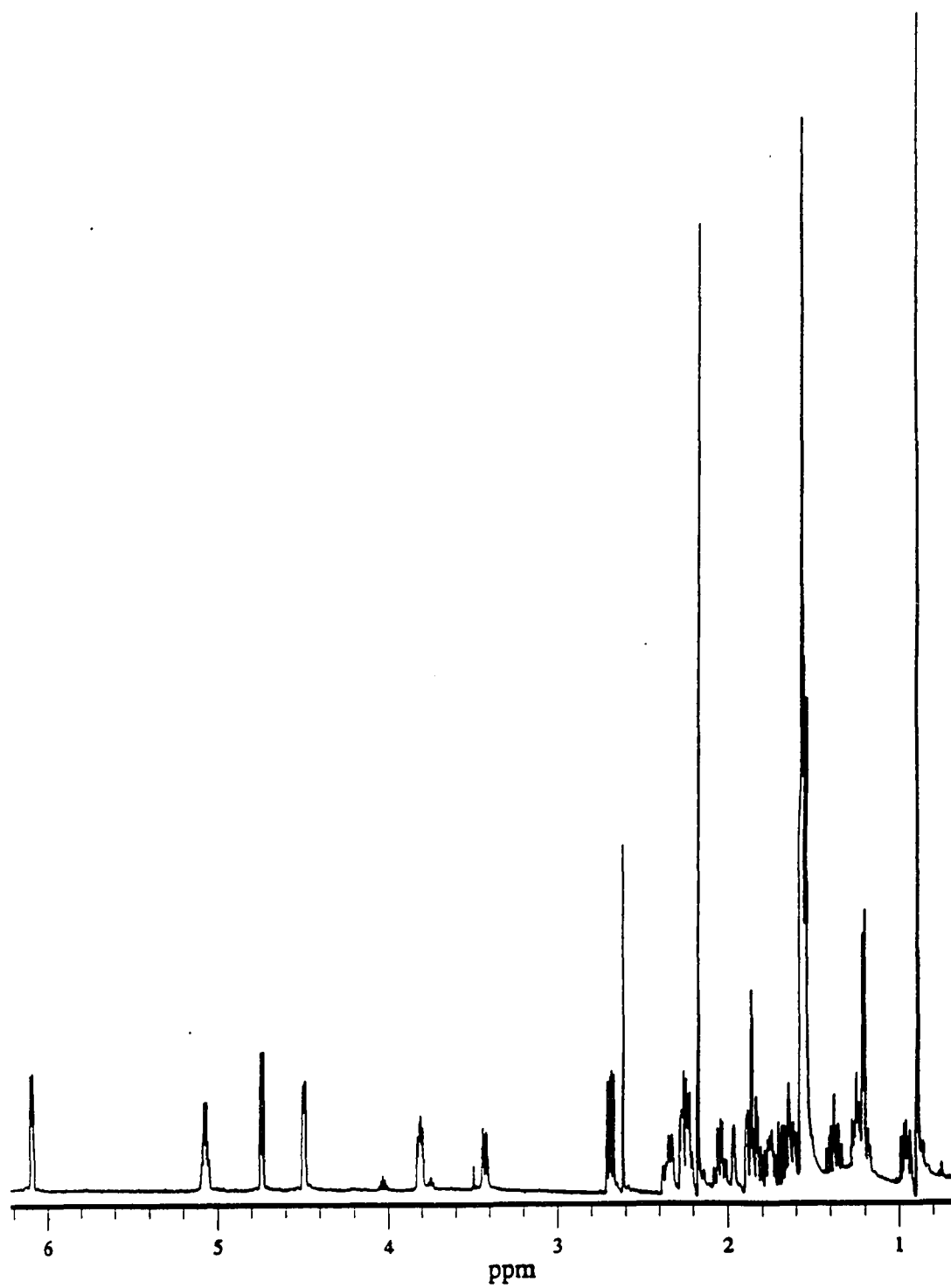
Figure 12. ^{1}H NMR spectrum of 3,4-dihydroxypregna-5,17-diene-10,2-
carbolactone (**16**) in $CDCl_3$ (500 MHz).
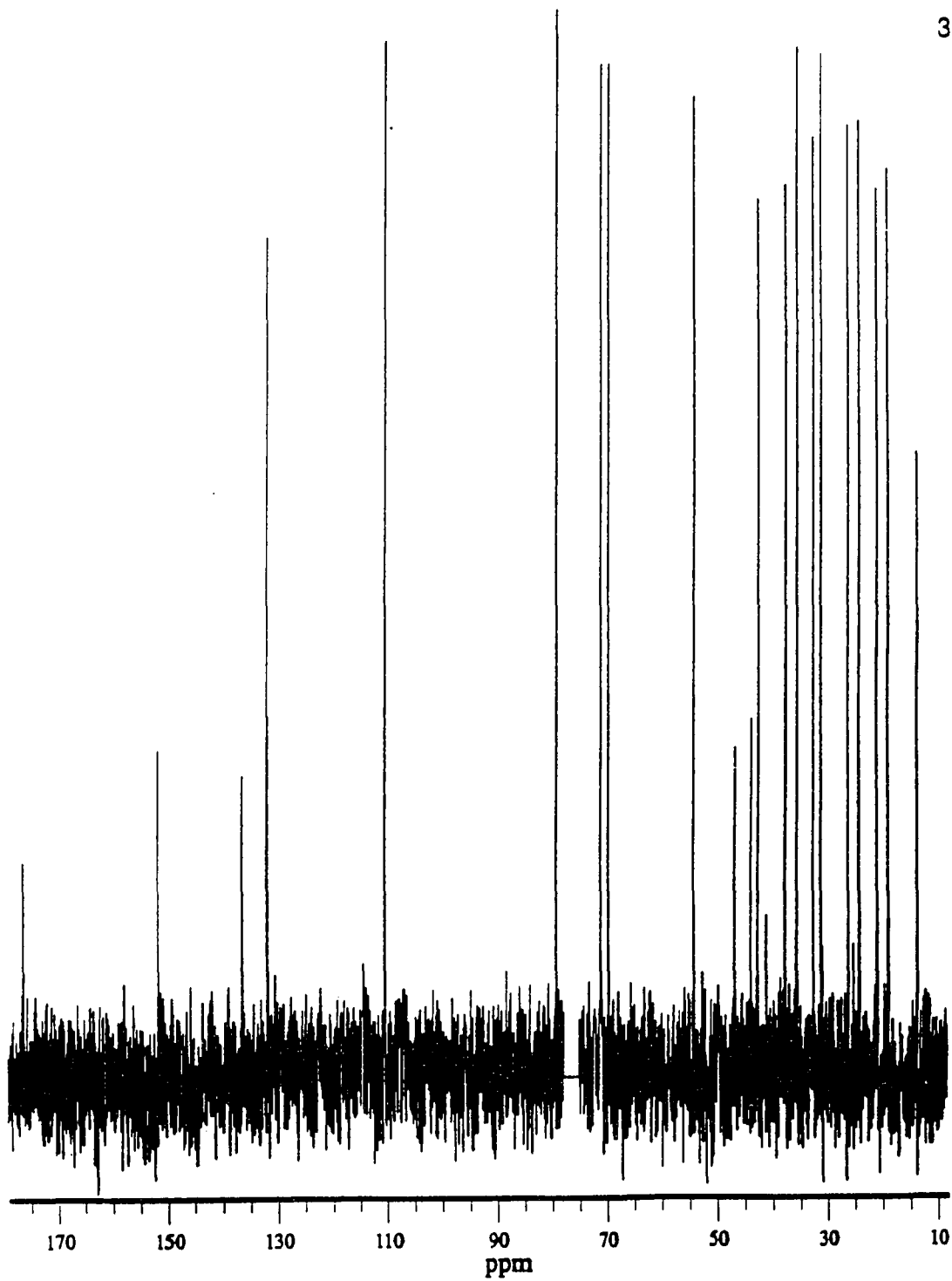
Figure 13. ^{13}C NMR spectrum of 3,4-dihydroxypregna-5,17-diene-10,2-carbolactone (**16**) in CDCl$_3$ (125 MHz).
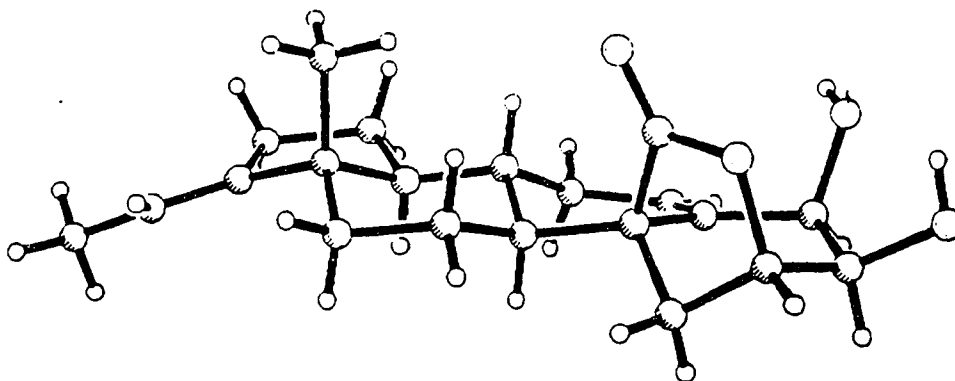
Figure 14. An ORTEP representation of 3,4-dihydroxypregna-5,17-diene-10,2-carbolactone (16).

This structure was confirmed by x-ray crystallography after 16 was crystallized from ethanol.[79] Figure 14 is an ORTEP drawing of the final structure.

The structure of the second pregnane, 2,3-dihydroxy-5,20-pregnadien-10,2-carbolactone (17) could be elucidated by comparing ^{1}H and ^{13}C NMR (Figs. 15 and 16) data of the two compounds. Substantial chemical shift differences for carbons and protons at C-16, 17, 18, 20 and 21 suggested that the C-17 olefin had shifted to C-20. Particularly revealing were the proton signals for the terminal (C-21) olefin: 5.00 ppm (ddd) and 4.97 ppm (ddd).
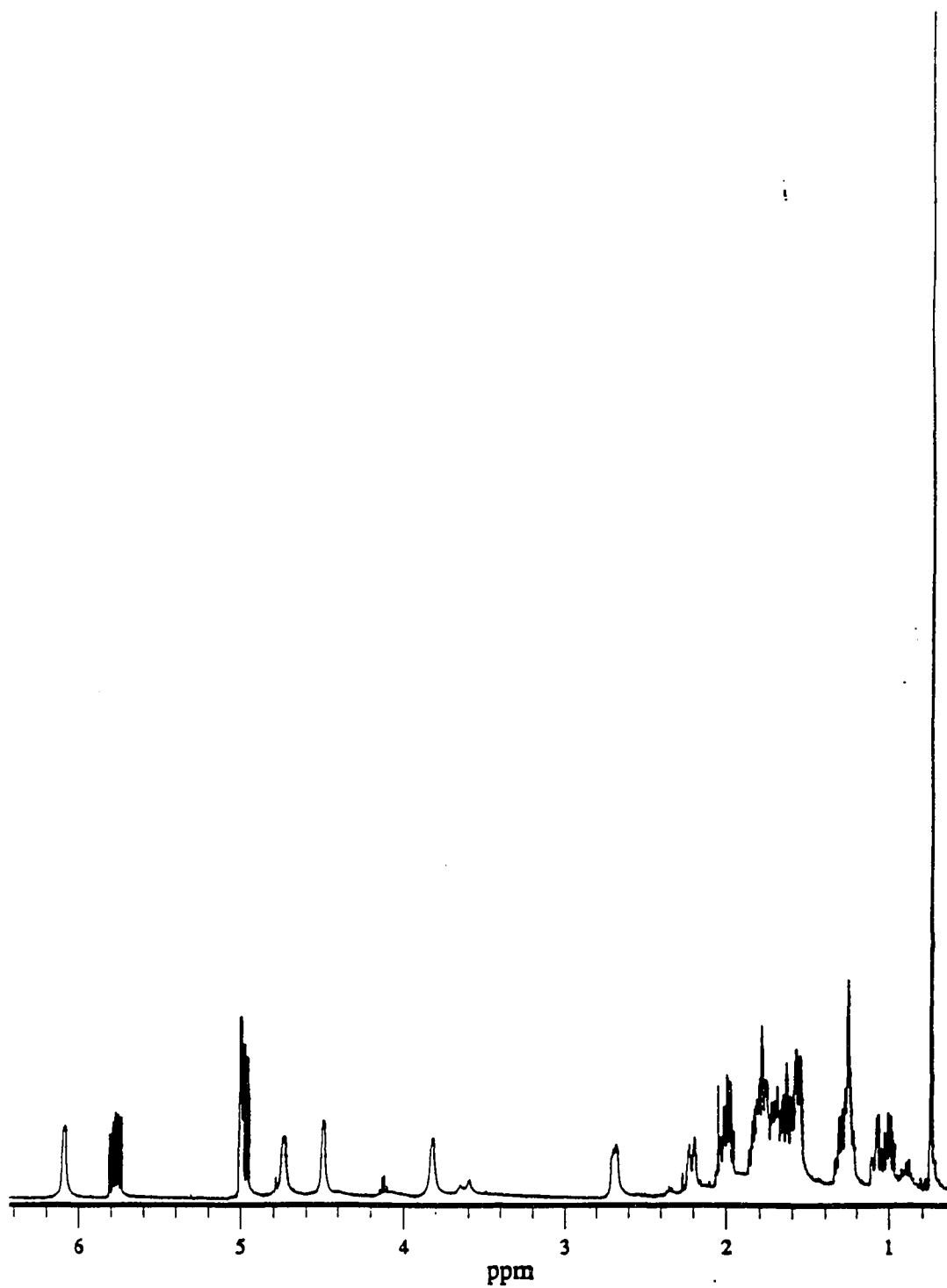
Figure 15. ^{1}H NMR spectrum of 3,4-dihydroxypregna-5,20-diene-10,2-carbolactone (**17**) in CDCl$_3$ (500 MHz).
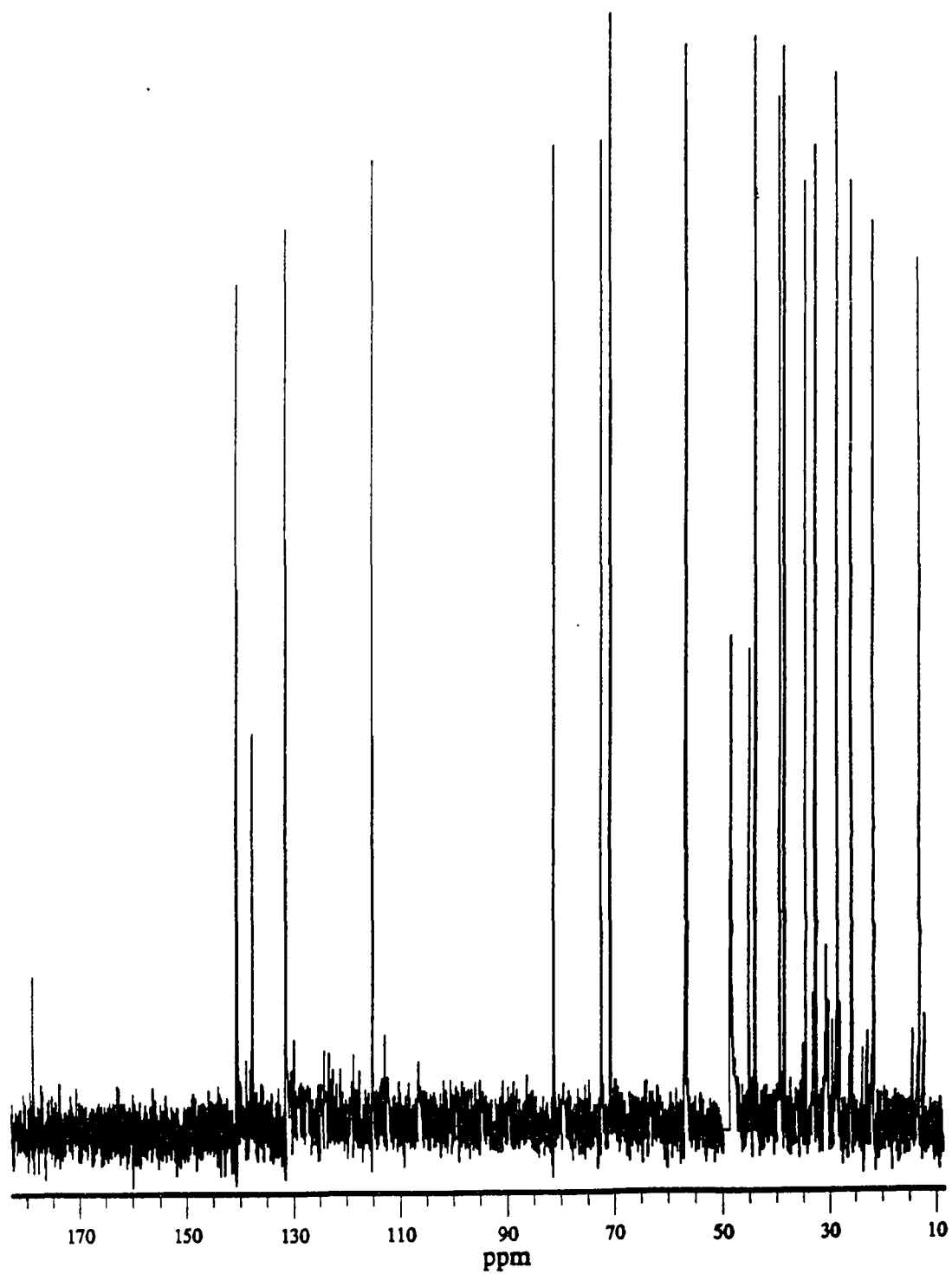
Figure 16. ^{13}C NMR spectrum of 3,4-dihydroxypregna-5,20-diene-10,2-carbolactone (**17**) in CDCl$_3$ (125 MHz).

The structure of 3,4-dihydroxy-5,16-pregnadien-20-one-10,2-carbolactone (18) was determined by comparison of the ^{1}H and ^{13}C spectra (Figs. 18 and 19) with those of 16 in CD_3OD and by 2-D NMR experiments (HMBC and HMQC). Deuterated methanol was used as the NMR solvent because of improved resolution. The αβ-unsaturated ketone was indicated by the H-16 resonance at 6.90 ppm, the H_3-21 singlet at 2.25 ppm and a new carbonyl band at 1790 cm^{-1} in the IR spectrum. The position of the αβ-unsaturated ketone of 18 was established by an HMBC experiment. The H_3-21 protons (2.25 ppm) showed a correlation in the HMBC NMR experiment to C-20 (200 ppm) and C-17 (156.2 ppm). The H-18 protons (1.03 ppm) also showed a correlation to C-17 (156.2) (see Fig. 17).
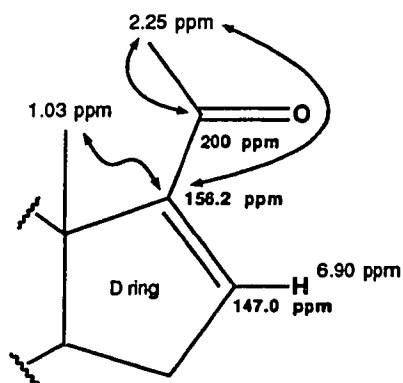
Figure 17. Significant HMBC correlations for 3,4-dihydroxypregna-5,15-dien-20-one-10,2-carbolactone (18).
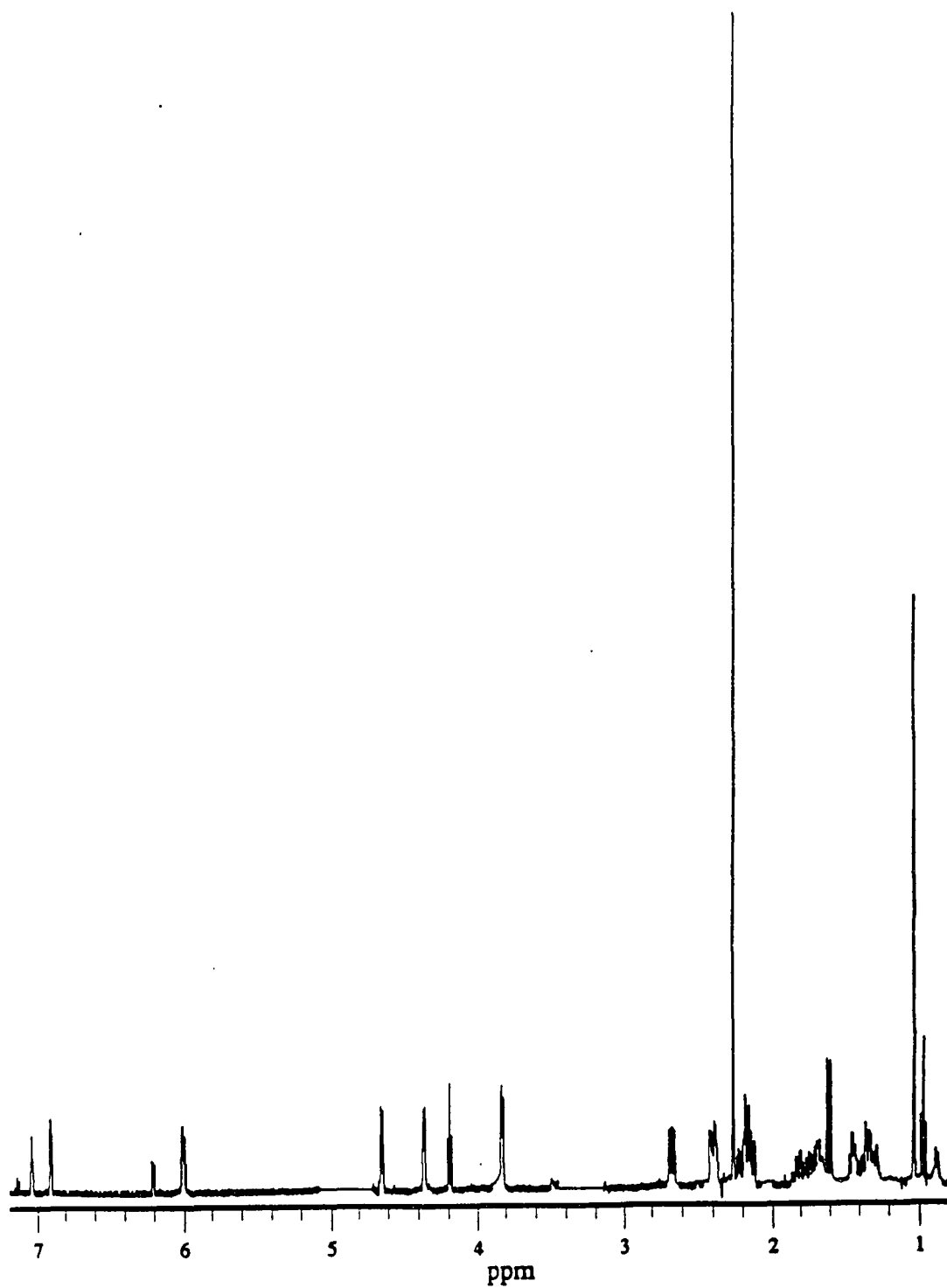
Figure 18. ¹H NMR spectrum of 3,4-dihydroxypregna-5,15-dien-20-one-10,2-carbolactone (**18**) in CDCl₃ (500 MHz).
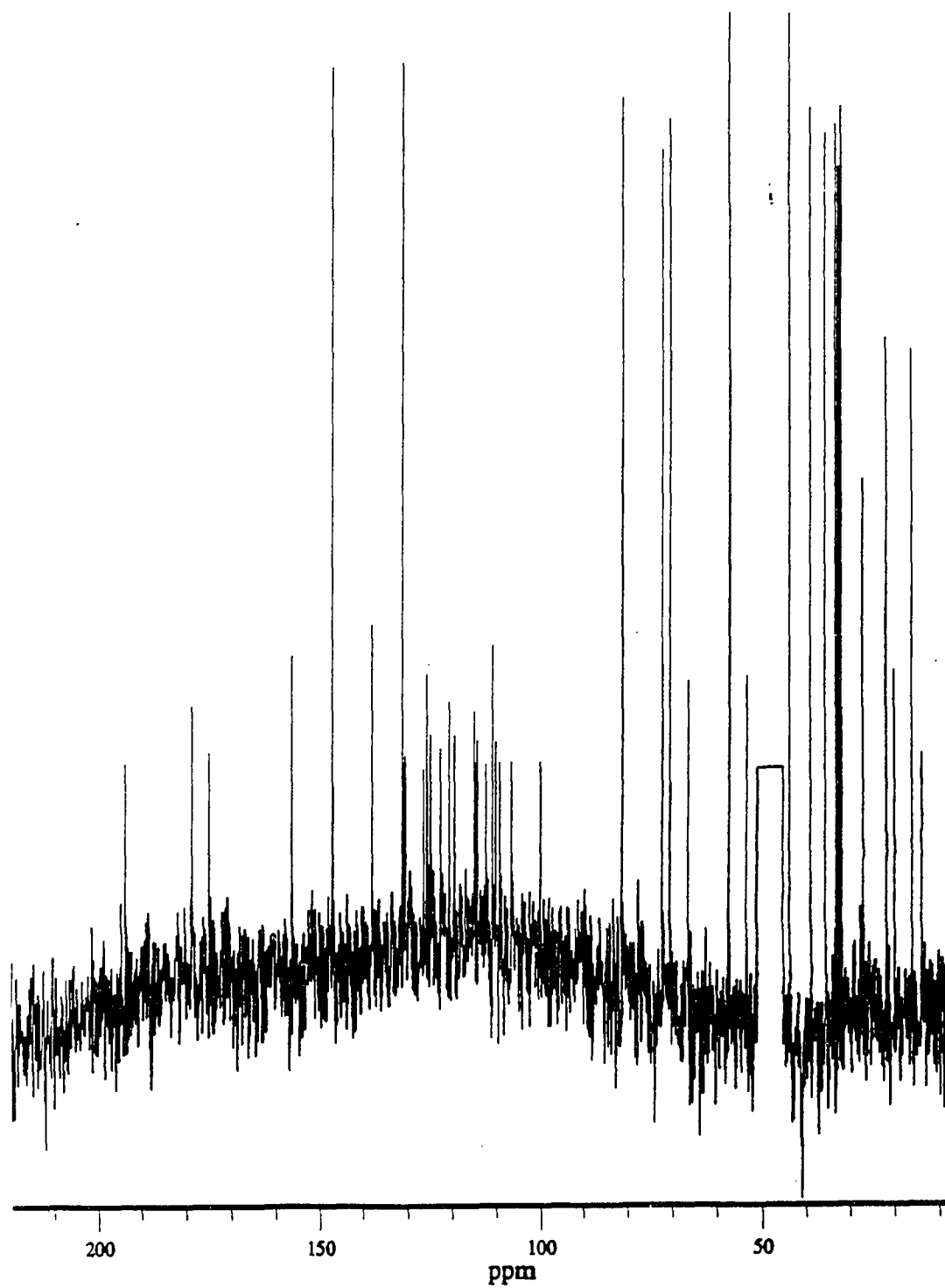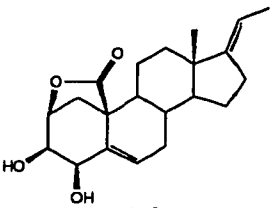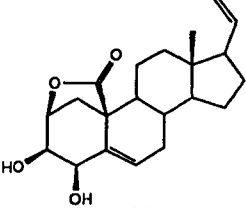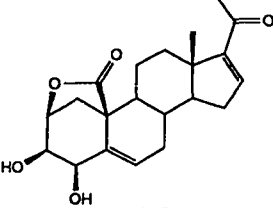
Figure 19. ^{13}C NMR spectrum of 3,4-dihydroxypregna-5,15-dien-20-one-10,2-carbolactone (**18**) in $CDCl_3$ (125 MHz).

An unprecedented structural feature of these molecules is the five-membered fused lactone that incorporates the oxidized C-19 position. Naturally occurring 19-hydroxysterols are unusual, having previously been isolated only from the alcyonacean coral *Litophyton viridis*[80,81] and from the sponge *Toxadocia zumi*[82] and are of interest as possible biosynthetic precursors of 19-norsterols.[83] The biosynthesis of the lactone is assumed to take place by the condensation of the triol and the free carboxylic acid or aldehyde. Formation of a 10-3 carbolactone by a sodium 3β-sulfate of cholest-5,24-dien-19-oic acid from *Toxadocia zumi* was observed when the sulfate group was hydrolyzed in refluxing methanolic hydrochloric acid.[81]

Conventional bioassays showed marginal cytotoxicity and some protein phosphotase inhibition for **18** and no biological activity for **16** and **17** (see Table 3).

Table 3. Biological Activities of Compounds **16** - **18**

	16	17	18
KB (MIC)	-	-	1 μg/mL
LoVo (MIC)	-	-	5 μg/mL
HIV	-	-	-
HSV II	>10 μg/mL	>10 μg/mL	>10 μg/mL
DHFR	-	-	-
TS	-	-	-
PI	-	-	58% @ 50 μg

C. Project AW: Organohalogen Constituents of the Acorn Worm *Balanoglossus aurantiacus*

Acorn worms (phylum Hemichordata, class Enteropneusta) are benthic, deposit-feeding invertebrates distributed throughout the world's oceans from the intertidal to the deep sea.[84,85] These animals are notable for their production of a host of halogenated compounds, notably phenols, hydroquinones, and indoles, many of which exhibit antimicrobial or toxic properties.[86,87] The first such report was Burkholder's comment on the antibacterial and antiyeast activity of an extract from *Saccoglossus* sp.[88] Higa and Sakemi tested organic extracts of *Ptychodera flava* and found that the ethyl acetate fraction inhibited the growth of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*.[89] King provided direct evidence that the secondary metabolites of an enteropneust can mediate the microbiology of its sedimentary habitat.[89,90] Dibromophenol, the predominant halogenated organic compound in *Saccoglossus kowalewskii*, inhibited the aerobic metabolism of sediment more than it interfered with anoxic processes. King also showed that microbes associated with the animal degraded dibromophenol and he suggested pathways of degradation.

Dr. Fred Dobbs, while at Florida State University, became interested in the ecology of microorganisms associated with an acorn worm believed to be *Pytchodera bahamensis*.[91] The animal has only recently been identified as *Balanoglossus aurantiacus*. Dr. Dobbs,

Ptychodera bahamensis (?)
(acorn worm)
140 g (worm tissue + gut contents)

Extracted with acetone,
50 mL distilled H_2O added
and partitioned

3 X 200 mL
Hexane
243.5 mg

3 X 200 mL
EtOAc
28.3 mg

Bulb to Bulb
distillation at $95^{\circ}C$
in vacuo

oil
4.0 mg
"EtOAc"

3 X 200 mL
n-BuOH
20.8 mg

Bulb to Bulb
distillation at $95^{\circ}C$
in vacuo

oil
1.6 mg
"n-BuOH"

Bulb to Bulb
distillation *in vacuo*

$75^{\circ}C$
crystals
5.1 mg
"Hex DB"

$75^{\circ}C$
oil
0.6 mg
"Hex oil"

$95^{\circ}C$
oil
8.1 mg

Formed two layers upon
addition of hexane

top layer

bottom layer

"95 Oil sol"

"95 Oil ins"

Figure 20. Separation scheme for the acorn worm *Balanoglossus aurantiacus.*

Table 4. Halogenated Constituents of the Acorn Worm

Balanoglossus aurantiacus

Fraction[a]	Retention Time (sec)	Molecular Weight (m/z)	Molecular Formula	Identification
Hex DB	1177	250	$C_6H_4Br_2O$	2,6 dibromophenol (19)
	1324	204	$C_6H_5BrO_3$	bromoepoxyhydroxycylohexenone (29)
	1409	264	$C_8H_6BrClO_3$	27 - acetate
	1492	266	$C_6H_4Br_2O_2$	dibromohydroquinone (26)
Hex Oil	1176	250	$C_6H_4Br_2O$	2,6 dibromophenol (19)
	1244	240	$C_6H_3BrCl_2O$	bromodichlorophenol (23)
	1311	284	$C_6H_3Br_2ClO$	dibromochlorophenol (24)
	1379	328	$C_6H_3Br_3O$	2,4,6-tribromophenol (22)
95 Oil sol	1178	250	$C_6H_4Br_2O$	2,6 dibromophenol (19)
	1402	264	$C_8H_6BrClO_3$	27- acetate
	1478	308	$C_8H_6Br_2O_3$	28
95 Oil ins	1177	250	$C_6H_4Br_2O$	2,6 dibromophenol (19)
	1238	188	$C_6H_5BrO_2$	bromohydroquinone (25)
	1401	264	$C_8H_6BrClO_3$	27 - acetate
	1411	246	$C_8H_7BrO_4$	19
	1426	222	$C_6H_4BrClO_2$	2,6-bromochlorohydroquinone (27)
	1477	308	$C_8H_6Br_2O_3$	28
	1519	266	$C_6H_4Br_2O_2$	dibromohydroquinone (26)
EtOAc	1177	250	$C_6H_4Br_2O$	2,6 dibromophenol (19)
	1259	230	$C_8H_7BrO_3$	25 - acetate
	1268	162	$C_6H_4Cl_2O$	3,4- or 3,5-dichlorophenol (20,21)
	1307	266	$C_6H_4Br_2O_2$	dibromocatechol
	1365	189	$C_7H_8ClNO_3$	?
	1379	328	$C_6H_3Br_3O$	2,4,6-tribromophenol (22)
	1389	226	?	contains Br
	1409	264	$C_8H_6BrClO_3$	27 - acetate
	1431	262	$C_6H_4Br_2O_2$	?
	1485	308	$C_8H_6Br_2O_3$	28
	1506	231	$C_9H_{10}ClNO_4$	(EtOAc 1365) - acetate
	1525	306	?	contains BrCl
	1533	266	$C_6H_4Br_2O_2$	dibromohydroquinone isomer
n-BuOH	1177	250	$C_6H_4Br_2O$	2,6 dibromophenol (19)

[a] see Fig. 20 for nomenclature

now in the Oceanography Department at the University of Hawaii, asked me to identify the volatile organohalogens present in the crude extracts of the acorn worm. Gas chromotography-mass spectrometry of the distillate from each extract (Fig. 20) revealed the presence of both brominated and chlorinated organics.[92]

Twenty compounds containing halogens were present in the samples (see Table 4). The compounds consisted of one chlorinated and twelve brominated aromatics, five aromatic compounds containing both chlorine and bromine, and two nitrogenous compounds containing chlorine. None of the chlorinated compounds had previously been reported from acorn worms. The compounds appear to be primarily phenols.

The phenols make up the majority (by weight) of the compounds. 2,6-Dibromophenol (19) crystallized in the receiving flask during distillation of the hexane fraction. It was identified by ^{1}H NMR spectroscopy and was present in all samples as judged by retention time and mass spectrum. A dichlorophenol was isolated from the EtOAc fracton eluting on GC after 2,6-dibromophenol. Comparison of retention times for 2,6-dibromophenol and the six isomers of dichlorophenol showed that isomers 20 and 21 were the only isomers to elute after 2,6-dibromophenol and thus are likely constituents of *Balanoglossus aurantiacus*.
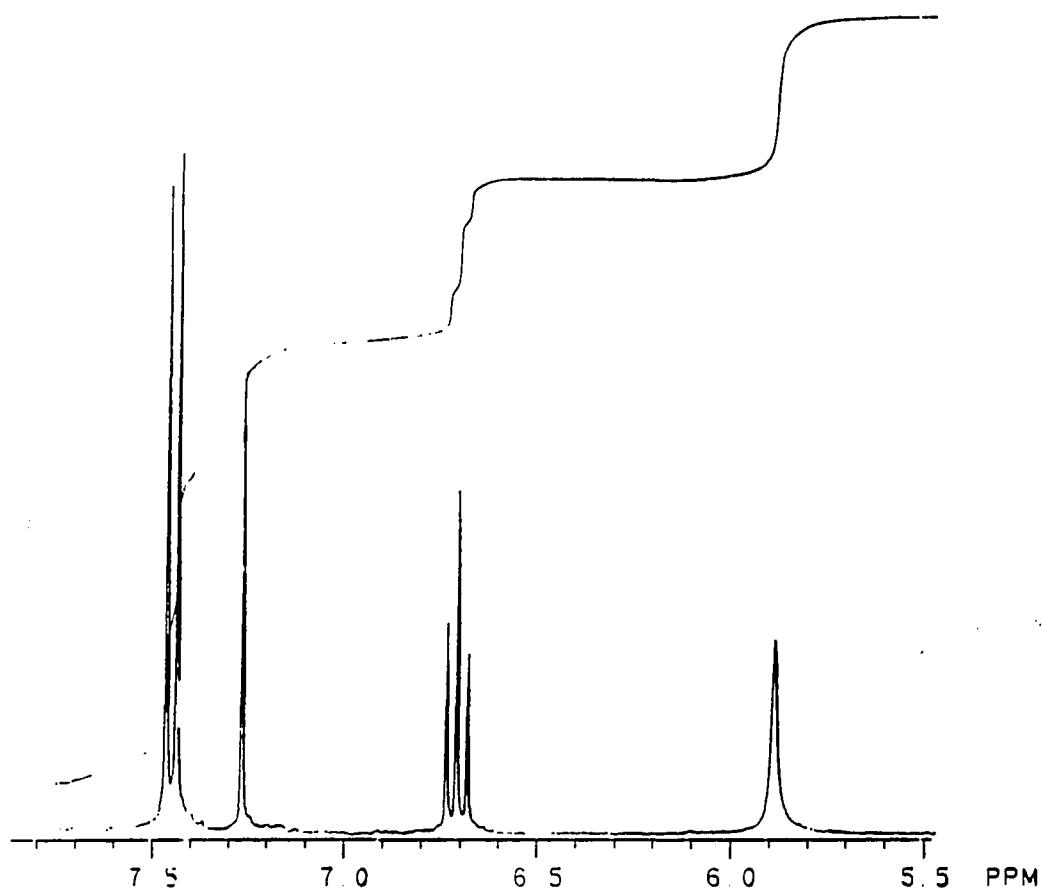
Figure 21. ^{1}H NMR spectrum of 2,6-dibromophenol (19) in $CDCl_3$ (300 MHz).

Structure 19 (2,6-dibromophenol), 20 (3,5-dichlorophenol), 21 (3,4-dichlorophenol)
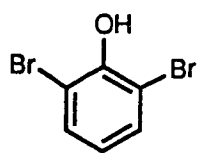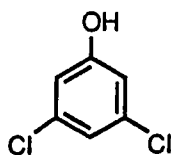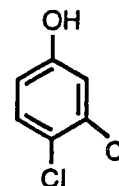
19   **20**   **21**

Three trihalogenated phenols were detected in the fraction designated "Hex Oil." Tribromophenol was identified as 2,4,6-tribromophenol (**22**) by comparison of retention time on GC and mass spectra of the two compounds. The structure was confirmed by co-injection with a known sample on GC-MS. 2,4,6-Tribromophenol was also isolated from the EtOAc fraction. Bromodichlorophenol **23** was compared with 4-bromo-2,6-dichlorophenol and found to have different retention times in gas chromatography.
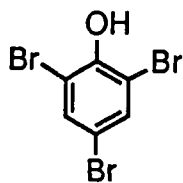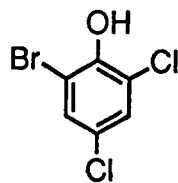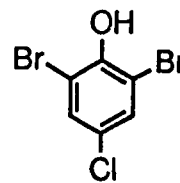
22   **23**   **24**

Assuming 2,4,6 substitution of the aromatic ring, the bromodichlorophenol would be 2-bromo-4,6-dichlorophenol. The regiochemistry of dibromochlorophenol **24** is unknown, but would also be expected to be 2,4,6-substituted.

Hydroquinones and their acetates were present in small amounts in all but the n-BuOH fraction. The compounds present include bromohydroquinone **25**, dibromohydroquinone **26** and bromochlorohydroquinone **27**. Also present are the acetates of these three compounds (e.g. **28**). Two additional isomers of dibromohydroquinone were detected. One of the isomers was identified as dibromocatechol by a pronounced M^+-18 peak in the mass spectrum.[93]
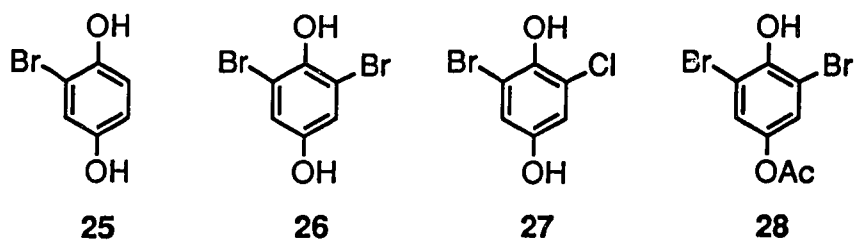
Two oxidized hydroquinones were detected, a bromoepoxyhydroxy-cylohexenone **29** in the "Hex DB" fraction and its acetate **30** in the "95 Oil ins" fraction.
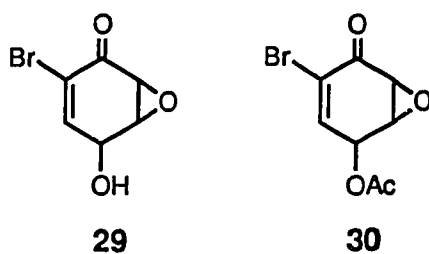
This is the first report of dibromocatechol, dichlorophenol, bromochlorophenol, bromochlorohydroquinone and acetylated hydroquinones in acorn worms. 2,4,6-Tribromophenol had previously been isolated from *Ptychodera flava laysanica*[94] and brominated hydroquinones have also been reported from acorn worms.[95] Bromoepoxyhydroxycyclohexenes have been described from an acorn worm of the genus *Ptychodera*.[96]

Chlorophenols have been reported previously only from terrestrial sources.[97] Intriguingly, 200,000 tons of chlorophenols are manufactured annually worldwide, mostly for use in the pulp and paper industry;[98] some have been classified as priority pollutants by the Environmental Protection Agency.[99]

Enteropneusts are widespread throughout the world, abundant in certain habitats, and may be a significant non-anthropogenic source of organohalogens in the marine environment. Higa and Sakemi estimated that acorn worms in a 1 km^2 habitat at Kattore Bay, Okinawa, annually discharge 4 tons of ethyl acetate-soluble materials, substantial amounts of which are halogen-containing compounds.[87] A similar analysis can be applied to the habitat of the present study, where *Balanoglossus aurantiacus* egest approximately 100 g (dry weight) of sediment per organism per day and occur in densities of two to five individuals per square meter.[100] Assuming that 2.4 X 10^{-3}% (by dry weight) of the fecal material is soluble in ethyl acetate,[87] and that the above density of animals extends 300 m from the low intertidal to the shallow subtidal,[101] *Balanoglossus aurantiacus* annually produce between 0.5 -

1.3 metric tons of ethyl acetate-soluble material per kilometer of coastline in the Eastern Gulf of Mexico.

Dr. Dobbs has exposed sedimentary bacteria from the environment of the acorn worms to extracts partitioned as described. Growth of many of the isolates was inhibited, but growth was promoted for some. These results suggest that certain bacteria can utilize one or more compounds in the extracts as a carbon or energy source. If organohalogens are among the compounds so utilized, which is a distinct possibility based on the results obtained by King,[89,90] bacteria associated with enteropneusts are promising candidates for bioremediation of anthropogenic organohalogens.[102]

D. Project 92-IND-169: Two Isomalabaricane Triterpenes from the Sponge *Rhabdastrella* sp.

Sponges have proven to be prolific producers of novel terpenoids that display a variety of biological activities.[103] A new isomalabaricane triterpenoid, stelliferinoside (31), containing an α-D-ribopyranose sugar moiety and the previously described stelliferin A (32) have been isolated from a sponge collected in Indonesia. This sponge has been identified as an undescribed species of *Rhabdastrella* (Order Choristida, Family Jaspidae).[104] Malabaricanes derive their name from the compound malabaricol, the first triterpene containing the unique tricarbocyclic ring structure, which was isolated from the resinous exudate of the tree *Ailanthus malabarica*.[105] This class of compounds was augmented by the discovery of related compounds from the sponges *Jaspis stellifera*[106] and *Stelletta* sp.[107] The stelliferins, isolated from *Jaspis stellifera*, exhibited "potent antineoplastic activities" versus KB and L1210 cancer cell lines.[106c, 108]

Two isomalabaricane-type triterpenes were isolated from a sponge, *Rhabdastrella* sp., collected near Monado, Indonesia on October 3, 1992. The sponge is round and reddish-brown-colored on the outside with a tan inside. The freeze-dried sponge was extracted with CH_2Cl_2 and the extract flash-chromatographed on silica with sequential elution of hexane, CH_2Cl_2, CH_2Cl_2 / EtOAc (2:1), EtOAc and MeOH. Stelliferinoside (31) was isolated from the EtOAc fraction using reverse phase C18 HPLC. Stelliferin A (32) was isolated from the CH_2Cl_2 / EtOAc (2:1) fraction by reverse phase C18 HPLC.
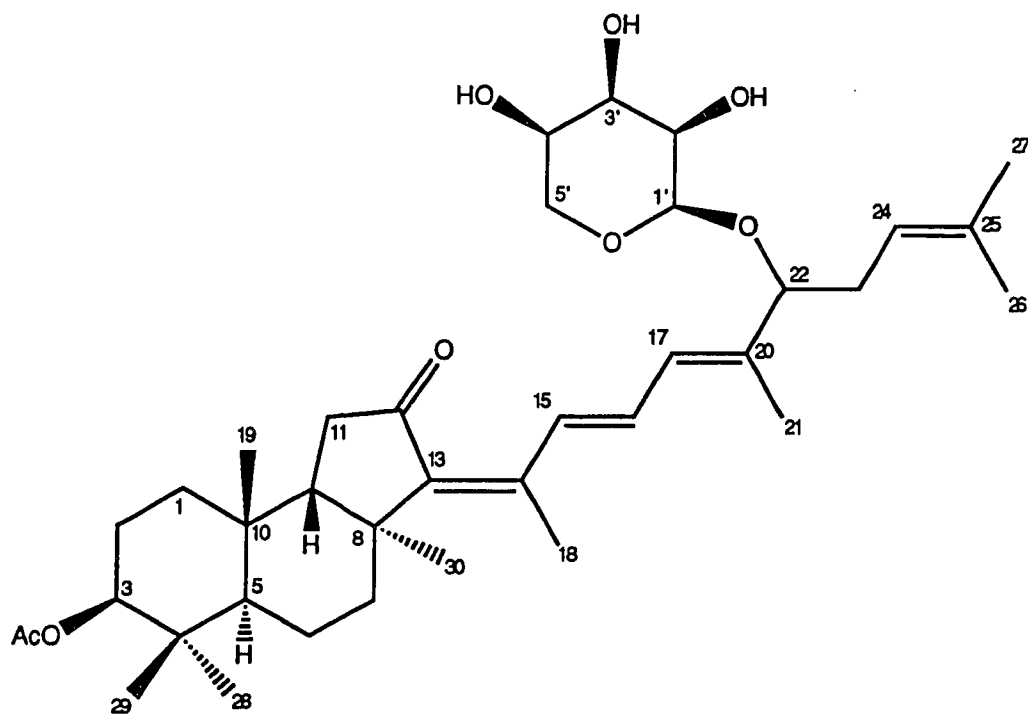
3 1
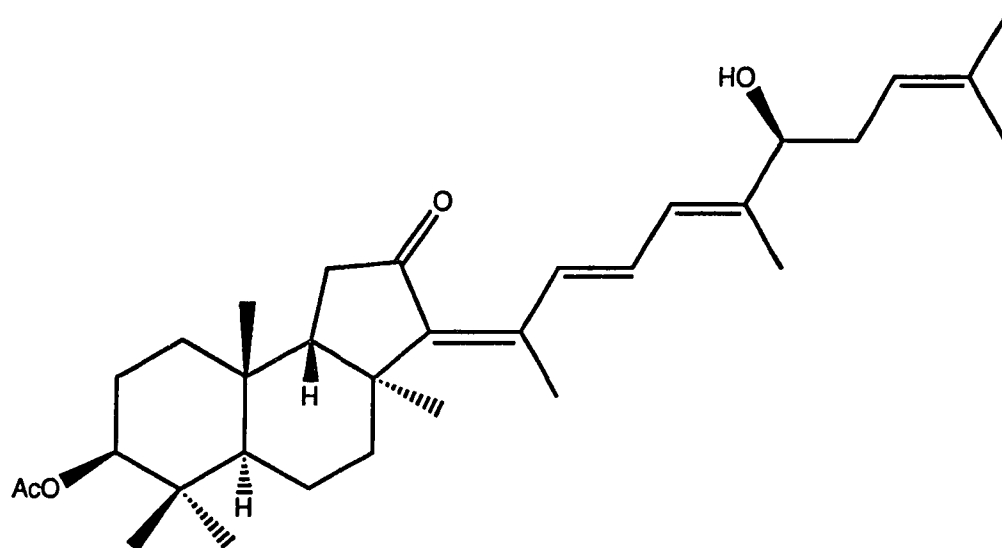
3 2

Stelliferinoside (31) was isolated and its structure determined prior to the isolation of stelliferin A because of larger quantities of the metabolite in the extracts. The structure of stelliferinoside was determined by NMR experiments. ^{1}H and ^{13}C NMR data were acquired and an HMQC experiment established the [1]H-^{13}C connectivities. A COSY experiment facilitated the elucidation of two partial structures and the final structure was determined using an HMBC experiment. The relative stereochemistry was established by a ROESY experiment. The glycoside was hydrolyzed and the absolute stereochemistry of the sugar determined by optical rotation.

The ^{13}C NMR spectrum (Fig. 22, Table 5) revealed the presence of 37 carbons: a ketone, an ester carbonyl, eight olefinic carbons, an acetal carbon, six oxygenated carbons and twenty alkyl carbons. An HMQC[78] experiment disclosed the alkyl carbons to consist of nine methyl carbons, six methylene carbons, two methine and three quaternary carbons.

^{1}H NMR (Fig. 23, Table 5) and COSY experiments provided two partial structures. Partial structure A (Fig. 24) represents a conjugated diene defined by ^{1}H NMR resonances at δ 8.00, δ 6.84 and δ 6.21. The stereochemistry of the diene was determined to be E by coupling constants of 16 Hz for δ 8.00, 16 and 11 Hz for δ 6.84 and 11 Hz for δ 6.21. Partial structure B is a sugar that was determined to be a pyranose by the ^{13}C chemical shift of C-4' (δ 70.2).[109] A furanose has a ^{13}C chemical shift of δ 85 at this carbon.[109] The coupling constants of the sugar protons range from 4 to 6 Hz indicating only equatorial - equatorial and axial - equatorial coupling.

Table 5. Comparison of ^{13}C and ^{1}H NMR Chemical Shifts for Stelliferinoside (31) and Stelliferin A.(32) with Literature Values.

	stelliferinoside (31)*		stelliferin A (32)	
Position	^{13}C δ	^{1}H δ (m, jHz)	^{13}C δ*,J.C.	^{13}C δ*,106C
1	29.7	a. 1.20 (m)	32.9	33.0
		b. 1.78 (m)		
2	24.7	a. 1.74 (m)	25.0	25.1
		b.2.04 (m)		
3	78.2	4.71 (t, 3)	80.7	80.8
4	37.7		38.1	38.2
5	41.9	2.31 (m)	46.5	46.5
6	18.4	a. 1.45 (m)	18.2	18.2
		b. 1.63 (m)		
7	38.9	2.10 (m)	38.1	38.1
8	45.0		44.5	44.5
9	50.8	1.83 (dd 15, 7)	50.2	50.2
10	35.9		35.4	35.4
11	37.0	a. 2.17 (dd 15, 7)	36.8	36.7
		b. 2.23 (d 15)		
12	207.0		206.3	206.5
13	147.2		145.8	146.0
14	142.2		142.4	142.4
15	133.4	8.00 (d 16)	132.1	132.2
16	129.6	6.84 (dd 16, 11)	130.2	130.1
17	130.7	6.21 (dd 11, 1)	126.2	126.3
18	16.2	2.02 (s)	15.9	15.9
19	22.5	1.02 (s)	22.3	22.3
20	138.8		143.0	143.0
21	12.0	1.77 (d 1)	12.7	12.7
22	82.3	4.17 (dt 7, 1)	76.8	76.8
23	32.8	a. 2.40 (dt 7, 15)	34.2	34.2
		b. 2.26 (m)		
24	120.0	5.07 (tt 7, 1)	119.5	119.5
25	134.5		134.6	135.3
26	18.0	1.62 (s)	17.9	18.0
27	25.8	1.68 (s)	25.8	25.8
28	27.9	0.89 (s)	28.9	28.9
29	21.4	0.92 (s)	16.9	16.9
30	24.2	1.43 (s)	24.6	24.7
C̲H̲₃CO	21.6	2.02 (s)	170.8	171.0
CH₃C̲O̲	170.0		21.1	21.2
1'	98.5	4.35 (d 5)		
2'	71.7	3.43 (dd 5, 6)		
3'	73.4	3.54 (t 6)		
4'	70.2	3.68 (dt 6, 4)		
5'	63.5	a. 3.33 (dd 12, 6)		
		b. 4.00 (dd 12, 4)		

*Recorded in CDCl₃
**Recorded in CD₂Cl₂
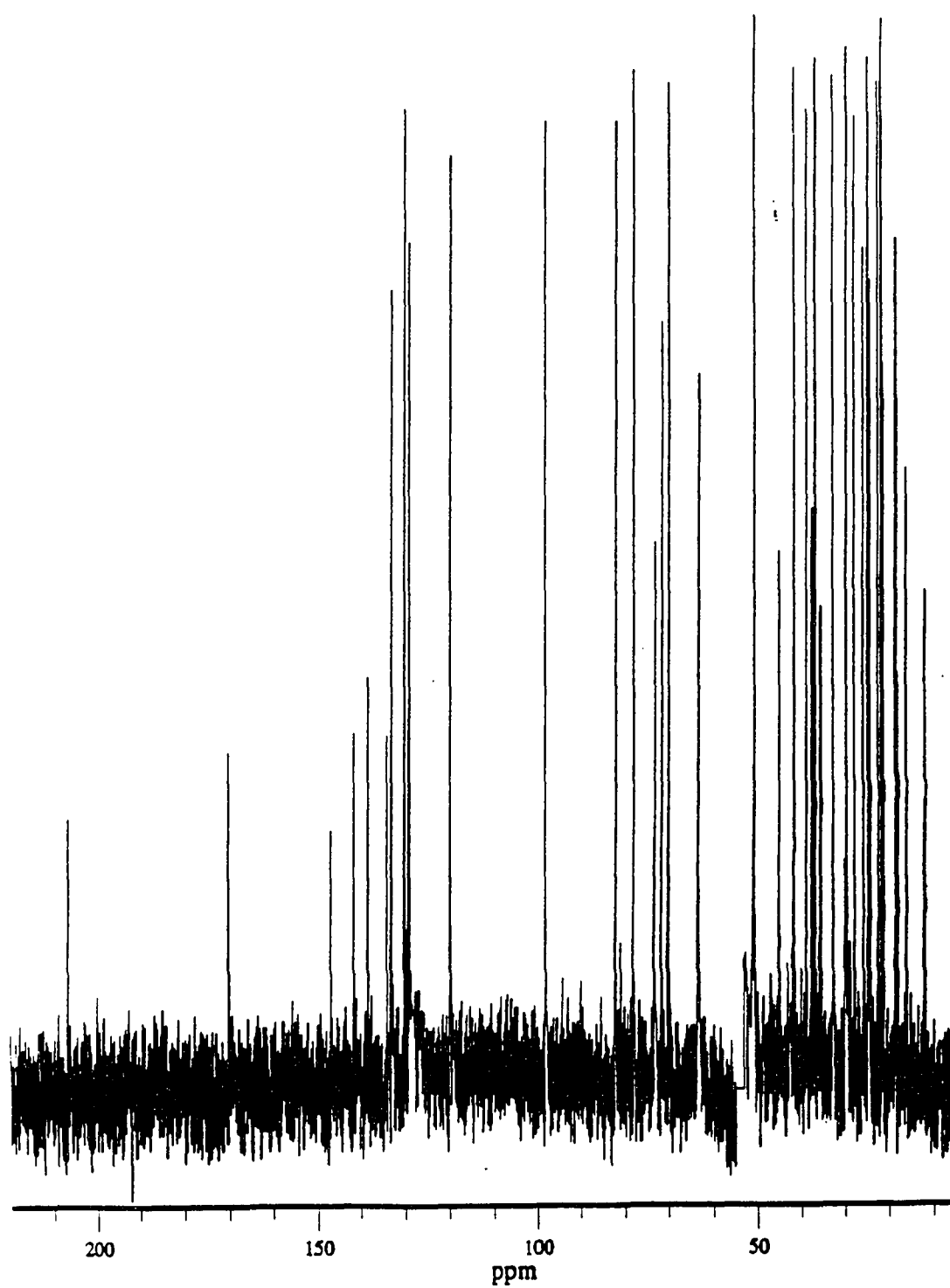
Figure 22. ^{13}C NMR spectrum of stelliferinoside (**31**) in $CDCl_3$ (125 MHz).
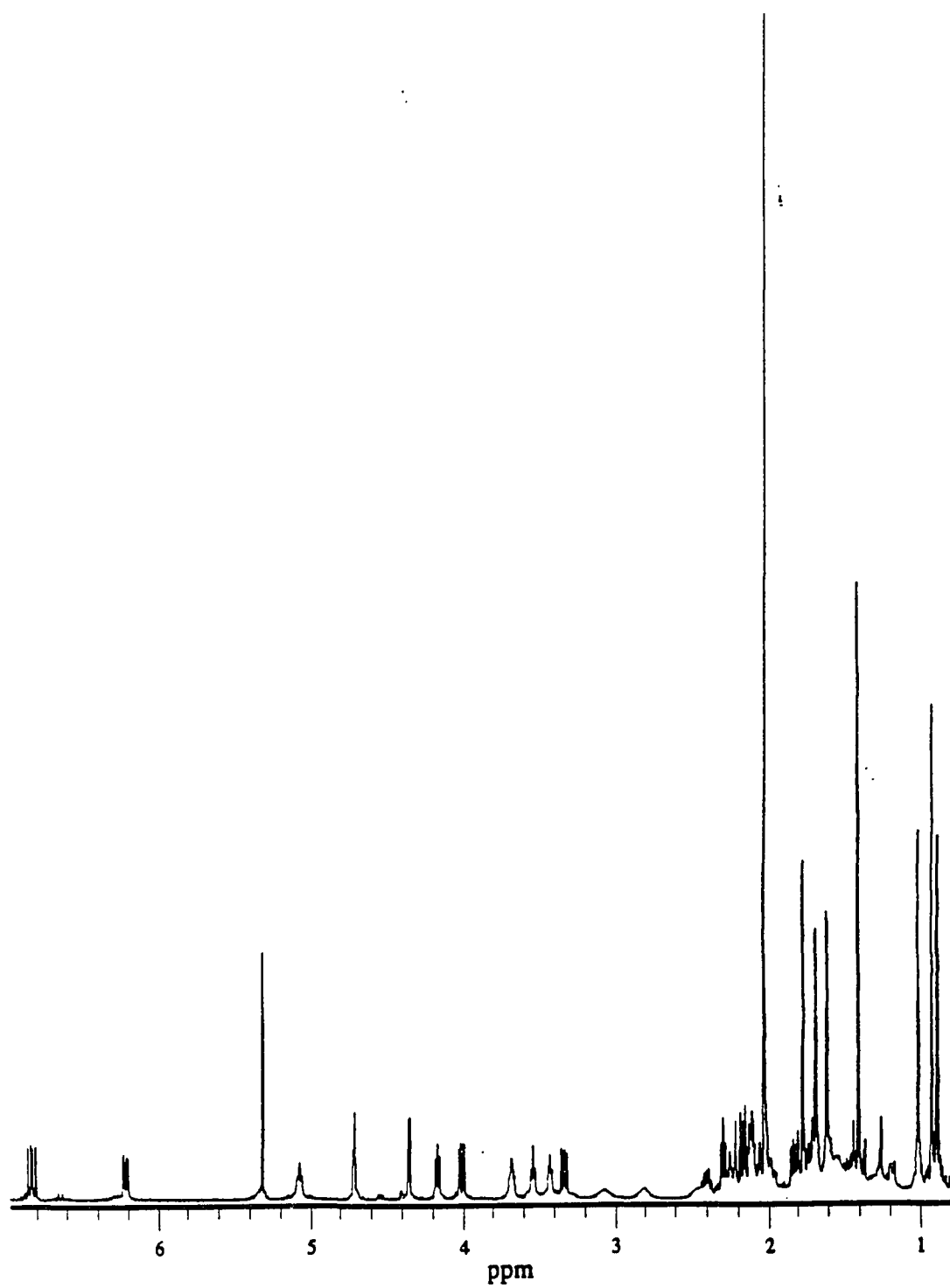
Figure 23. ¹H NMR spectrum of stelliferinoside (31) in CDCl₃ (500 MHz).
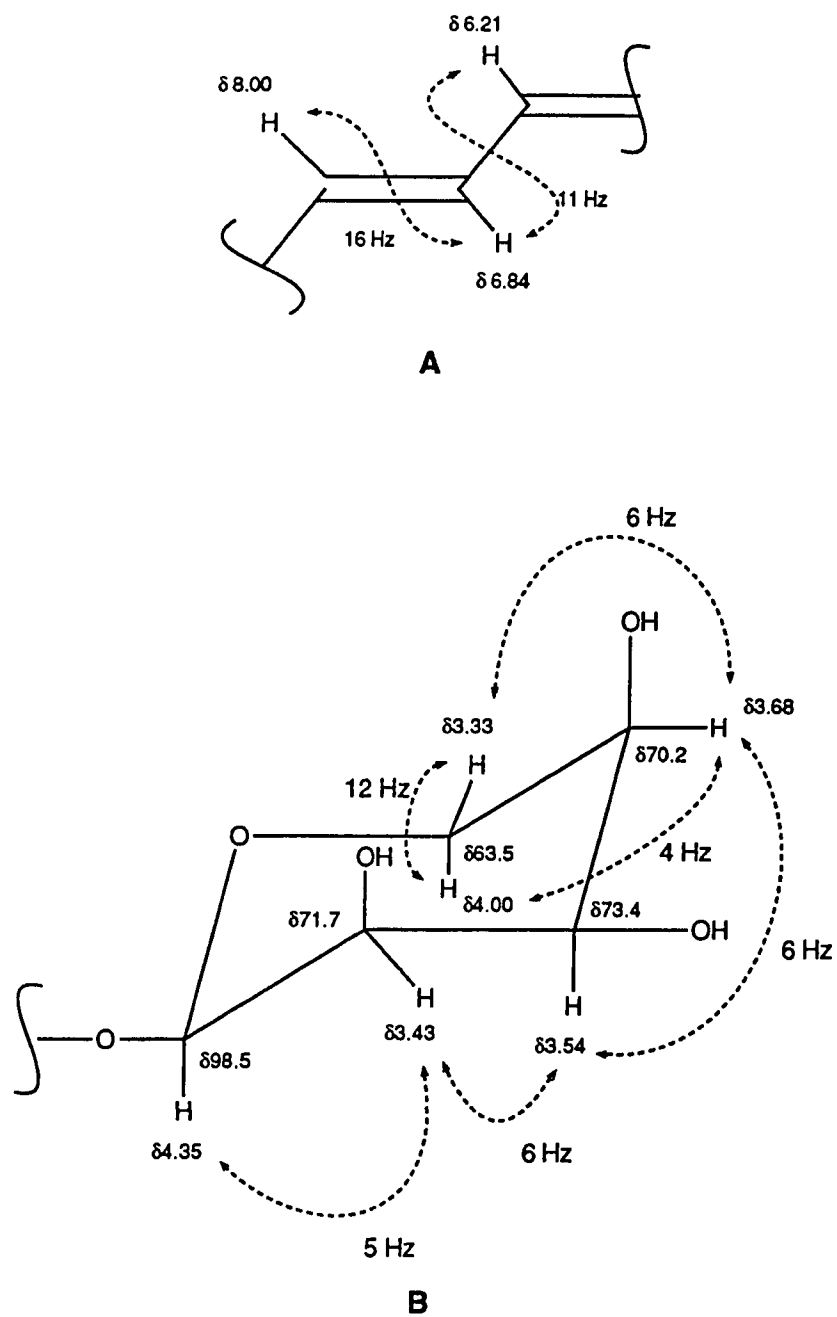
Figure 24. Important ^{1}H-^{1}H coupling constants for partial structures **A** and **B** of stelliferinoside (**31**).

The final structure was elucidated using an HMBC experiment. A terminal dimethyl olefin was established by mutual coupling of the methyl proton resonances at δ 1.62 and δ 1.68 to the corresponding methyl carbon (δ 25.8 and δ 18.0, respectively) and to the olefin ^{13}C resonances at δ 120.0 and δ 134.5. The methylene protons at δ 2.26 and δ 2.40 were coupled to two carbon signals at δ 138.8 and δ 82.3 and to both terminal methyl groups. A third methyl group at δ 1.77 also was coupled to these two carbon resonances at δ 82.3 and δ 138.8 and to a signal at δ 130.7. This was a signal of a carbon in the conjugated diene that was interpreted by COSY correlations and coupling constants (**A**, Fig. 24). The fourth methyl signal at δ 2.02 was coupled to carbons resonating at δ 134.4, δ 142.2 and to a signal at δ 147.2, thus completing the partial structure shown in Figure 25.
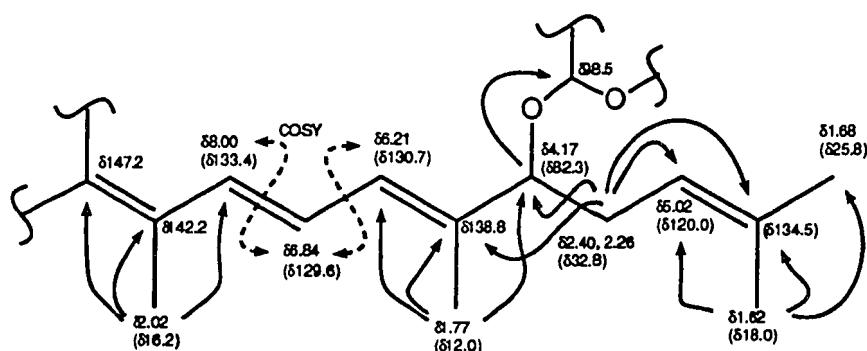
Figure 25. Important HMBC correlations for the sidechain of stelliferinoside (**31**).

The tricyclic partial structure shown in Figure 26 was determined by an HMBC experiment. A methyl signal at δ 1.43 was coupled to carbon signals at δ 38.9,

δ 45.0, δ 50.2 and δ 145.8. A methine signal at δ 2.30 was coupled to carbon resonances δ 38.9, δ 18.0, δ 37.7, δ 21.4, δ 27.9 and δ 29.7. Both methyl signals at δ 0.92 and δ 0.89 were coupled to carbon resonances δ 41.9, δ 37.7 and δ 78.2. A methyl signal at δ 1.02 was coupled to carbon resonances δ 29.7, δ 41.9, δ 35.9 and δ 50.8. A methine signal at δ 1.83 was correlated to carbon resonances δ 29.7, δ 35.9, δ 38.9, δ 45.0, δ 24.2 and δ 37.0. The methylene signals at δ 2.17 and δ 2.23 were coupled to the ketone carbon at δ 207.0. A ^{1}H of the remaining methylene at δ 2.04 was coupled to a carbon resonating at δ 29.7 completing the partial structure shown in Figure 26.
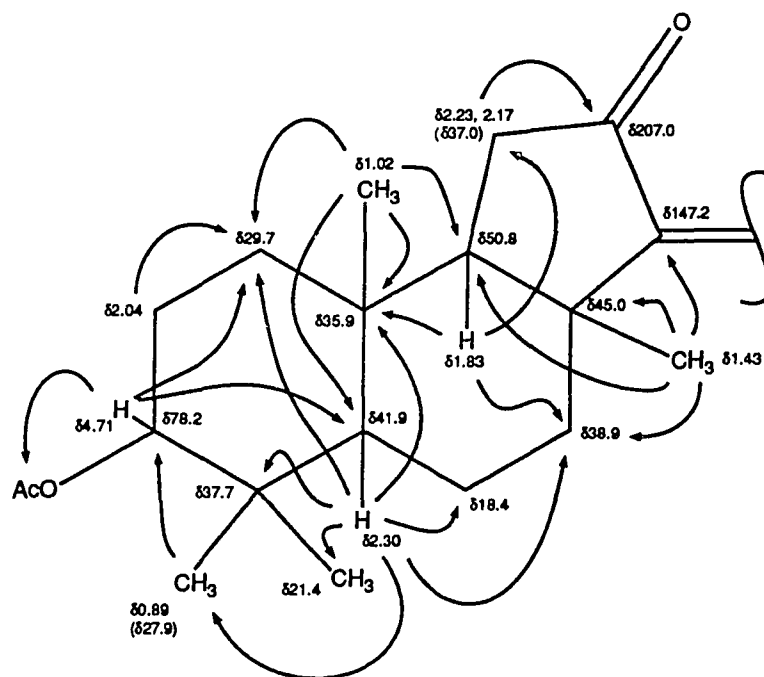
Figure 26. Important HMBC correlations for the tricyclic portion of stelliferinoside (**31**).

The HMBC experiment also showed connectivity from the proton signal at δ 4.17 to the carbon at δ 98.5 and from the proton signal at δ 4.71 to the acetate carbonyl at δ 170.6 establishing the location of the pyranose and the acetate in the molecule (Fig. 25).

The relative stereochemistry of the molecule was determined by a ROESY NMR experiment. The sugar moiety showed NOE correlations between H-1' (δ 4.35), H-3' (δ 3.54) and H-5b' (δ 4.00) indicating that these protons are axial. This, along with the ^1H-^1H coupling constants, establishes the sugar as a ribopyranose.[110] The aglycone showed ROESY correlations consistent with the reported relative stereochemistry of stelliferin A (Fig. 27).[106c]

The absolute stereochemistry of the ribopyranose was determined to be D by comparison of the optical rotation of the monosaccharide with published values. The sugar of 31 was hydrolyzed in refluxing aqueous acetic acid. The reaction mixture became bright yellow during heating, slowly fading to a light yellow color. The resulting reaction mixture was partitioned with CH_2Cl_2 and both extracts were concentrated to dryness. The residue of the aqueous extract was identified as the free monosaccharide by ^1H NMR spectroscopy (Fig. 28). The residue of the CH_2Cl_2 extract was a complex mixture, as indicated by ^1H NMR spectroscopy, with none of the aglycone present. It appears that the aglycone dehydrated at C-23 to form a conjugated pentaenone, as witnessed by the color change during the reaction. This compound then degraded into a complex mixture. The optical rotation of the sugar was determined to be -25°, in agreement with the published value of D-ribose.[111]

The structure of stelliferin A (32) was determined by comparison of ^{1}H and ^{13}C chemical shift (Figs 29 and 30 and Table V) and optical rotation data with published data for stelliferin A.[106c]

Conventional bioassays against human epidermoid carcinoma (KB) and human colorectal adenocarcinoma (LoVo) cell lines showed marginal cytotoxicity for stelliferinoside (31). Stelliferinoside had an IC_{50} of 2 μg/mL in the KB assay and 3 μg/mL in the LoVo assay. An antiviral assay against Herpes simplex II virus showed activity at 5 μg/mL (98.5% reduction) and toxicity to a mink lung cell line, in which the virus is cultured, at 10 μg/mL.
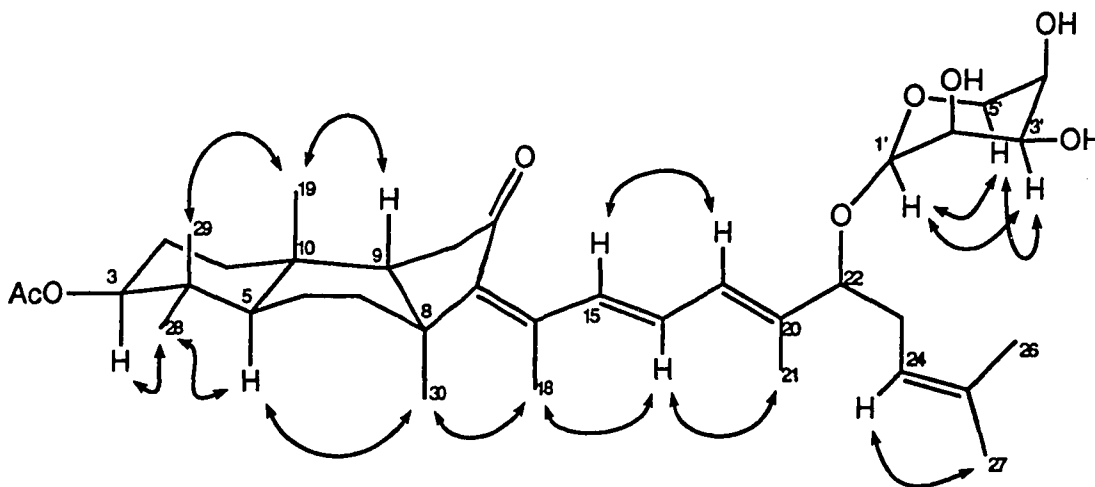
Figure 27. Important ROESY NMR correlations for stelliferinoside (31).

A

B

5.3   5.1   4.9   4.7   4.5   4.3   4.1   3.9   3.7   3.5
                              ppm
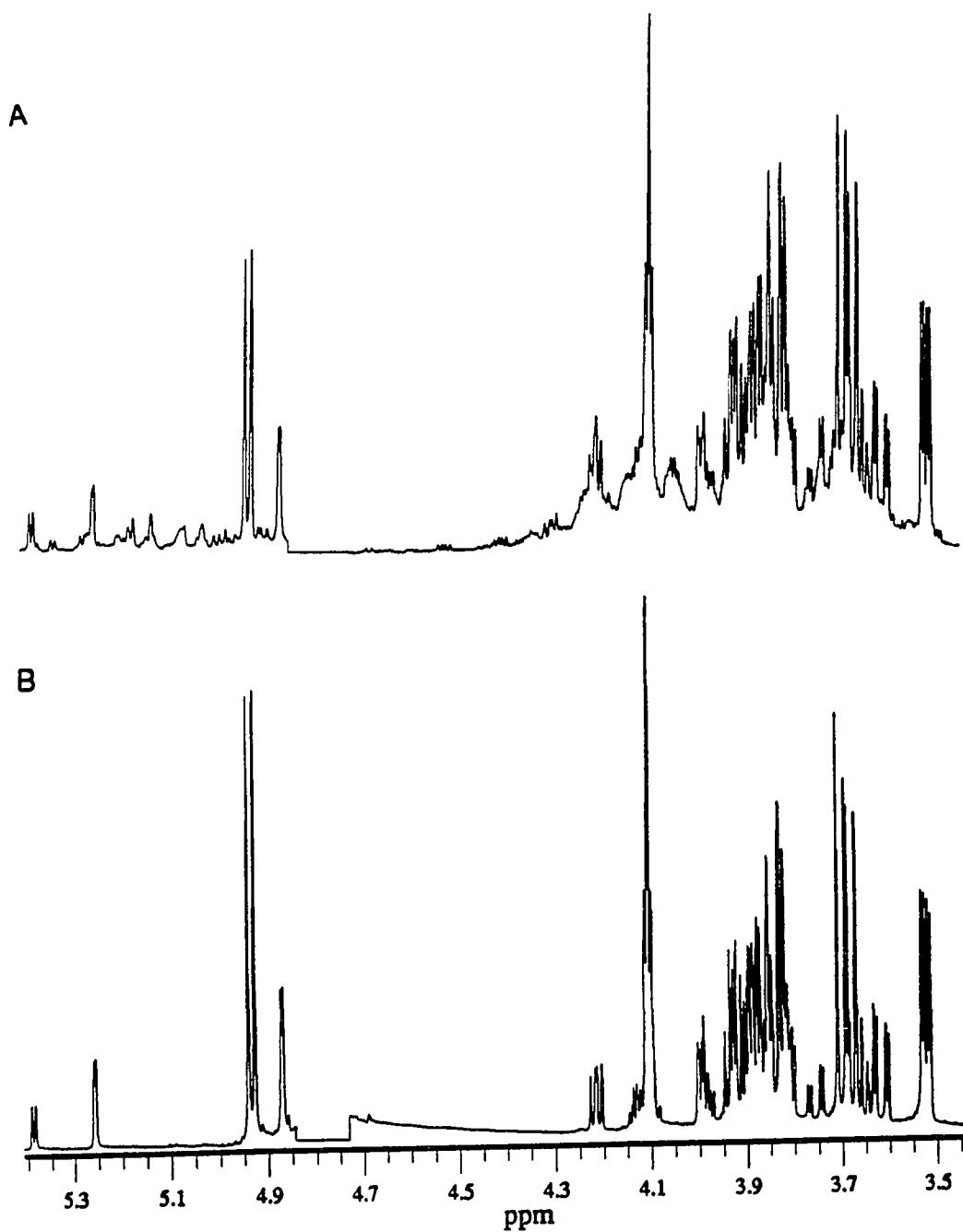
Figure 28. Comparison of[1]H NMR spectrum of D-ribose from **31** (A) and a

reference sample[112] (B) in D_2O (500 MHz).
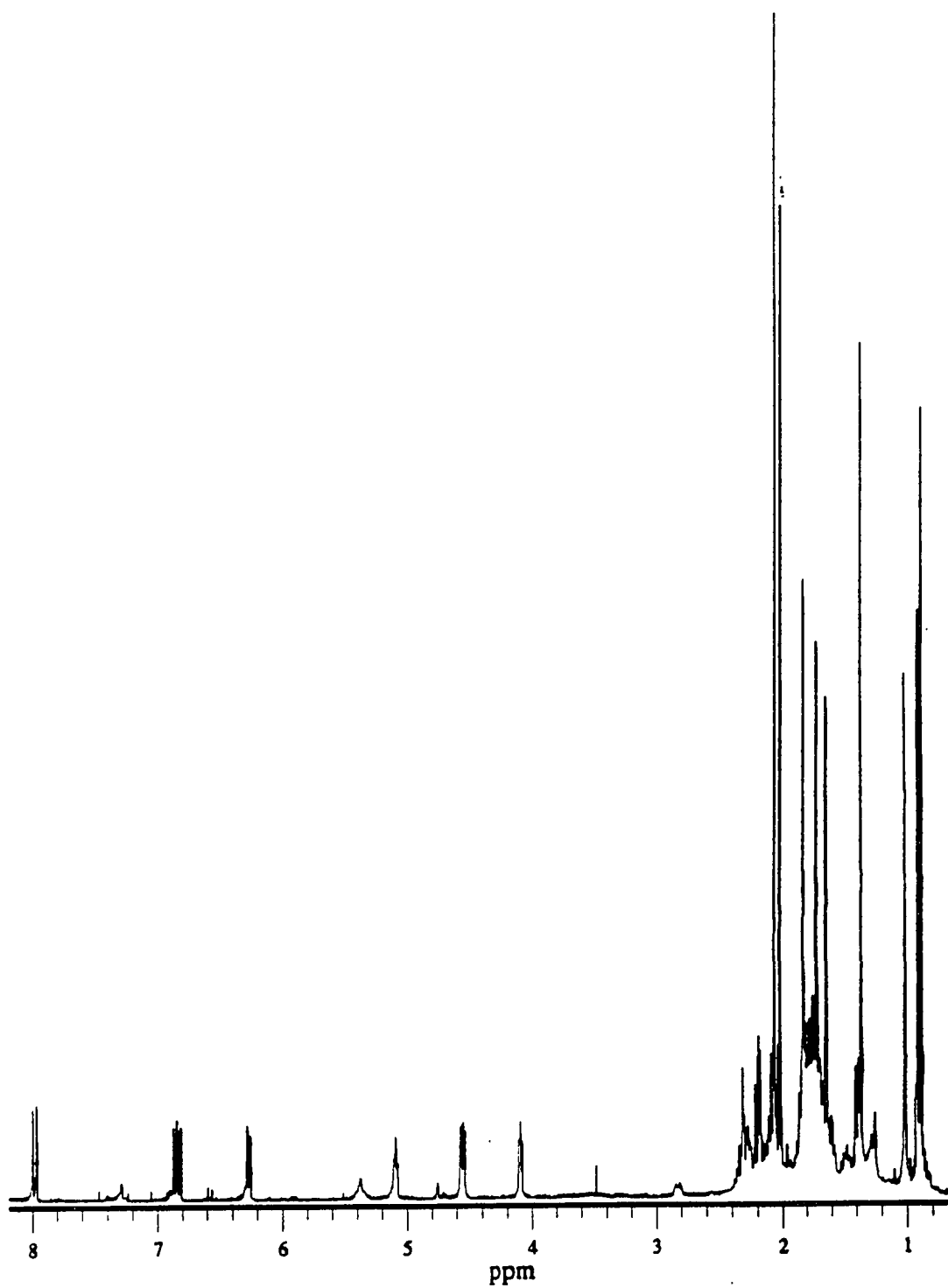
Figure 29. ^{1}H NMR spectrum of stelliferin A (**32**) in $CDCl_3$ (500 MHz).
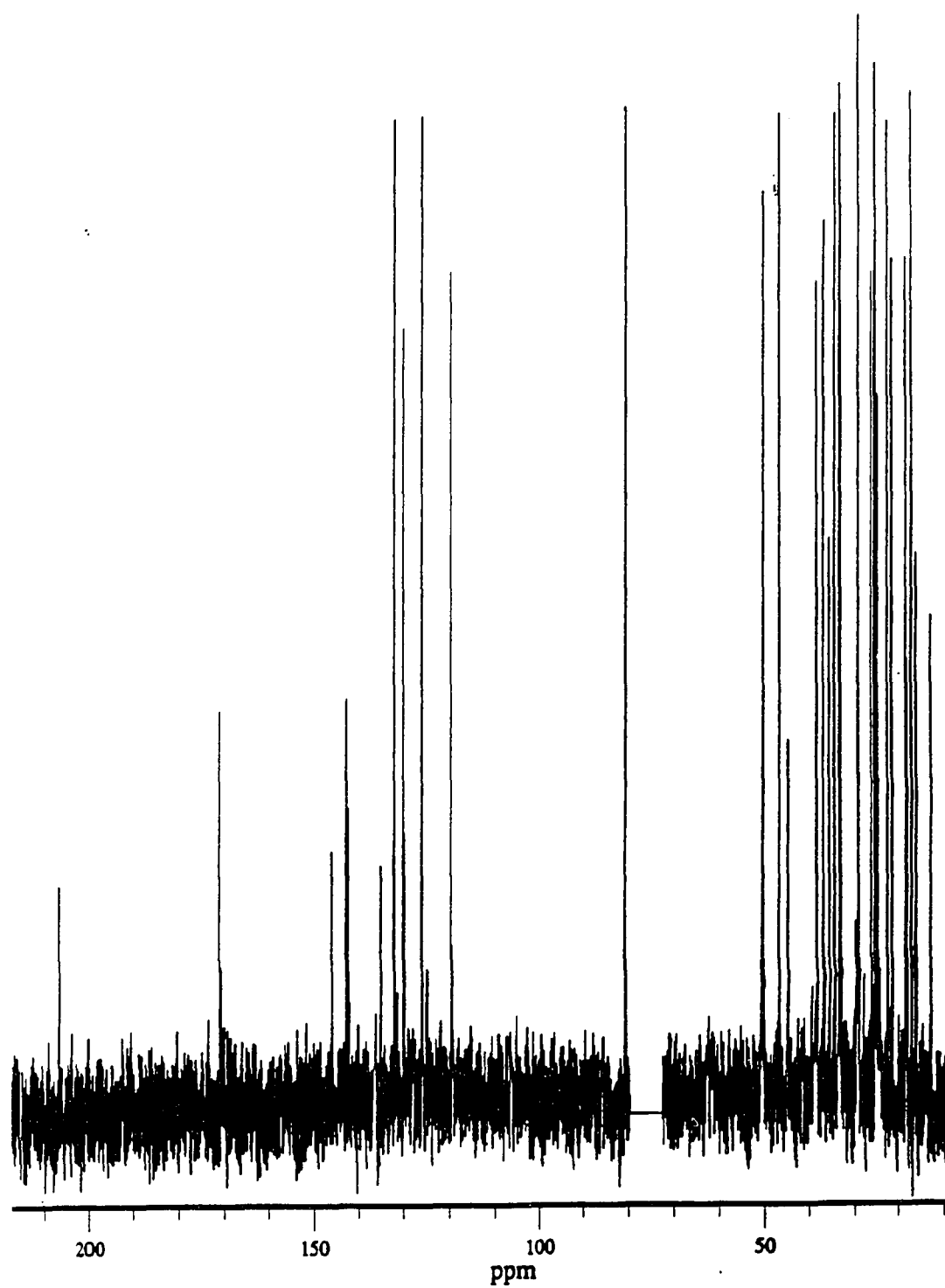
Figure 30. ^{13}C NMR spectrum of stelliferin A (**32**) in $CDCl_3$ (125 MHz).

E. Project 92-IND-293

4-(6'-Acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (**33**) was isolated from a sponge collected near Monado, Indonesia. The compound is similar to curcuphenol (**34**), the first marine natural product shown to inhibit H, K-ATPase activity.[113]
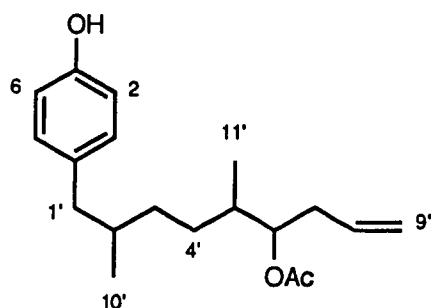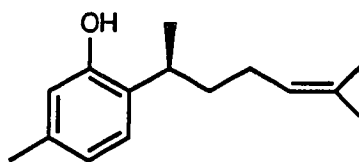
33   **34**

A red spiculated sponge was collected by SCUBA near Bunaken in Sulawesi, Indonesia. A specimen has been sent to Dr. Michelle Kelly-Borges of the Harbor Branch Oceanographic Institute for identification. Extraction with MeOH of the freeze-dried sponge (34.8 g dry weight), addition of H_2O, partitioning with CH_2Cl_2 followed by n-BuOH, and chromatography of the dichloromethane solution led to the isolation of 4-(6'-acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (**33**). The crude lipophilic extract showed moderate immunosuppression (LcV/MLR = 109), no cytotoxicity and no antiviral activity. The crude aqueous extract showed no bioactivity in our conventional assays.
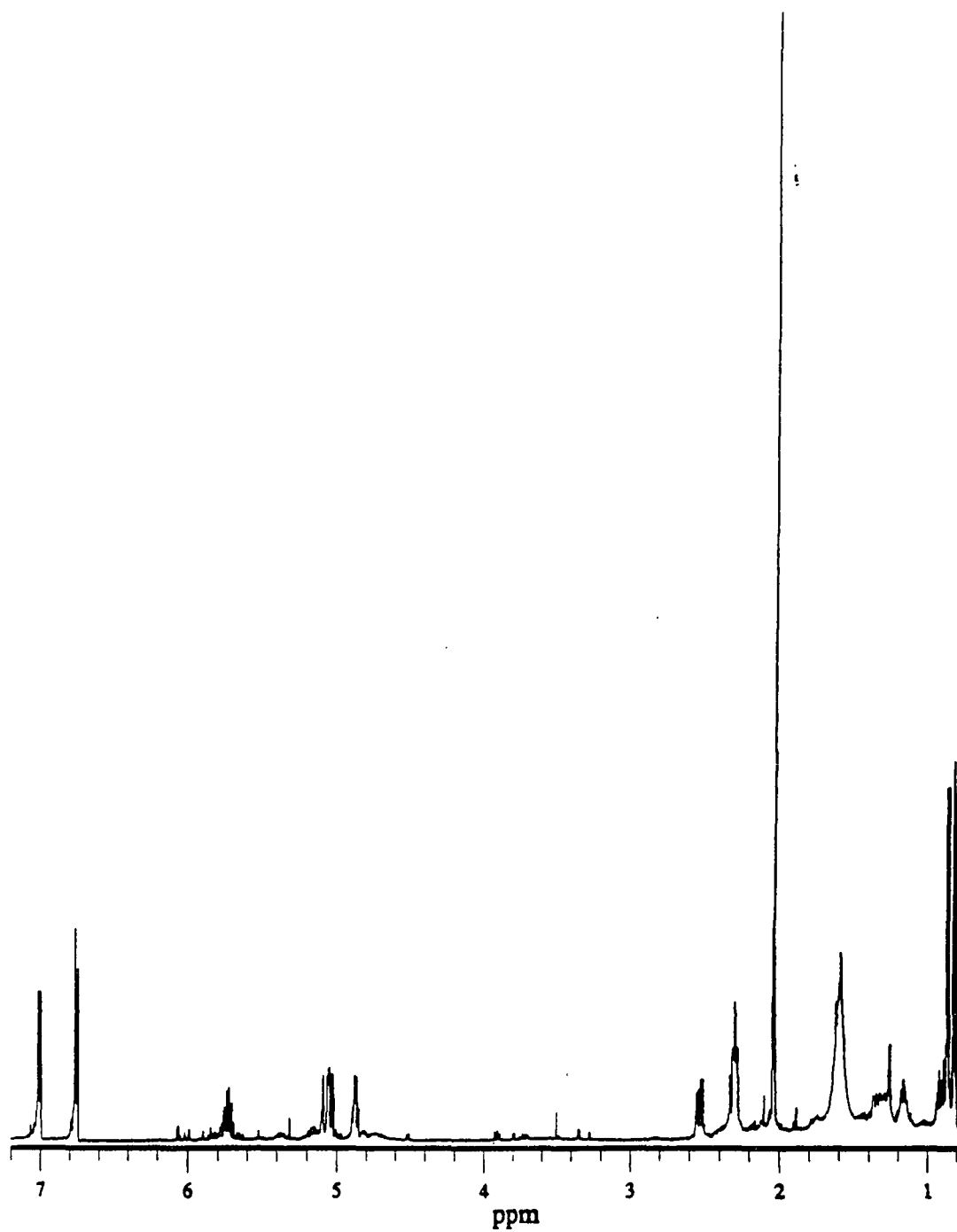
Figure 31. ¹H NMR spectrum of 4-(6'-acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (**33**) in CDCl₃ (500 MHz).
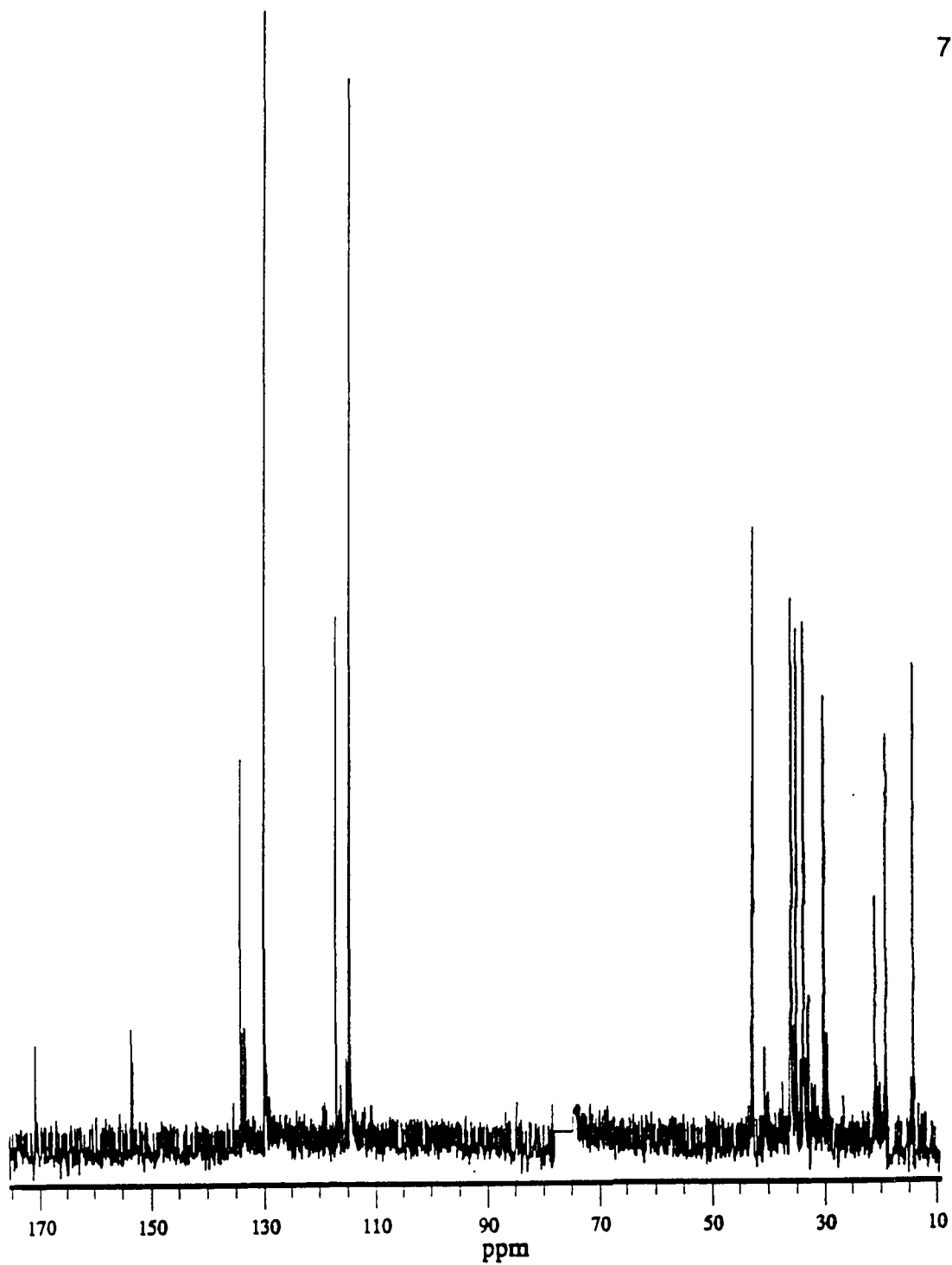
Figure 32. ^{13}C NMR spectrum of 4-(6'-acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (**33**) in CDCl$_3$ (125 MHz).

^{1}H (Fig. 31), ^{13}C (Fig. 32), mass spectrometry and 2-D NMR experiments (HMQC, HMBC, and COSY) established the structure of **33**. HREIMS and ^{13}C NMR measurements of **33** suggested a molecular formula of $C_{19}H_{28}O_3$. The ^{13}C NMR spectrum revealed the presence of an ester carbonyl, six aromatic carbons, two olefinic carbons, an oxygenated carbon and nine alkyl carbon. A HMQC experiment disclosed the alkyl carbons to consist of three methyl carbons, five methylene carbons, and a methine.

^{1}H NMR and COSY experiments provided two partial structures, an aliphatic chain and a para-substituted phenol. Two doublet signals in the ^{1}H NMR spectrum at δ 6.99 and δ 6.74 integrating for two protons each and having a coupling constant of 9 Hz pointed to a para-substituted phenol. COSY and HMBC experiments allowed unambiguous assignment of all ^{1}H and ^{13}C signals in the aliphatic chain. An HMBC experiment (Fig. 33) showed connectivity of the aliphatic chain to the phenol. The HREI mass spectrum (Fig. 34) showed a M$^+$ peak at m/z 304 and a peak at m/z 244 corresponding to loss of acetic acid. This was followed by loss of 41 amu, corresponding to C_3H_5. A signal at m/z 134 corresponds to a loss of the side chain starting at C-3' (Fig. 35). A base peak at m/z 107 (C_7H_7O) corresponds to a p - methylphenol ion. These mass spectral data confirmed the suspected structure.

The stereochemistry of the compound is unknown. The compound has three chiral carbons, but it displayed no optical rotation at the sodium D line, thus suggesting the unlikely possibility that the compound exists as a racemate, unless it shows rotation at a shorter wavelength. The absolute configuration of

the chiral center at C-6' can be determined by hydrolysis of the acetate, esterification of the resulting secondary alcohol with (R)-2-methoxy-2-phenyl-2-(triflouromethyl)acetyl chloride ((R)-MTPA chloride) and (S)-MPTA chloride and comparison of the ^{1}H NMR spectra of the resulting compounds (Mosher's method).[114] If the compound is a racemate, the resulting diastereomers should be separable.
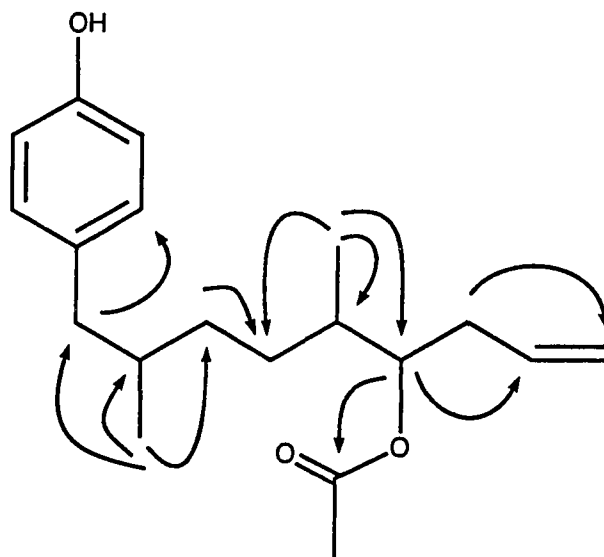
Figure 33. Important HMBC correlations for 4-(6'-Acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (33).
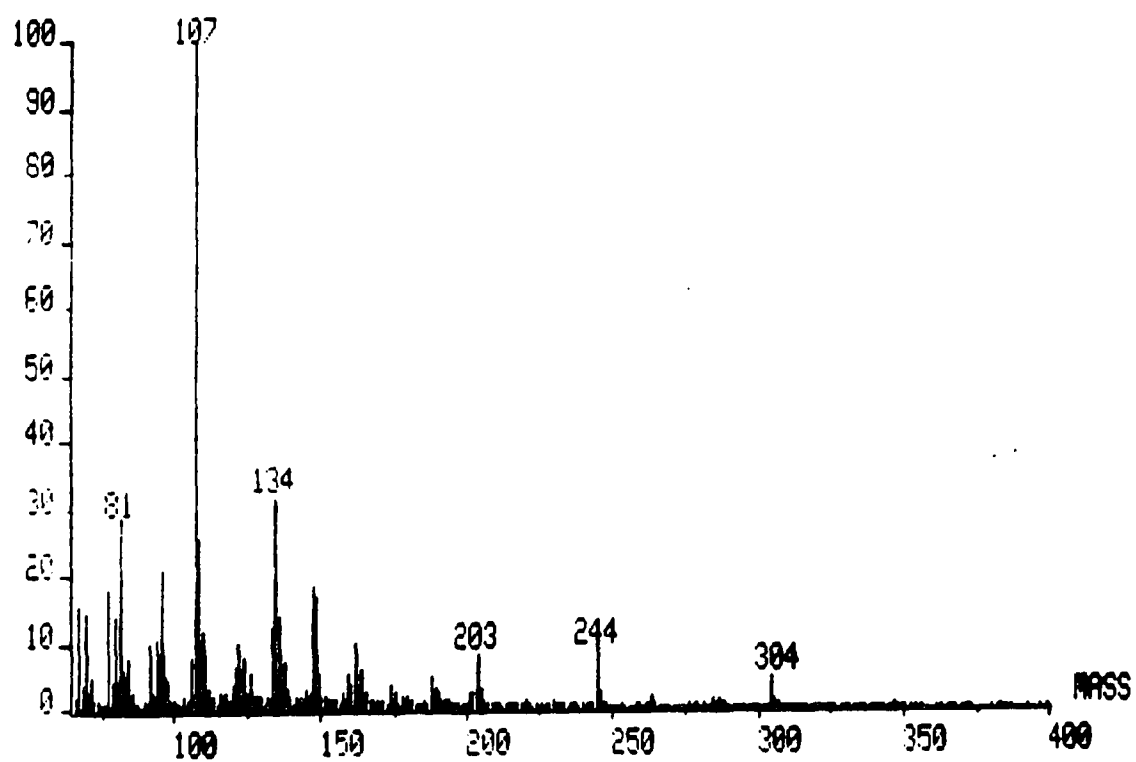
Figure 34. HREI mass spectrum of 4-(6'-acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (**33**).
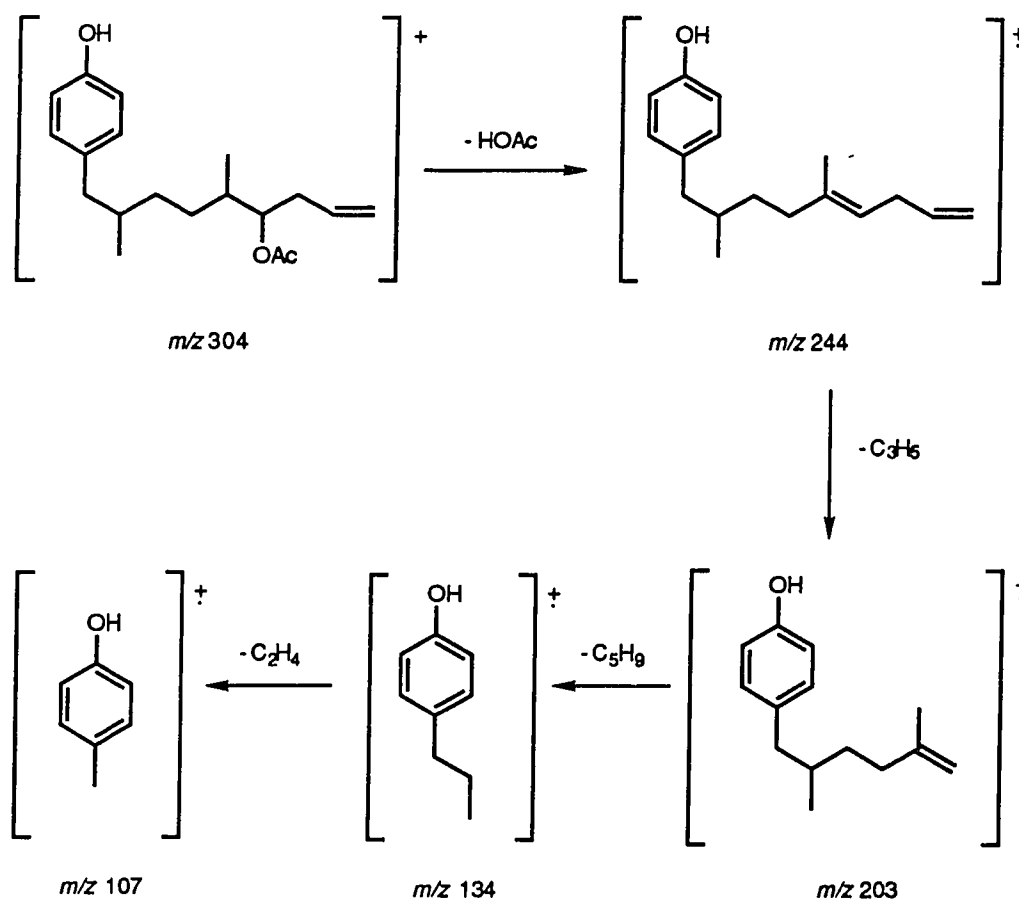
Figure 35. Structures corresponding to the mass fragmentation pattern of 4-(6'-acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (**33**).

Hydrolysis of **33** was accomplished with K_2CO_3 in MeOH (Fig. 36). A ^1H NMR spectrum of the product showed a 1.3 ppm upfield shift of H-6' and the disappearance of the acetate methyl at δ 2.03. The resulting secondary alcohol was derivatized with the (*R*)-MTPA chloride in $CDCl_3$ with Et_3N and a catalytic amount of DMAP. Flash chromatography on silica of the resulting product yielded the (*S*)-MTPA diester of **32** as evidenced by the appearance of two methyoxy groups (at δ 3.49 and at δ 3.69) and a 1.6 ppm downfield shift of H-6'.
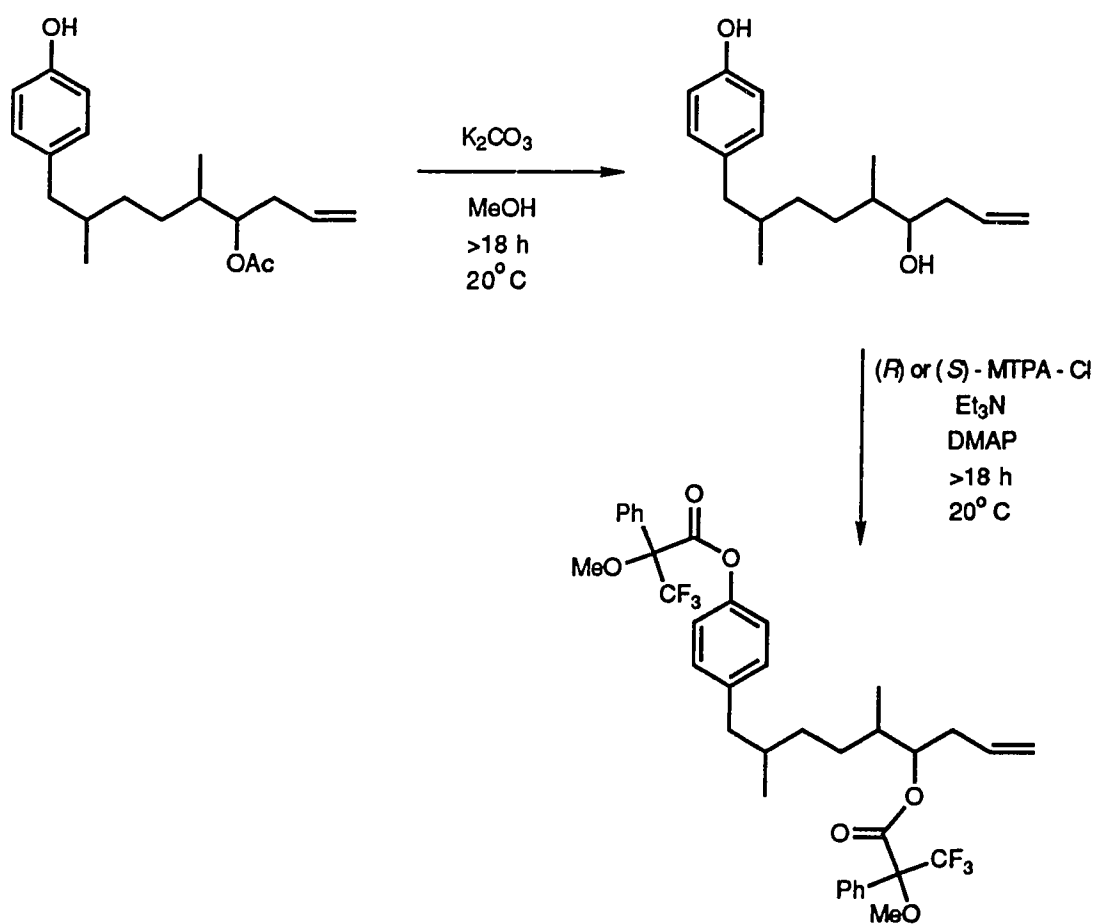
Figure 36. The synthesis of the Mosher's diester from 4-(6'-acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (**33**).

The (R)-MTPA diester was made in a like fashion. Both MTPA diesters of **32** were determined to be enantiomerically pure by ^{1}H NMR and TLC. The ^{1}H chemical shift values of the (R)-MTPA diester (Fig. 37) were subtracted from the corresponding values of the (S)-MTPA diester (Fig. 38) and the differences are given in ppm (Fig. 39). The differences in the ^{1}H NMR chemical shifts of the MTPA derivatives used to determine the absolute stereochemistry of C-6' were inconclusive. While the compound appears to be enantiomerically pure, the flexible side chain containing the secondary alcohol may be preventing the diester from assuming a conformer that allows anisotropic effects to be expressed in a meaningful way.

The biogenesis of 4-(6'-acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (**33**) is not readily obvious. Many of the previously isolated phenols containing aliphatic side chains such as curcuphenol (**34**) appear to be of isoprenoid origin. A deviation from this is navenone C (**35**), a phenolic alarm pheromone of the marine opisthobranch *Navanax inermis*.[115]
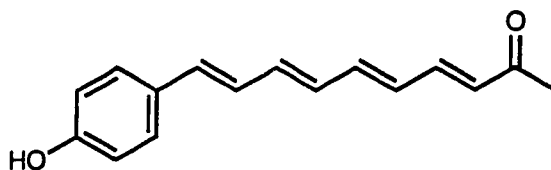
3 5
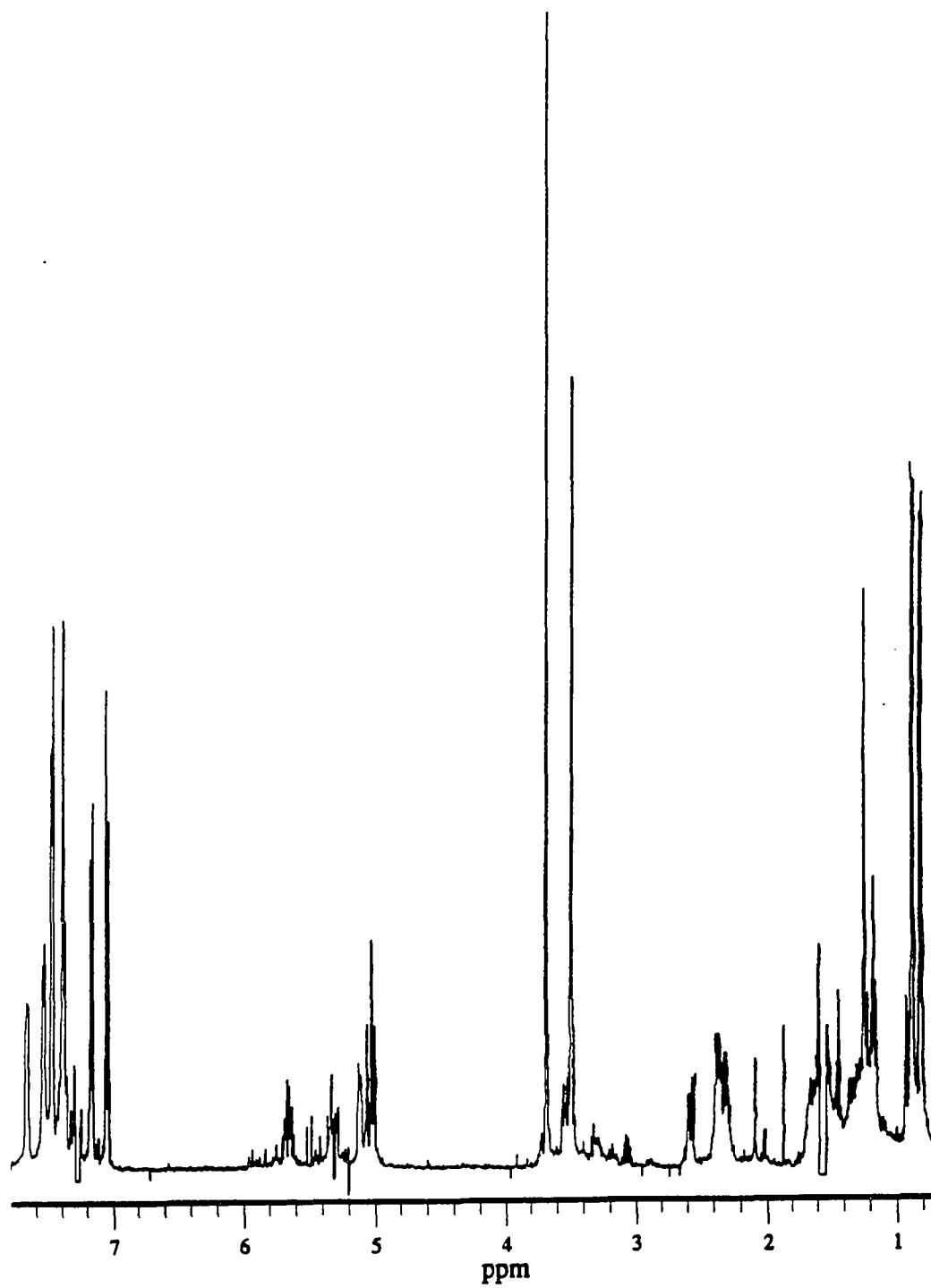
Figure 37. ^{1}H NMR spectrum of the (R)-MTPA diester of 4-(2',5'-dimethyl-non-8'-en-6'-ol)phenol in CDCl$_3$ (500 MHz).
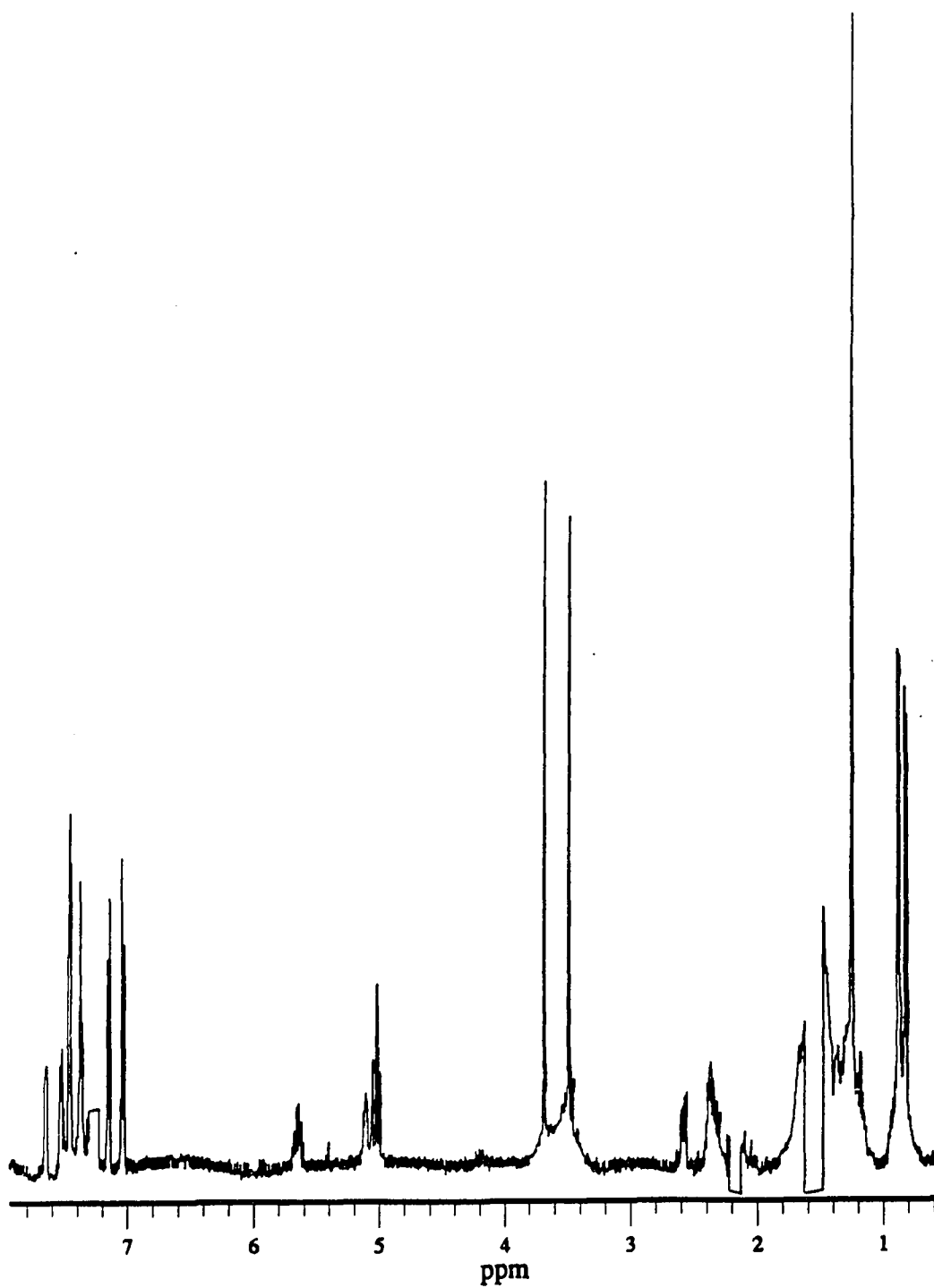
Figure 38. ^{1}H NMR spectrum of the (S)-MTPA diester of 4-(2',5'-dimethyl-non-8'-en-6'-ol)phenol in CDCl$_3$ (500 MHz).

OMTPA

-0.01

0.00

0.00

-0.04,0.00

0.00

0.00

0.00

0.00

0.00

-0.05

0.00

0.00

0.00

0.00

-0.01

0.00

OMTPA

0.00
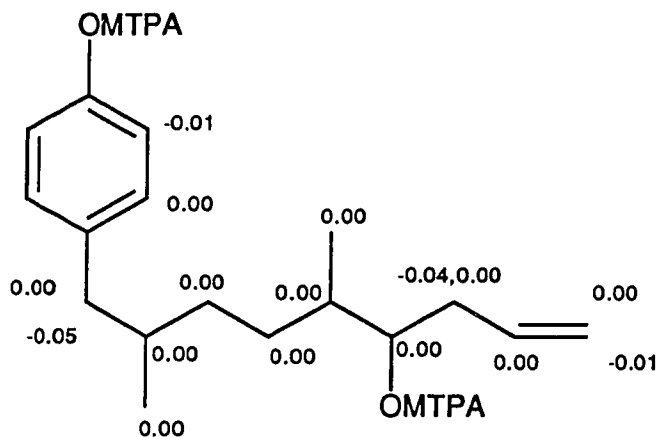
Figure 39. Results of Mosher's method (differences expressed in ppm).

Radiolabelling studies of navanone C (35) with ^{14}C-labelled sodium acetate illustrated that it was produced, at least in part, from acetate. While compound 32 does not appear to be of isoprenoid origin, a reasonable biogenesis can be hypothesized by a mixed biosynthetic pathway.[116] Figure 40 shows a possible biogenesis. The phenol can be constructed via the shikimic acid pathway and the remaining carbons of the aliphatic chain may be derived from acetate. The C-10' methyl may come from the acetate methyl as in the biosynthesis of the cyclophanes[116] and oncorhyncolide[117] or from reduction of the carboxylic acid, while the C-11' methyl may be of methylmethionine origin or by substitution of a propionate for an acetate. The terminal olefinic carbon may originate from the methyl group of an acetate that has lost its carbonyl carbon by oxidation followed by decarboxylation.
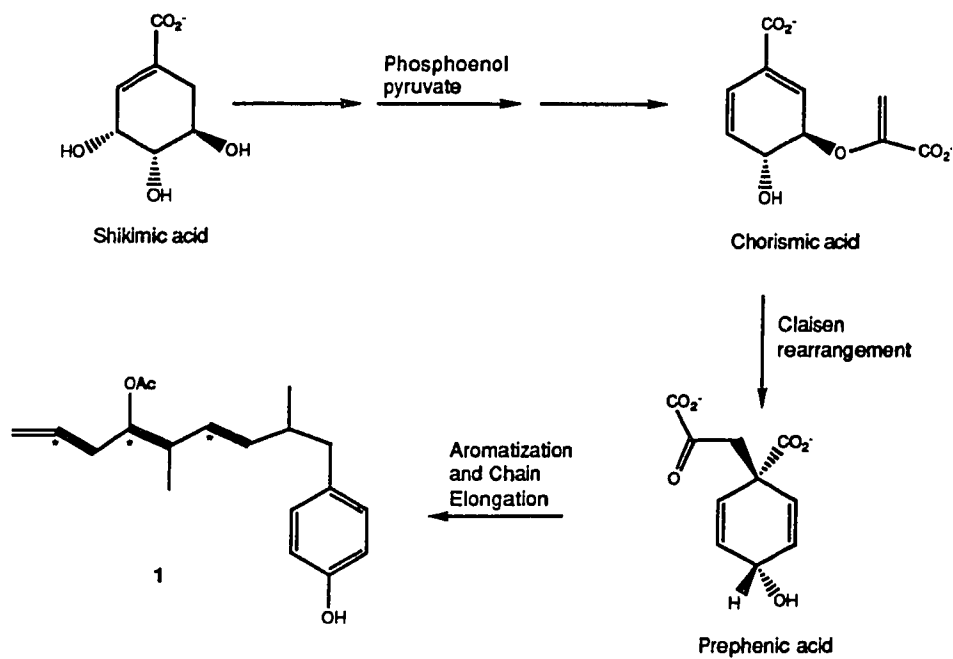
Figure 40. A biogenesis of 4-(6'-acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (**33**). (Two carbon units originating from acetate are in bold and the carbonyl is starred.)

F. Summary

The investigation of the secondary metabolites associated with three sponges, A *Strongylophora* sp., a *Rhabdastrella* sp. and an unidentified species from Indonesia, has led to the characterization of six compounds five of which are new. These compounds consist of three steroids, two triterpenes and a phenol. A carbon isotope study was undertaken to determine if the compound ulapualide was sequestered by the nudibranch *Hexabranchus sanguineus* and passed to its eggmass. Twenty halogenated organics were detected in the acorn worm *Balanoglossus aurantiacus*. This was the first report of chlorinated aromatics in a natural source from the marine environment.

1. Project IRMS: A Carbon Isotope Study of Ulapualide B

This study was the first attempt to determine if secondary metabolites are being sequestered by comparing the natural carbon isotopic composition of a compound between different trophic levels. The technique of measuring carbon isotopic compositions to a degree of accuracy, where differences in natural abundances can be seen, is relatively new. Although the results of the experiment were inconclusive, the technique shows promise as a sensitive probe of suspected, but for the most part unexplored, natural phenomena including symbiosis, sequestering and biosynthesis. This technique has the potential to become an important tool in marine natural product chemistry.

2. Project BIP 6: Three Pregnane-10,2-Carbolactones from a Sponge, *Strongylophora* sp.

The chemical investigation of the Hawaiian sponge, *Strongylophora* sp. has led to the identification of three new marine pregnane steroids. These compounds are the first steroids, marine or terrestrial, to possess a five membered fused lactone that incorporates an oxidized C-19. These compounds are of interest because of the hormonal activity common to pregnane steroids and as possible biosynthetic precursors of 19-norsterols.

3. Project AW: Organohalogen Constituents of the Acorn Worm *Balanoglossus aurantiacus*

This collaborative project with Dr. Fred Dobbs of the Oceanography Department involving the acorn worm *Balanoglossus aurantiacus* is significant for several reasons. Twenty halogenated organics were detected in extracts from the organism that had previously displayed both inhibition and promotion of growth of bacterial isolates in the habitat of the acorn worms. Promotion of growth implies that the bacteria are utilizing one or more of the compounds as a carbon or energy source. If organohalogens are among the compounds so utilized, bacteria associated with enteropneusts are promising candidates for the bioremediation of anthropogenic organohalogens. Also significant is the identification of chlorophenols in the extracts. This is the first report of chlorinated aromatics from the marine environment. Also detected for the first time from enteropneusts were dibromocatechol, bromochlorophenols,

bromochlorohydroquinone and acetylated hydroquinones. An analysis of the amount of ethyl acetate-soluble material being discharged by the animal showed the organism egests between 0.5 - 1.3 metric tons per kilometer of coastline per year.

4. Project 92-IND-169: Two Isomalabaricane Triterpenes from the Sponge, *Rhabdastrella* sp.

The chemical investigation of the Indonesian sponge, *Rhabdastrella* sp., resulted in the isolation of the known compound stelliferin A and the previously unreported stelliferinoside (31), a glycosylated isomalabaricane triterpene. Isomalabaricane triterpenes have shown both cytotoxic and antiviral activity. Stelliferinoside displayed cytotoxic and antiviral activity *in vitro* similar to the previosly described isomalabaricane compounds. The addition of a sugar to the isomalabaricane skeleton without significantly affecting bioactivity provides a clue to the structural features essential to bioactivity.

5. Project 92-IND-293

This project has led to the isolation of a phenolic compound of apparently non-terpenoid biosynthetic origin. This compound is reminiscent of curcuphenol, the first marine natural product to show H, K-ATPase inhibition. Also of interest is the absolute stereochemistry of the compound. although enantiomerically pure and possessing three stereocenters, the compound shows no optical rotation or CD maxima at the concentrations tested.

CHAPTER 3. EXPERIMENTAL PART

A. General Considerations

Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Infrared spectra were recorded on a Nicolet MX-5 FTIR spectrometer. Gas chromatography was accomplished using a Hewlett-Packard Model 5890 instrument. Mass spectra were measured on a VG-70SE magnetic sector mass spectrometer. NMR spectra were measured on a General Electric QE-300 or a GN OMEGA 500 instrument. ^{1}H NMR chemical shifts are reported in ppm with the chemical shift of the residual protons of the solvent used as an internal standard. ^{13}C NMR chemical shifts are reported in δ by using the natural abundance ^{13}C of the solvent as an internal standard. Ultraviolet spectra were recorded on a Hewlett-Packard Model 8452A diode array spectrophotometer. All solvents were distilled from glass before use.

B. Project IRMS: A Carbon Isotope Study of Ulapualide B

1. General procedure for the isolation of ulapualide B

Ulapualide B was isolated by a modification of the method used by Roesener and Scheuer.[4] All nudibranchs and eggmasses were collected at Pupukea on the north shore of Oahu. The nudibranchs were dissected and the gut and its contents removed. The mantle of the nudibranch, weighing between 5 and 13 g, was ground with quartz sand in a mortar and pestle in MeOH. Methanol was

decanted and the procedure repeated until the solvent was clear and colorless. Methanol was concentrated on a rotary evaporator; distilled water was added; and the sample was partitioned with hexane, then CCl_4. The CCl_4 extract was concentrated and chromatographed on either a semi-preparative or an analytical reverse phase C-18 column (Phenomenex), depending on the sample size. Isotopic fractionation can occur during isolation of a compound using HPLC.[118] To avoid isotopic separation during sample isolation, base line separation of all compounds was achieved by maintaining a mobile phase of $MeOH/H_2O$ (4:1) at a flow rate of 0.5 mL/min for the analytical column, and 2.0 mL/min for the semi-preparative column. The retention time on both columns for ulapualide A was ~22 min and ulapualide B ~28 min. Pure ulapualide B was transferred to quartz tubes for pyrolysis.

Eggmasses (60 g) of *Hexabranchus sanguineus* were steeped in MeOH for 24 h. Methanol was filtered and concentrated; distilled water was added and the sample partitioned with hexane, then CCl_4. The CCl_4 extract was concentrated and chromatographed as described above.

Thirty-nine specimens of *Favorinus japonicus*, ranging in length from 0.5 to 1.5 mm, were collected from the eggmasses of *Hexabranchus sanguineus* at Pupukea. *Favorinus japonicus* lays its eggmasses on the eggmasses of *Hexabranchus sanguineus* and these were collected when present. The 39 specimens of *Favorinus japonicus* were steeped in MeOH for 48 h. Methanol was decanted, concentrated and partitioned with CH_2Cl_2 to collect the complete lipid fraction. The CH_2Cl_2 extract was chromatographed using the

conditions described above. The ulapualides were identified by HPLC retention times.

2. Isotopic Analysis

All samples submitted for carbon isotopic analysis were placed in 9 mm o.d. quartz glass tubes and freeze-dried overnight. Excess cupric oxide was added and the quartz tubes evacuated and sealed. The sealed tubes were placed in an oven at 850^0 C for at least 8 h. The gases generated by pyrolysis were distilled cryogenically (Fig. 41). The gases (A) were released into an
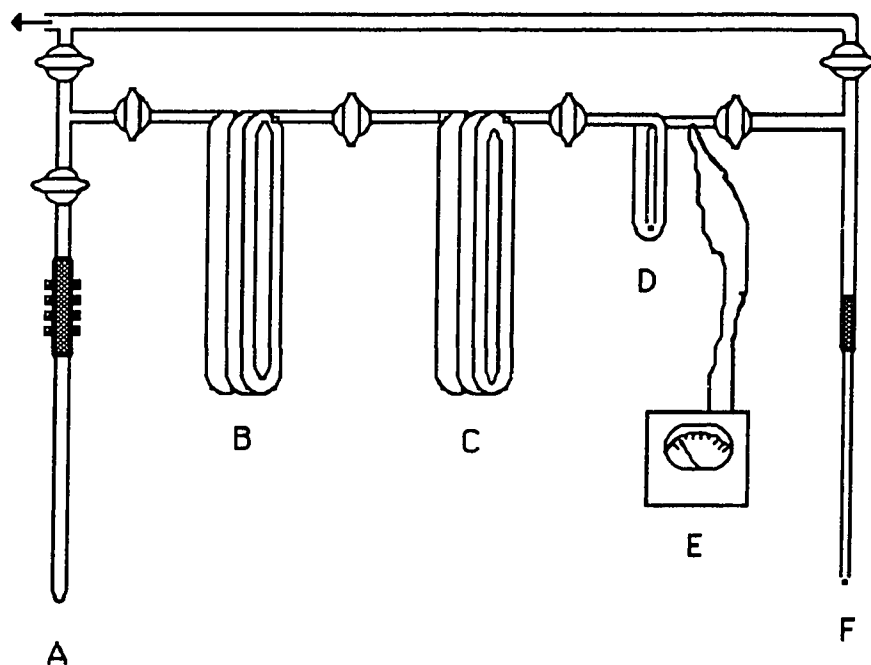
Figure 41. A cryogenic distillation apparatus.

evacuated glass line; water was trapped in coils (B) immersed in a liquid nitrogen/pentane slush; CO_2 was frozen in coils (C) immersed in liquid nitrogen; and nitrogen cleared from the system by evacuation. The amount of CO_2 present was determined manometricaly (D,E). Purified CO_2 was collected by condensing the gas in a 6 mm o.d. glass tube (F) immersed in liquid nitrogen and the tube was flame-sealed. The CO_2 samples were sent to the Biogeochemical Laboratories at Indiana University where they were analyzed isotopically on either a Delta E or Nuclide 6-60 mass spectrometer.

3. Methods

Isotopic compositions are referred to as δ values and given units of ‰ (per mil) because they are differential measurements and differences in naturally occurring organic compounds are expressed at the third significant figure and beyond.[119] The δ value is defined as:

$$\delta(‰) = [(R_x - R_{std})/ R_{std}] \times 10^3 \tag{1}$$

where R_x is the isotopic ratio or the amount of "heavy" isotope vs. the amount of "light" isotope and R_{std} is the isotopic ratio of a standard. The standard that is used for carbon is PeeDee belemnite, a limestone. The source of this standard was exhausted long ago, but many secondary standards exist and carbon isotopic values are still reported as "vs PDB."[119]

Mass-balance equations are used frequently in isotopic analysis. They allow calculation of isotopic abundances derived from isotopically different sources. The mass balance equation takes the form:

$$n_t F_t = n_1 F_1 + n_2 F_2 + ...$$ (2)

where the n term represents molar quantities and the F term represents fractional isotopic abundances and the subscript t refers to the total sample. The fractional abundances can be replaced with δ values for a good approximation. For carbon, the error associated with this approximation is typically less than 0.02 ‰ for most calculations.[119]

If a sample is contaminated during its preparation by contributions from an analytical blank, the isotopic composition determined is that of the sample plus the blank.[119] Analytical blanks can result from an undetected co-eluting compound during HPLC, contamination by solvents, or improper handling. The true isotopic composition of the sample can be calculated using the mass balance equation:

$$n_t \delta_t = n_s \delta_s + n_b \delta_b$$ (3)

where t represents the sample prepared for analysis and s and b represent the sample and the blank. Substituting $n_s = n_t - n_b$ and rearranging yields:

$$\delta_t = \delta_s - n_b(\delta_s - \delta_b)/n_t$$ (4)

an equation of the form y = a + bx. If the size and isotopic composition of the analytical blank are constant, a plot of δ_t vs. $1/n_t$ will yield a straight line with a slope of $n_b(\delta_s - \delta_b)$ and the y-intercept will yield the blank-corrected carbon isotopic composition of the sample.

4. Isotopic Values

See Tables 1 and 2 for the isotopic composition of ulapualide B isolated from *Hexabranchus sanguineus* and its eggmasses.

C. Project BIP 6: Three Pregnane-10,2-Carbolactones from a Sponge, *Strongylophora* sp.

On November 19, 1989, a sponge (1 kg wet) of, identified as a *Strongylophora* sp. (Haplosclerida), was collected by SCUBA at Puako, Hawai'i. The sponge was extracted with EtOH (3 X 200 mL) for >24 h (Fig. 9). The EtOH extract of the sponge was concentrated and then diluted with water and partitioned with CH_2Cl_2 (3 X 100 mL). Upon concentration of the CH_2Cl_2 extract, two layers formed. The top layer was chromatographed using HPLC on a silica stationary phase (Rainin Microsorb Si, 2.5% iPrOH in CH_2Cl_2) yielding a white powder (16, 4.3 mg, 4.3 x 10^{-4}% of wet sponge weight) (Fig. 9). Pregnane 16 co-crystallized from EtOH with a disordered water molecule in the asymmetric unit. Figure 14 depicts an ORTEP drawing of the crystal structure.

On April 9, 1991, the sponge (2 kg wet) was recollected at the same location. The sponge was extracted with EtOH (3 X 200 mL) for >24 h (Fig 10). The EtOH extract of the sponge was concentrated and then diluted with water and partitioned with CH_2Cl_2 (3 X 100 mL). Upon concentration of the CH_2Cl_2 extract, two layers formed. The top layer was chromatographed using HPLC on a silica stationary phase (Rainin Microsorb Si, 2.5% iPrOH in CH_2Cl_2). A new pregnane (17) co-eluted with 16 during silica HPLC. Pregnane 17 (12.1 mg, 1.21 x 10^{-3}% of wet sponge weight) was separated from 16 (8.6 mg, 4.3 x 10^{-4}% of wet sponge weight) using reverse phase HPLC (Phenomenex Ultracarb 5 ODS 30, MeOH/H_2O (50:3)).

Pregnane 18 was isolated from the combined CH_2Cl_2 bottom layers with 12.0 mg of 16 (24.9 mg, 8.3 x 10^{-4}% of total wet sponge weight) and 10.2 mg of 17 (22.1 mg, 7.4 x 10^{-4}% of total wet sponge weight) using C-18 reverse phase HPLC. Reverse phase HPLC (Phenomenex Ultracarb 5 ODS 30, MeCN/H_2O (10:1)) of the combined CH_2Cl_2 precipitates yielded an early fraction that contained 18 and two later fractions that contained pure 16 and 17. Pregnane 18 (1.0 mg, 3.3 x 10^{-5}% of wet sponge weight) was isolated by twice repeated reverse phase HPLC (Phenomenex Ultracarb 5 ODS 30, MeCN/H_2O (1:1)) (Fig. 10).

3,4-Dihydroxypregna-5,17-diene-10,2-carbolactone (16): $[\alpha]^{20}_D$ -82° (c 1.2, CHCl$_3$); ^{13}C NMR (125 MHz, CDCl$_3$, Fig. 13): δ 176.0 (C-19), 151.0 (C-17), 136.0 (C-5), 132.0 (C-6), 110.6 (C-20), 79.4 (C-2), 71.2 (C-4), 69.8 (C-3), 54.3 (C-14), 46.7 (C-10), 43.7 (C-13), 42.6 (C-9), 37.6 (C-1), 35.6 (C-12), 32.5 (C-8),

31.2 (C-7), 26.3 (C-16), 24.2 (C-15), 21.1 (C-11), 18.9 (C-18), 13.8 (C-21); ^{1}H NMR (500 MHz, CDCl$_3$, Fig. 12): δ 6.07 (br dd, J=6.2, 1.7 Hz, H-6), 5.06 (tq, J=6.6, 2.5 Hz, H-20), 4.73 (dt, J=6.7, 1.3 Hz, H-2), 4.48 (br d, J=6.0 Hz, H-4), 3.80 (ddd, J=9.6, 6.0, 1.3 Hz, H-3), 3.42 (br d, J=9.6 Hz, OH), 2.70 (dd, J=12.1, 6.7 Hz, H-1), 2.35 (br dd, J=17.0, 9.4 Hz, H-16), 2.26 (m, H-16), 2.25 (ddd, J=18.4, 6.3, 4.6 Hz, H-7), 2.05 (qd, J=11.0, 4.6 Hz, H-8), 1.96 (br d, J=2.0 Hz, OH), 1.86 (m, H-1), 1.85 (m, H-12), 1.84 (m, H-11), 1.75 (m, H-15), 1.67 (ddd, J=18.4, 11, 1.7 Hz, H-7), 1.62 (m, H-11), 1.56 (d, J=6.6 Hz, H-21), 1.38 (m, H-15), 1.24 (dt, J=11.0, 5 Hz, H-9), 1.20 (m, H-12), 0.95 (ddd, J=12.5, 11.0, 6.3 Hz, H-14), 0.90 (s, H-18); IR neat (KBr): 3300 (m, br), 1775 (s), 1455 (m), 1375 (m), 1300 (m), 1215 (m), 1085 (s); mass spectrum HREI, m/z (relative intensity): 344.1996 (M$^+$,1.2) (calc for C$_{21}$H$_{28}$O$_4$: 344.1988), 326(42), 298(25), 282(65), 257(30), 213(100), 159(42), 128(40), 105(58), 91(68); UV (CHCl$_3$) λ_{max} 246 nm (ε = 1,080).

Crystal data for **15** were measured by Dr. Jorge L. Rios Steiner at Cornell University: C$_{21}$H$_{28}$O$_4$·H$_2$O; Mw=360.4; Hexagonal space group P6$_5$; a = 21.511(5) and c = 7.176(1) Å, α = β= 90.0° and γ= 120.0°; Z = 6; D$_c$ = 1.249 g/cm^3. A colorless crystal with dimensions of 0.10 X 0.14 X 0.52 mm was selected. Data collection was carried out on a Siemens R3m/V diffractometer using Cu Kα radiation (λ = 1.54178) at room temperature. The ω scan mode was used at a variable rate from 2.00 to 29.30°/min. A total of 2,606 independent reflections were measured, of which 2,052 (F > 4.0σ(F)) were used in the refinement.

The structure was solved by direct methods with Siemens SHELXTL PLUS (VMS). The positional and thermal parameters of all non-hydrogen atoms were refined anisotropically, and the hydrogen atoms were introduced in fixed positions with a fixed isotropic thermal parameter. The final refinement included 233 variables. The converged model had unweighted and weighted R agreement factors of 8.12% and 10.76%, respectively, $w^{-1} = \sigma^2(F) + 0.0010F^2$. Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK.

3,4-Dihydroxypregna-5,20-diene-10,2-carbolactone (**17**): $[\alpha]^{20}{}_D$ -96° (c 5.0, CHCl$_3$); ^{13}C NMR (125 MHz, CDCl$_3$, Fig. 16): δ 177.2 (C-19), 139.5 (C-20), 135.5 (C-5), 132.5 (C-6), 114.8 (C-21), 79.8 (C-2), 71.8 (C-4), 69.7 (C-3), 55.2 (C-14,C-17), 46.8 (C-10), 43.7 (C-13), 42.2 (C-9), 37.4 (C-1), 36.9 (C-12), 32.7 (C-8), 31.2 (C-7), 27.3 (C-16), 24.6 (C-15), 20.8 (C-11), 12.7 (C-18); ^{1}H NMR (500 MHz, CDCl$_3$, Fig. 17): δ 6.08 (br d, J=4.82 Hz, H-6), 5.76 (ddd, J=15.1, 10.7, 7.5 Hz, H-20), 5.00 (ddd, J=7.5, 2.1 Hz, H-21), 4.97(ddd, J=15.1, 2.1, 1.0 Hz, H-21), 4.73 (br d, J=4.0 Hz, H-2), 4.49 (br d, J=1.9 Hz, H-4), 3.82 (s, H-3), 2.68 (dd, J=11.2, 4.0 Hz, H-1), 2.20 (dt, J=17.9, 4.2 Hz, H-7), 2.03 (qd, J=11.0, 4.2 Hz, H-8), 1.98 (q, J=8.0 Hz, H-17), 1.80 (m, H-16), 1.76 (m, H-12), 1.75 (m, H-11), 1.70 (m, H-15), 1.65 (m, H-7), 1.60 (m, H-16), 1.56 (m, H-1), 1.55 (m, H-11), 1.38 (dt, J=11.0, 5.9 Hz, H-15), 1.25 (dt, J= 11.0, 4.8 Hz, H-9), 1.07 (dt, J=13.5, 4.0 Hz, H-12), 1.00 (dt, J=11.0, 7.5 Hz, H-14), 0.75 (s, H-18); IR neat (KBr): 3390 (m, br), 1772 (s), 1450 (m), 1375 (m), 1285 (m), 1215 (m), 1083 (s);

mass spectrum HREI, *m/z* (relative intensity): 344.1963 (M+, 5) (calc for $C_{21}H_{28}O_4$: 344.1988), 326(40), 298(20), 282(62), 257(28), 213(100), 159(38), 129(40), 91(55); UV (CHCl$_3$) λ_{max} 244 nm (ε = 634).

3,4-Dihydroxypregna-5,15-dien-20-one-10,2-carbolactone (18): $[\alpha]^{20}_D$ -130° (*c* 1.5, CHCl$_3$); ^{13}C NMR (125 MHz, MeOH, Fig 19): δ 200.0 (C-20), 178.7 (C-19), 156.2 (C-17), 147.0 (C-16), 138.0 (C-5), 131.3 (C-6), 81.3 (C-2), 72.4 (C-4), 70.6 (C-3), 57.2 (C-14), 48.0 (C-10), 47.5 (C-13), 43.7 (C-9), 38.9 (C-1), 35.7 (C-12), 33.2 (C-8), 32.6 (C-7), 32.0 (C-15), 27.1 (C-21), 21.9 (C-11), 16.2 (C-18); ^{1}H NMR (500 MHz, MeOH, Fig. 18): δ 6.90 (dd, *J*=3.4, 1.9 Hz, H-16), 6.00 (dd, *J*=6.2, 1.7, Hz, H-6), 4.65 (dt, *J*=1.2, 6.8 Hz, H-2), 4.36 (d, *J*=5.8 Hz, H-4), 3.82 (dd, *J*=5.8, 1.2 Hz, H-3), 2.67 (dd, *J*=12.1, 6.8 Hz, H-1), 2.48 (m, H-12), 2.41 (m, H-15), 2.25 (s, H-21), 2.16 (m, H-15), 2.14 (m, H-8), 2.12 (m, H-7),1.82 (m, H-11), 1.73 (m, H-7), 1.68 (m, H-11), 1.61 (m, H-1), 1.43 (m, H-12), 1.41 (m, H-14), 1.35 (m, H-9), 1.03 (s, H-18); IR neat (KBr): 3450 (m, br), 1790 (s), 1760 (m), 1655 (m), 1260 (m), 980 (s); mass spectrum HREI, *m/z* (relative intensity): 358.1786 (M+, 1.7) (calc for $C_{21}H_{26}O_5$: 358.1791), 340 (45), 325 (35), 297 (100),175 (30), 159 (50), 129 (35), 91 (71); UV (MeOH) λ_{max} 260(2,280), 204 nm (ε = 10,620).

D. Project AW: Organohalogen Constituents of the Acorn Worm *Balanoglossus aurantiacus*

Body fragments of *Balanoglossus aurantiacus* (140 g wet mass including ingested sand) were collected by Dr. Fred Dobbs in July, 1987 from an intertidal sand flat 500 m west of the Florida State University Marine Laboratory at Turkey Point (29^0 55.00' N, 84^0 30.36' W) and kept at -70^oC until extraction. The sample was extracted overnight in 400 mL of acetone and filtered. The sample was re-extracted with 300 mL of acetone and the extracts were combined. The combined acetone extracts were concentrated to 350 mL; 50 mL of H_2O was added; and the mixture was successively partitioned with hexane (3 X 200 mL), EtOAc (3 X 200 mL) and n-BuOH (3 X 200 mL). These fractions were dried in a stream of N_2 and stored at -20^0 to -70^0 C until further use.

The hexane fraction (243.5 mg) was distilled using a Kugelrohr apparatus under vacuum at 75^0 C, then at 95^0 C. At 75^0 C, 5.1 mg of white crystals formed in the collecting flask and were designated "Hex DB." Further heating of the hexane fraction at 75^0 C yielded 0.6 mg of a colorless oil designated "Hex oil." The temperature was increased to 95^0 C and 8.1 mg of a colorless oil was collected ("95 Oil"). The EtOAc (28.3 mg) and the n-BuOH fractions (20.8 mg) were distilled as above at 95^0 C. The EtOAc fraction yielded 4.0 mg of a colorless oil ("EtOAc"), while the n-BuOH fraction gave 1.6 mg of a colorless oil ("nBuOH").

Comparison samples of chlorinated phenols and 2,4,6-tribromophenol were purchased from Aldrich Chemical Co., Milwaukee, WI. Bromohydroquinone and 4-bromo-2,6-dichlorophenol were purchased from Chem Service Inc., West Chester, PA. Solvents were freshly distilled before use.

Gas chromatography - mass spectrometry was carried out with hexane solutions of each sample. A 25 m microbore OV-1 column was used for gas chromatography. The injector was set at 210° C and the initial temperature of the column was set at 40° C for 5 min. The temperature was increased to 100° C at a rate of 10° C/min. After 5 min the temperature was increased to 200° C at a rate of 20° C/min and held for 10 min. The "95 oil" from the hexane extract formed two layers upon addition of hexane. The bottom layer ("95 Oil ins") did not contain hexane and was injected neat. The top layer was designated "95 Oil sol."

Comparison of samples with known compounds was carried out under identical GC column conditions as before, except for different temperature ramp parameters. The initial temperature of the column was set at 40° C for 6 min. The temperature was increased to 110° C at a rate of 7° C/min. After 5 min the temperature was increased to 200° C at a rate of 20° C/min and held for 10 min.

2,6-Dibromophenol (19): ^{1}H NMR (300 MHz, CDCl$_3$, Fig. 21): 7.44 (d, J=12 Hz) 6.71   (t, J=12 Hz), 5.88 (br s, OH); mass spectrum EI, m/z (relative intensity):

254 (M⁺+ 4, 60), 252 (M⁺+ 2, 100), 250 (M⁺, 60), 225 (10), 223 (20) 221(10), 172 (40), 170 (40), 145 (55), 143 (55), 117 (40), 119 (40)

Dichlorophenol (**20, 21**): mass spectrum EI, *m/z* (relative intensity): 164 (M⁺+ 2, 15), 162 (M⁺, 40), 146 (12),144 (50), 128 (20), 126 (100), 110 (90)

2,4,6-Tribromophenol (**22**): mass spectrum EI, *m/z* (relative intensity): 334 (M⁺+ 6, 25), 332 (M⁺+ 4, 95), 330 (M⁺+ 2, 100), 328 (M⁺, 25), 143 (20), 141(20)

2-Bromo-4,6-dichlorophenol (**23**): mass spectrum EI, *m/z* (relative intensity): 244 (M⁺+ 4, 40), 242 (M⁺+ 2, 100), 240 (M⁺, 60), 133 (20)

Dibromochlorophenol (**24**): mass spectrum EI, *m/z* (relative intensity): 290 (M⁺+ 6), 288 (M⁺+ 4, 85), 286 (M⁺+ 2, 100), 284 (M⁺, 60), 206 (15), 178 (20), 143 (10), 126 (10)

Bromohydroquinone (**25**): mass spectrum EI, *m/z* (relative intensity): 190 (M⁺+ 2, 95), 188 (M⁺, 100), 119 (20), 105 (20)

Dibromohydroquinone (**26**): mass spectrum EI, *m/z* (relative intensity): 270 (M⁺+ 4, 45), 268 (M⁺+ 2, 100), 266 (M⁺, 50), 119 (20), 105 (35)

Bromochlorohydroquinone (**27**): mass spectrum EI, *m/z* (relative intensity): 226 (M⁺+ 4, 30), 224 (M⁺+ 2, 100), 222 (M⁺, 75), 206 (20), 204 (25), 160 (10), 158 (10), 126 (20), 114 (30)

Dibromochatechol: mass spectrum EI, *m/z* (relative intensity): 270 (M⁺+ 4, 50), 268 (M⁺+ 2, 100), 266 (M⁺, 50), 252 (15), 250 (30), 248 (15)

Acetoxybromohydroquinone: mass spectrum EI, *m/z* (relative intensity): 232 (M⁺+ 2, 10), 230 (M⁺, 10), 190 (100), 188 (100), 108 (10)

Acetoxydibromohydroquinone (28): mass spectrum EI, *m/z* (relative intensity): 312 (M⁺+ 4, 5), 310 (M⁺+ 2, 10), 308 (M⁺, 5), 270 (60), 268 (100), 266 (55), 228 (80), 226 (85), 147 (25), 118 (15)

Acetoxybromochlorohydroquinone: mass spectrum EI, *m/z* (relative intensity): 268 (M⁺+ 4, 10), 266 (M⁺+ 2, 40), 264 (M⁺, 40), 226 (80), 224 (100), 222 (95), 160 (20), 158 (25), 142 (25), 114 (60)

Bromoepoxyhydroxycyclohexenone (29): mass spectrum EI, *m/z* (relative intensity): 206 (M⁺+ 2, 65), 204 (M⁺, 65), 177 (40), 175 (35), 105 (75)

Acetoxybromoepoxycyclohexenone (30): mass spectrum EI, *m/z* (relative intensity): 248 (M⁺+ 2, 8), 246 (M⁺, 10), 206 (95), 204 (100), 126 (15)

E. Project 92-IND-169: Two Isomalabaricane Triterpenes from the Sponge, *Rhabdastrella* sp.

The sponge 92-IND-169 was collected off Monado, Indonesia on October 5, 1992, at a depth of 80-130'. A description by Dr. Michelle Kelly-Borges follows.

"The sponge is spherical, firm, with a granular tuberculate surface. The sponge exterior is reddish-brown, and is tan internally, in life. In ethanol preservative, the sponge is beige and exudes a brilliant yellow pigment. The cortex is packed with spherasters of a great size range, and these are extremely common throughout the choanosome, where oxyasters are also common. The choanosome contains abundant oxeas and plagio/orthotriaenes with short rhabds, the clads of which are occasionally malformed, and aligned along the cortex-choanosome boundary. The sponge is an undescribed species of *Rhadbastrella* (Order Choristida, Family Jaspidae). This is a very unusual example that perhaps demonstrates the "intermediate" position of this genus between the stelletid (with triaenes) and jaspid (without triaenes) condition. A voucher specimen has been deposited at the Harbor Branch Oceanographic Museum, Fort Pierce, Florida."

The sponge (0.10 kg wet weight) was kept frozen until it was freeze-dried (14.2 g). The freeze-dried sponge was extracted with CH_2Cl_2, the extract concentrated and flash chromatographed on silica by sequential elution with

CH$_2$Cl$_2$, 2:1 CH$_2$Cl$_2$/EtOAc, EtOAc and MeOH. The EtOAc fraction was subjected to reverse phase HPLC (Phenomenex Ultracarb 5 ODS 30, MeOH/H$_2$O (20:1)) yielding impure stelliferinoside (31). Final purification of 31 (3.3 mg, 0.023% of dry sponge weight) was achieved by reverse phase HPLC (Phenomenex Ultracarb 5 ODS 30, MeCN). Stelliferin A (32, 1.7 mg, 0.012% of dry sponge weight) was isolated from the CH$_2$Cl$_2$/EtOAc (2:1) fraction by reverse phase HPLC (Phenomenex Ultracarb 5 ODS 30, MeOH/H$_2$O (20:1)).

Stelliferinoside (1 mg) was hydrolyzed in ~5 mL of refluxing aqueous acetic acid (AcOH/H$_2$O (2:1)) for 2 h. The solution turned a deep yellow color. The resulting reaction mixture was partitioned three times with 2 mL of CH$_2$Cl$_2$ and the aqueous extract was dried *in vacuo*. The residue of the aqueous extract (0.2 mg) was identified as the free monosacharide by ^{1}H NMR spectroscopy by comparison with a known sample (Fig. 28). The free sugar was placed in 100 μL of distilled H$_2$O and allowed to equilibrate for 24 h before the optical rotation was determined. The optical rotation for the ribose was determined to be -25 ± 2° at a concentration of 0.2 g/100mL.and a temperature of 20° C. The Merck Index gives an optical rotation of -25° at a concentration of 1 g/100 mL and a temperature of 20° C.[110]

Stelliferinoside (31): A light yellow oil; [α]$_D$ -115° (c 2.0, CHCl$_3$); ^{13}C NMR (125 MHz, CDCl$_3$, Fig. 22): δ 207.0 (C-12), 170.0 (CH$_3$CO), 147.2 (C-13), 142.2 (C-14), 138.8 (C-20), 134.5 (C-25), 133.4 (C-15), 130.7 (C-17), 129.6 (C-16), 120.0 (C-24), 98.5 (C-1'), 82.3 (C-22), 78.2 (C-3), 73.4 (C-3'), 71.7 (C-2'), 70.2 (C-4'), 63.5 (C-5'), 50.8 (C-9), 45.0 (C-8), 41.9 (C-5), 38.9 (C-7), 37.7 (C-4), 37.0

(C-11), 35.9 (C-10), 32.8 (C-23), 29.7 (C-1), 27.9 (C-28), 25.8 (C-27), 24.7 (C-2), 24.2 (C-30), 22.5 (C-19), 21.6 ($\underline{C}$H3CO), 21.4 (C-29), 18.4 (C-6), 18.0 (C-26), 16.2 (C-18), 12.0 (C-21); ^{1}H NMR (500 MHz, MeOH, Fig. 23): δ 8.00 (d, J=16 Hz, H-15), 6.84 (dd, J=16, 11 Hz, H-16), 6.21 (dd, J=11, 1 Hz, H-17), 5.07 (tt, J=7, 1 Hz, H-24), 4.71 (t, J=3 Hz, H-3), 4.35 (d, J=5 Hz, H-1'), 4.17 (dt, J=7, 1 Hz, H-22), 4.00 (dd, J=12, 4 Hz, H-5'), 3.68 (dt, J=6, 4 Hz, H-4'), 3.54 (t, J=6 Hz, H-3'), 3.43 (dd, J=5, 6 Hz, H-2'), 3.33 (dd, J=12, 6 Hz, H-5'), 2.40 (dt, J=15, 7 Hz, H-23), 2.31 (m, H-5), 2.26 (m, H-23), 2.23 (d, J=15 Hz, H-11), 2.17 (dd, J=15, 7 Hz, H-11), 2.10 (m, H-7), 2.04 (m, H-2), 2.02 (s, C$\underline{H}$3CO), 2.02 (s, H-18), 1.83 (dd, J= 15, 7 Hz, H-9), 1.78 (m, H-1), 1.77 (d, J=1 Hz, H-21), 1.74 (m, H-2), 1.68 (s, H-27) 1.63 (m, H-6), 1.62 (s, H-26), 1.45 (m, H-6), 1.43 (s, H-30), 1.20 (m, H-1), 1.02 (s, H-19), 0.92 (s, H-29), 0.89 (s, H-28); IR thin film (NaCl): 3600-3200 (m, br), 2925 (s), 1731 (s), 1375 (m), 1244 (s); mass spectrum FAB, m/z (relative intensity): 651 (M$^+$+Na, 20) (calc. for C37H55O8Na: 651), 479(85), 427(35), 357(30), 135 (75); 85 (100); UV (MeOH) 348 nm (ε = 39,100), 336 nm (ε = 39,600), 234 nm (ε = 22,000), 206 nm (ε = 16,800).

Stelliferin A (32): A light yellow oil; [α]D -129.4° (c 1.7, CHCl3) (reported as -126.6° (c 0.54, C6H6));[107c] ^{13}C NMR (125 MHz, CDCl3, Fig. 30): δ 206.3 (C-12), 170.8 (CH3$\underline{C}$O), 145.8 (C-13), 143.0 (C-20), 142.4 (C-14), 134.6 (C-25), 132.1 (C-15), 130.2 (C-16), 126.2 (C-17), 119.5 (C-24), 80.7 (C-3), 76.8 (C-22), 50.2 (C-9), 46.5 (C-5), 44.5 (C-8), 38.1 (C-7), 38.1 (C-4), 36.8 (C-11), 35.4 (C-10), 34.2 (C-23), 32.9 (C-1), 28.9 (C-28), 25.8 (C-27), 25.0 (C-2), 24.6 (C-30), 22.3 (C-19), 21.1 ($\underline{C}$H3CO), 18.2 (C-6), 17.9 (C-26), 16.9 (C-29), 15.9 (C-18), 12.7 (C-21); IR thin film (NaCl): 3600-3200 (m, br), 2934 (s), 1732 (s), 1375 (m),

1245 (s); UV (MeOH) 346 nm (ε = 15,400), 306 nm (ε = 20,700), 234 nm (ε = 12,100), 206 nm (ε = 15,300).

F. Project 92-IND-293

On October 9, 1992, a red spiculated sponge (34.8 g, dry) was collected by SCUBA near Bunaken in Sulawesi, Indonesia. A concentrated MeOH extract of the sponge was diluted with water and partitioned consecutively with CH_2Cl_2 and n-BuOH. The CH_2Cl_2 extract was chromatographed using a silica gravity column with CH_2Cl_2 as the mobile phase. A resulting fraction was subjected to HPLC on a silica stationary phase (Rainin Microsorb Si, CH_2Cl_2/EtOAc (1:1)) yielding a light yellow oil (33, 4.7 mg, 1.3×10^{-2}% of dry sponge weight).

The acetate of 33 was hydrolyzed in weak base. Compound 33 (0.5 mg, 0.0016 mmol) was dissolved in 1 mL of MeOH with excess K_2CO_3 and stirred for >18 h. The resulting reaction mixture was acidified with glacial acetic acid, and partitioned with CH_2Cl_2. The CH_2Cl_2 extract was concentrated and ^{1}H NMR spectroscopy of the resulting residue showed complete conversion of 33 to the secondary alcohol (Fig. 42). A solution of Et_3N (4 µL, 0.03 mmol), DMAP (a small crystal) and (R)-MTPA chloride (1.5 mg, 0.006 mmol) was added to the secondary alcohol in $CDCl_3$ (100 µL) and stirred for >18 h at room temperature. Both (S) and (R)-MTPA chloride where purchased from Aldrich Chemical Co., Milwaukee, WI. The resulting reaction mixture was flash chromatographed on silica with sequential elution of hexane, hexane / CH_2Cl_2 (1:1), CH_2Cl_2 and EtOAc. The hexane / CH_2Cl_2 (1:1) fraction was concentrated to dryness and a
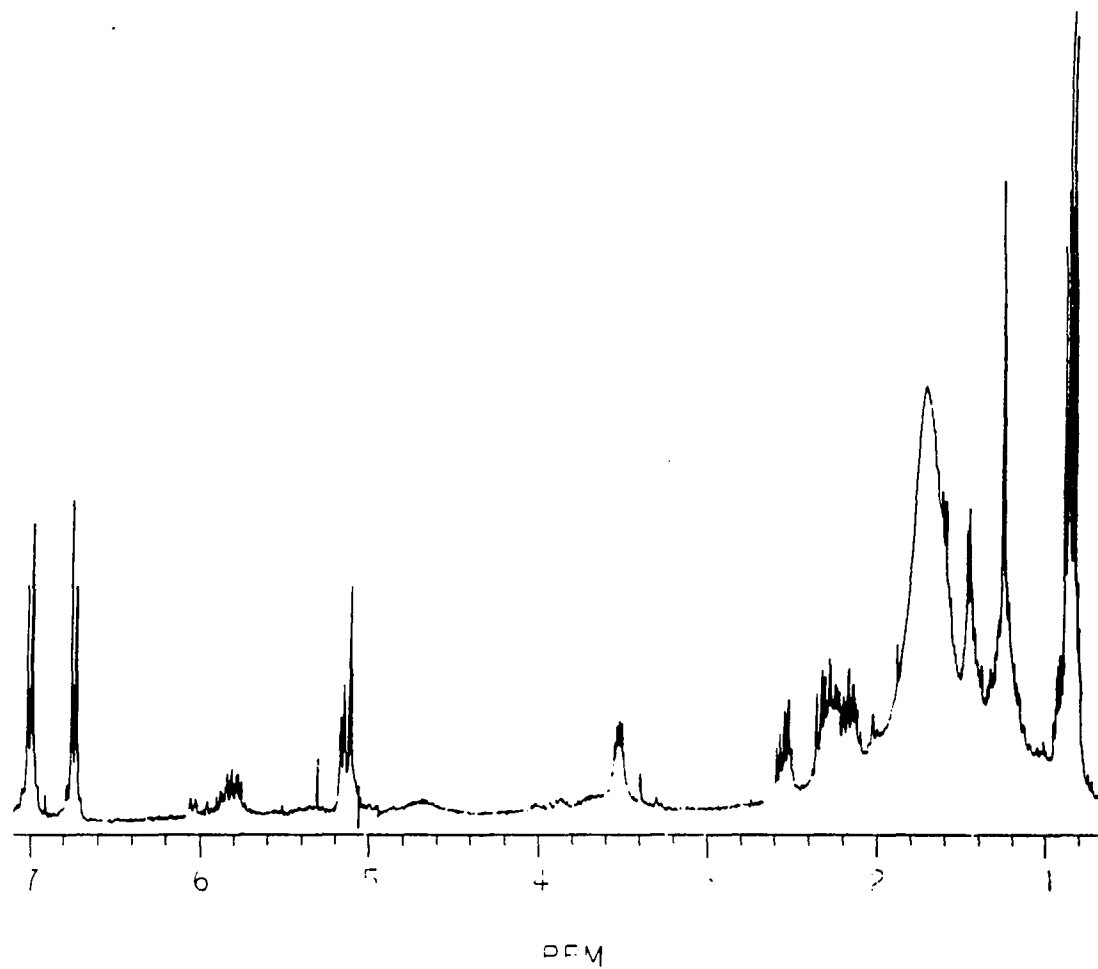
Figure 42. ^{1}H NMR spectrum of 4-(2',5'-dimethyl-non-
8'-en-6'-ol)phenol in CDCl₃ (300 MHz).

^{1}H NMR spectrum showed the compound to be the (S)-MTPA diester of **33** (Fig. 38). Thin layer chromatography of the (S)-MTPA diester of **33** on silica with hexane / CH$_2$Cl$_2$ (1:1) as a mobile phase showed only a single UV active compound. The (R)-MTPA diester of **33** was synthesized from 0.5 mg in the same fashion.

4-(6'-acetoxy-2',5'-dimethyl-non-8'-enyl)phenol (**33**): [α]$_D$ 0° (c 3.5, CHCl$_3$, Fig 32); ^{13}C NMR (125 MHz, CDCl$_3$): δ 170.9 (CH$_3$$\underline{C}$O), 153.5 (C-1), 134.3 (C-8'), 133.6 (C-4), 130.2 (C-3,5), 117.3 (C-9'), 114.9 (C-2,6), 76.3 (C-6'), 42.8 (C-1'), 36.1 (C-7'), 35.9 (C-5'), 35.1 (C-2'), 33.8 (C-3'), 30.2 (C-4'), 21.1 ($\underline{C}$H$_3$CO), 19.2 (C-10'), 14.2 (C-11'); ^{1}H NMR (500 MHz, CDCl$_3$, Fig. 31): δ 6.99 (d, J= 9 Hz, H-3,5), 6.74 (d, J= 9 Hz, H-2,6), 5.72 (ddt, J= 17, 10, 7 Hz, H-8'), 5.06 (ddd, J= 17, 3, 1 Hz, H-9'), 5.03 (ddd, J= 10, 3, 1 Hz, H-9'), 4.86 (dt, J= 6, 5 Hz, H-6'), 2.52 (dd, J= 13, 6, H-1'), 2.30 (m, H-1'), 2.28 (m, H-7'), 2.26 (m, H-7'), 2.02 (s, C$\underline{H}$$_3$CO), 1.65 (m, H-2'), 1.62 (m, H-5'), 1.36 (m, H-4'), 1.35 (m, H-3'), 1.30 (m, H-4'), 1.18 (m, H-3'), 0.86 (d, J= 7 Hz, H-11'), 0.82 (d, J= 7 Hz, H-10'); IR thin film (NaCl): 3417 (m, br), 1734 (OAc, s), 1514.3 (s), 1241.2 (s), 1023 (w); mass spectrum HREI, m/z (relative intensity): 304.1996 (M$^+$, 5) (calc. for C$_{19}$H$_{28}$O$_3$: 304.2038), 244(10), 203(8), 147(17), 134(28), 107 (100); UV (MeOH) λ$_{max}$ 278 nm (ε = 3225).

(S)-MTPA diester of **33**: ^{1}H NMR (500 MHz, CDCl$_3$, Fig. 38): δ 7.4-7.7 (phenyl of MTPA), 7.14 (d, J= 9 Hz, H-3,5), 7.03 (d, J= 9 Hz, H-2,6), 5.65 (ddt, J= 17, 10, 7 Hz, H-8'), 5.11 (dt, J= 6, 5 Hz, H-6'), 5.03 (ddd, J= 17, 3, 1 Hz, H-9'), 5.00 (ddd, J= 10, 3, 1 Hz, H-9'), 3.69 (methoxy of MTPA), 3.49 (methoxy of MTPA), 2.58

(dd, J= 13, 6, H-1'), 2.34 (m, H-1'), 2.28 (m, H-7'), 2.26 (m, H-7'), 1.65 (m, H-2'), 1.62 (m, H-5'), 1.36 (m, H-4'), 1.36 (m, H-3'), 1.30 (m, H-4'), 1.18 (m, H-3'), 0.88 (d, J= 7 Hz, H-11'), 0.82 (d, J= 7 Hz, H-10')

(R)-MTPA diester of **33**: ^{1}H NMR (500 MHz, CDCl$_3$, Fig. 38): δ 7.4-7.7 (phenyl of MTPA), 7.15 (d, J= 9 Hz, H-3,5), 7.03 (d, J= 9 Hz, H-2,6), 5.65 (ddt, J= 17, 10, 7 Hz, H-8'), 5.11 (dt, J= 6, 5 Hz, H-6'), 5.03 (ddd, J= 17, 3, 1 Hz, H-9'), 5.01 (ddd, J= 10, 3, 1 Hz, H-9'), 3.69 (methoxy of MTPA), 3.49 (methoxy of MTPA), 2.58 (dd, J= 13, 6, H-1'), 2.39 (m, H-1'), 2.32 (m, H-7'), 2.26 (m, H-7'), 1.65 (m, H-2'), 1.62 (m, H-5'), 1.36 (m, H-4'), 1.36 (m, H-3'), 1.30 (m, H-4'), 1.18 (m, H-3'), 0.88 (d, J= 7 Hz, H-11'), 0.82 (d, J= 7 Hz, H-10')

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