



Biologically significant metabolites of several marine invertebrates
by Ken F Kinzer

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Biochemistry
Montana State University
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Abstract:

In this work, secondary metabolites were isolated and characterized from three marine invertebrates. These were a coelenterate, *Ptilosarcus gurneyi*, a limpet, *Siphonaria alternata*, and a tunicate, *Eudistoma olivaceum*. Twelve of these compounds were found to be novel.

From *P. gurneyi*, homarine, 1, and trigonelline, 2, were isolated via reverse phase, propylamine HPLC and identified. A literature search and our bioassay work have shown that these two compounds most likely act as benign storage forms for picolinic acid, nicotinic acid, and/or methyl groups.

From stressed *S. alternata*, twelve compounds were isolated using reverse phase ODS and silica HPLC systems. Spectral data had shown these compounds to be polypropionate siphonarins A through L, 3, 4, 9-16, 18, and 19. Of these, siphonarins A, B, and F had been previously identified. Spectral and physical data indicate that siphonarins C through L are open chain and cyclic ketal stress metabolites originating only in stressed limpets from their precursors siphonarins A and B.

From *E. olivaceum*, seven β -carbolines were isolated using a propyl amine HPLC system. Novel eudistomins R, S, and T, 26-28, were shown to contain an α -toluidone moiety in place of the pyrrolidine ring found in previously characterized eudistomins G, H, I, and P, 20-23-

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Master of Science

in

Biochemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

November 1986

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of a thesis submitted by

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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DEDICATED TO:

Connie,

for being her,

for being there.

ACKNOWLEDGMENTS

As in all works of this magnitude, the author is merely one of a large number of individuals responsible for its completion. But to single out just those who have gone beyond the call of duty to help me in my work: I have to give special thanks to Joe Sears for the many hours he spent obtaining HRMS data for me. Extra thanks must be given to Rob West, Rob Hendrickson, Dave Barnekow, Mike Raub, Andrea Stierle, and Tim Schram for their work on equipment and/or bioassay work that allowed me to fill in and collect data. And finally a big "right on" to the "leader," John Cardellina, for never failing in an abundance of optimism and his own brand of concern.

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ABSTRACT

In this work, secondary metabolites were isolated and characterized from three marine invertebrates. These were a coelenterate, Ptilosarcus gurneyi, a limpet, Siphonaria alternata, and a tunicate, Eudistoma olivaceum. Twelve of these compounds were found to be novel.

From P. gurneyi, homarine, 1, and trigonelline, 2, were isolated via reverse phase, propylamine HPLC and identified. A literature search and our bioassay work have shown that these two compounds most likely act as benign storage forms for picolinic acid, nicotinic acid, and/or methyl groups.

From stressed S. alternata, twelve compounds were isolated using reverse phase ODS and silica HPLC systems. Spectral data had shown these compounds to be polypropionate siphonarins A through L, 3, 4, 9-16, 18, and 19. Of these, siphonarins A, B, and F had been previously identified. Spectral and physical data indicate that siphonarins C through L are open chain and cyclic ketal stress metabolites originating only in stressed limpets from their precursors siphonarins A and B.

From E. olivaceum, seven β -carbolines were isolated using a propylamine HPLC system. Novel eudistomins R, S, and T, 26-28, were shown to contain an α -toluidone moiety in place of the pyrrolidine ring found in previously characterized eudistomins G, H, I, and P, 20-23.

OVERVIEW AND OBJECTIVES

The natural products chemist is primarily concerned with isolation and structure elucidation of the secondary metabolites of an organism. At this point, to clarify the aim of this thesis, an important distinction must be made. Primary metabolites have a broad distribution throughout the ecosphere, are generally essential to most life forms, and for the most part, their chemistry is at least nominally understood. On the other hand, secondary metabolites have a restricted distribution, are often characteristic of a specific genus or species, are formed through specialized metabolic pathways and are therefore not important except to the specific group of organisms in which they are found (1). But most importantly, the structures and more often the functions of these products are largely unknown. It is through chemical and spectroscopic analysis of separated and purified compounds that structures may be assigned. This information is then coupled with biochemical as well as pharmacological screening to allow the ecological implications of the specific secondary metabolites to be understood or surmised. A further and often more important emphasis dealing with the work on these compounds is their use as biologically active agents by man (2). The beneficial uses of secondary metabolites include application as insecticides

and herbicides, as well as drugs for antiviral, cancer, and hypertensive therapy, to name a few. This general emphasis on ecology and biological activity summarizes the mode and focus of the research which is detailed in this thesis.

In this study, secondary metabolites of three different species of marine invertebrates were isolated and examined. The first of these marine fauna was a coelenterate of the class Anthozoa, Ptilosarcus gurneyi, a sea pen. The second was a gastropod mollusk of subclass Pulmonata, Siphonaria alternata, or the false limpet. The final organism was Eudistoma olivaceum, a chordate of subphylum Tunicata. In all cases, ecological considerations were the initial basis for the study. In the first two species, the interest in specific isolated compounds remained ecological; while in the third organism attention was turned toward a potentially beneficial class of compounds for human use. In all three investigations, a major emphasis was on separation techniques, especially high performance liquid chromatography, to purify structurally homologous or isomeric groups of molecules.

Previous and ongoing work on the sea pen was primarily concerned with the organic soluble extracts. Here several types of biological activity, which included insecticidal tendencies, have been attributed to a group of novel diterpenes (3,4). From an ecological standpoint, previous work had been done on this orange, fluorescent organism by Shimomura where a type of luciferin, coelentraine, was isolated and determined to be the

major bioluminescent component of P. gurneyi (5). In addition to its luminescent properties, this coelenterate is soft bodied and generally devoid of any physical defense mechanisms, yet it maintains itself in an absence of nearly all predation as well as observable encroachment and macroscopic fouling. There was no clear indication that the previously identified organic soluble components were responsible for this allomonal effect. But it had been previously reported that the aqueous extracts induced apparent anesthesia in fish (6). Therefore, attention was directed toward the isolation and characterization of unique components of the water soluble fraction of P. gurneyi which were not simple salts or common amino acids in composition.

The inquiry into ecologically significant molecules from the false limpet was done from a different perspective. Previous work had been done on pulmonates of the genus Siphonaria implicating a series of polypropionates in antimicrobial activity as well as a chemical defense against predators (7,8). In S. alternata, it had been shown that the organic soluble polypropionates, siphonarins A and B, were possibly being employed as a trail marker by these slow moving, intertidal mollusks for possible use as alarm pheromones, sex attractants, or food location (9). Since the structures of these two compounds had been determined contemporaneously by the Faulkner group (10) and our group (11), the main emphasis for this part of the research was to determine if there were different quantities of these marker molecules produced in

limpets which were slowly terminated upon collection and presumably would be in an alarmed state for a greater period of time as opposed to their quickly terminated counterparts. The presence of more polypropionates per organism in the former group could then be used as an indicator as to the use of these molecules as an alarm pheromone. Concomitant with the quantification analysis, a major objective was to develop a better chromatographic technique to resolve the siphonarin compounds from each other as well as other organic components.

Like the sessile P. gurneyi, the tunicate E. olivaceum was initially of ecological interest because of its apparent dependence on chemical defense agents due to its lack of physical protective systems. Unlike the coelenterate, interest in this case shifted from ecology to biological activity. The colonial tunicate from the Caribbean region came under study in the late 1970's. By 1983 a number of new compounds of the oxathiazepinotetrahydro- β -carboline (12) and β -carboline ring systems (13, 14) had been isolated and characterized. It was found that a number of these compounds were antiviral, especially against Herpes simplex (13, 15). While the β -carbolines of this organism were of interest due to their pharmacological activity as opposed to their ecological uses, the separation objectives and techniques were very similar to those employed in the siphonarin project. In this case, our group (14) had partially purified and characterized several β -carbolines from E. olivaceum but had been unable to separate

completely the various brominated and hydroxylated isomers. And because the techniques employed by Rinehart, et al. were not reproducible with our fractions, a project was undertaken to develop a chromatographic system whereby the β -carboline isomeric mixtures could be cleanly resolved.

RESULTS AND DISCUSSION

Ptilosarcus gurneyi:

Isolation. The crude aqueous extract of Ptilosarcus gurneyi exhibited interesting aromatic signals rather far downfield in the ^1H NMR and ^{13}C NMR spectra. Even though the signals were aromatic and might lead one to believe they were due to an organic soluble compound, the high solubility in water as well as its rapid elution with a 3:1 water:methanol eluent from a C_{18} reverse phase column indicated the presence of a rather polar, if not ionic compound. On the other hand, an 85:15 methanol:water system caused this compound to be eluted in the second fraction, over 30 minutes after the void volume. This lent evidence to the fact that the target aromatic molecules were extremely polar and "preferred" the aqueous eluent system over adsorption onto uncapped segments of the nonpolar octadecylsilane packing. By decreasing the polarity of the solvent system, the target molecules were no longer as soluble as they were in the more polar system; therefore, they were not as easily removed from their adsorption onto the C_{18} packing.

This fraction was then run on a Sephadex LH-20 column. Based on its elution from this column in the third of six fractions, it was inferred that the compounds in question were

of a relatively small size. It was also at this time that it became apparent that the aromatic compounds under study included a major component as well as a far less abundant minor constituent. This was based on the presence of a major as well as a minor set of signals in the ^1H NMR spectrum of an otherwise clean compound corresponding to the major set of signals. The minor component could only be removed from the major compound by use of an analytical propylamine HPLC column run under reverse phase conditions, from which both compounds were obtained in pure form.

Characterization of homarine. The major aromatic component proved to be N-methyl picolinic acid, or homarine, **1**. A key set of data for this assignment were based on ^1H NMR as seen in Figure 1a. The rather low field nature of four proton signals between 8.0 and 8.8 ppm indicated a heteroaromatic compound, a pyridine system appearing most likely. Analysis of the decoupling pattern, Figure 1b, where there are two doublet and two triplet signals in which each of the doublet hydrogens is coupled to a different triplet hydrogen and each of the triplet protons are coupled to each other; pointed toward a substitution at the 2 position. Finally, the existence of an uncoupled three hydrogen singlet at 4.4 ppm indicated a highly deshielded methyl group indicative of a 1-methyl pyridinium type ion which tended to correlate well with the observed polarity of this compound.

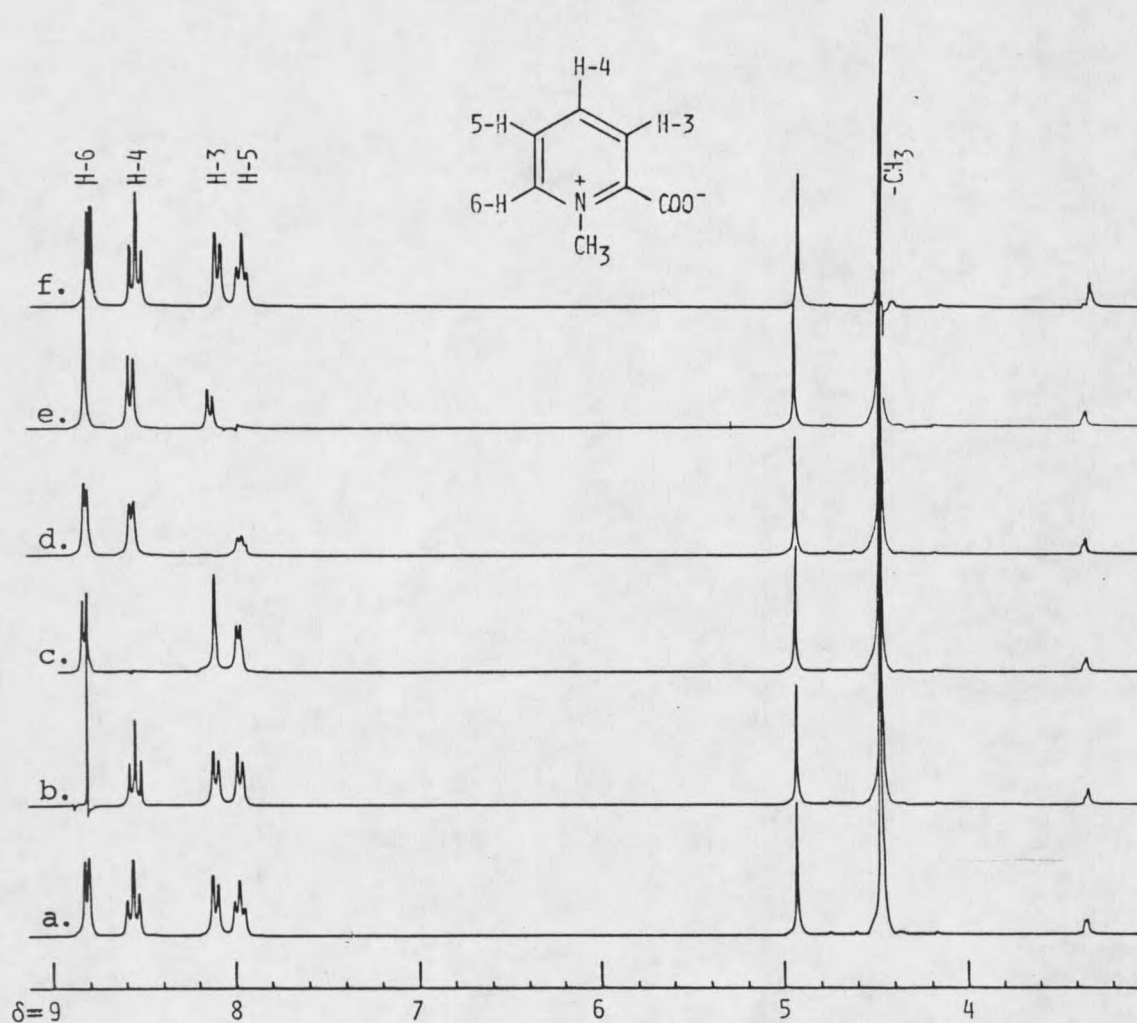
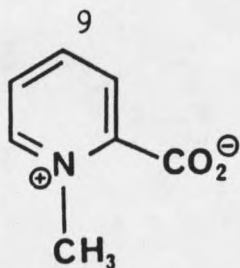


Figure 1. ^1H NMR spectrum of homarine, coupled (a) and decoupled (b-f).



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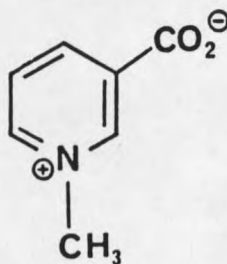
Gated ^{13}C NMR provided confirmatory as well as new structural evidence. As expected, this spectrum showed four aromatic doublet signals between 124 and 146 ppm and one methyl quarter at 47 ppm. Evidence for the as yet unassigned one site of substitution was found in the final two signals. A substituted, weak carbon singlet signal was found at 152 ppm. This shift also fits well for the heteroaromatic carbon further justifying our choice of a pyridine type structure. The final carbon signal was found as a singlet at 166 ppm. This shift along with the known polarity of this compound gave good evidence for the substituent being a carboxylate functionality.

The infrared spectrum showed a number of bands. Most important were strong bands at 1636 and 1358 cm^{-1} which indicated the presence of a carboxylate anion. Also present was a weak but broad band at 3394 cm^{-1} which was most likely due to interaction of some of the carboxylate anion with atmospheric water vapor resulting in the formation of a small amount of the acid form of the anion.

Confirmatory evidence for a 2-carboxy-1-methyl pyridinium salt was obtained from FAB mass spectrometry which showed a parent ion (MH^+) m/z 138 and a second prominent peak at m/z 94

corresponding to a loss of CO_2 . Final structure proof came via preparation of this inner salt by the methylation of picolinic acid with iodomethane to yield a product with ^1H NMR and UV data identical to the compound isolated from P. gurneyi.

Characterization of trigonelline. Upon completing the structure elucidation of homarine, the characterization of the minor component, 3-carboxy-1-methyl pyridinium salt, trigonelline 2, was rapidly accomplished. Again ^1H NMR, Figure 2, provided the initial key data. The proton signals for this substituted nicotinic acid were in the same general range as were those of homarine. But in this case the low field region contained a one hydrogen singlet at 9.2 ppm pointing toward a substitution at the 3 position. Also present were a pair of overlapping one hydrogen doublets at 8.9 ppm and a one hydrogen doublet of doublets at 8.1 ppm indicating the presence of three adjacent hydrogens. The high field zone, like homarine, contained a methyl singlet at 4.4 ppm.



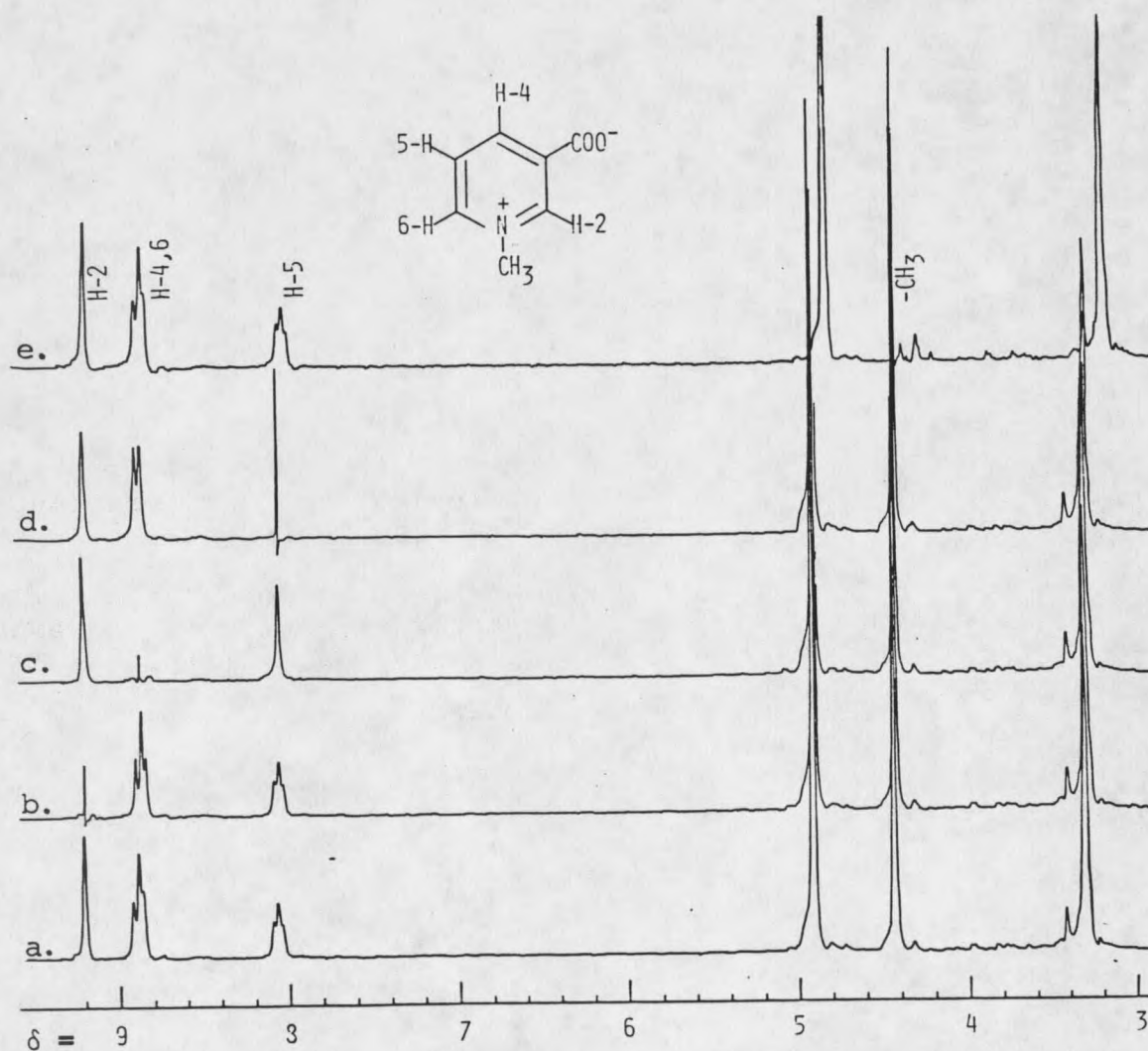


Figure 2. ^1H NMR spectrum of trigonelline, coupled (a) and decoupled (b-e).

The ^{13}C NMR was also analogous to that of homarine. Here the carbon methyl signal was at 49 ppm, the aromatic carbon four doublets and one singlet were in the 128 to 146 ppm range, while the carboxylate singlet was found at 168 ppm. Further, the mass spectrometry data gave a (MH^+) at m/z 138 with a CO_2 loss correlating with a 94 mw fragment. Finally, IR and UV data were similar to that of homarine, but here the λ_{max} was shifted 8 nm shorter with a corresponding 45% decrease in absorbance.

Like its picolinic acid counterpart, structure proof was secured through product synthesis. In this case, trigonelline was synthesized by the use of iodomethane to methylate nicotinic acid. The synthesized product was identical in all respects to naturally occurring trigonelline. Interestingly, in the presence of contaminant Na^+ and/or I^- , the overlapping doublets separated so that one of the hydrogen signals moved .2 ppm to higher field, thereby lending clearer support for the assigned structure for trigonelline.

Ecological functions of homarine. In 1933 homarine was identified as an isomer of trigonelline. This compound received its trivial name based on where it was first found by Hoppe-Seyler, that being the genus Homarus, or the lobster (16). Since that time N-methyl picolinic acid has been found almost exclusively in marine invertebrates. These include Annelidia, Mollusca, Arthropoda, Echinodermata, Echinoidea, and rarely the marine Chordata (17, 18). Further, our group has detected

homarine in several species of Porifera (19). Finally, this compound has been isolated from one non-marine invertebrate, the fungus Polyporus sulfureus (20, 21). Our 0.4% dry weight recovery for homarine from P. gurneyi may appear large but this value is the rule rather than the exception in marine invertebrates (17). Some examples of high N-methyl picolinic acid concentrations include the lobster (H. americanus) with 0.18% wet weight (17), the blue crab with 0.25% wet weight (22), sea urchin eggs with homarine accounting for 1.0% of their dry weight (23), and in the octopus, Molgula manhattensis, homarine accounts for a high 7.2% of renal fluid (24).

With this relatively large quantity of a secondary metabolite present in an organism, the main question is: what is it doing there? Historically, this has been the focus of attention for a number of researchers on the homarine question. Because this molecule is found almost exclusively in marine organisms but not in their fresh water counterparts, it might be logical to assume that homarine is involved in osmoregulatory processes (17). But work by Dall (25) appeared to discount any direct rôle for this compound in osmoregulation. Recently, work on shrimp homogenates by Netherton, et al. (26) has shown that homarine acts as a methyl reservoir as well as a methylating agent. Further, it has been shown by him that this molecule is one of the initial agents used in the formation of amino acid betaines, especially glycine betaine. Possible methyl donor mechanisms are illustrated in Figure 3. This methylation

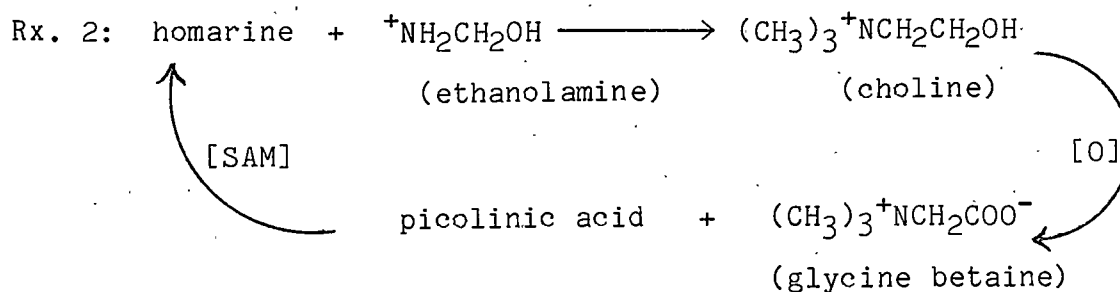
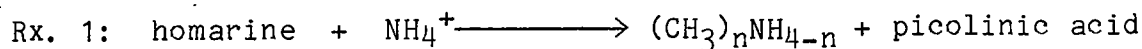


Figure 3. Homarine as a methylating agent.

Reaction 1 holds for the general case. Reaction 2 is specific for brine shrimp homogenates (26) and presumably is valid for all homarine containing marine invertebrates.

process is interesting in light of the fact that glycine betaine is widely distributed among plants and animals (27) and is very common among marine invertebrates (17, 28, 29). Further, it appears that one of the functions of glycine betaine in marine organisms is that of osmoregulation (30). Therefore, homarine may be important in control of cell volume in such organisms as P. gurneyi indirectly as an intermediate methylating agent of amino acids to form betaines. In this sense, homarine may be functioning as the more common methylating agent, S-adenosylmethionine (SAM), in those marine organisms where N-methyl picolinic acid is present.

A second role of homarine in P. gurneyi may be direct or indirect action as an antifouling agent. Evidence for this comes from work by Targett et al. on diatoms (31), who have

shown that nicotinic acid, picolinic acid, and homarine, in that order, are effective against diatomaceous growth on gorgonians. The gorgonians, which are phylogenetically related to P. gurneyi, are also similar in that neither is found overgrown with epiphytes. Unfortunately, no evidence of nicotinic or picolinic acid in the sea pen was observed by us, nor has there been any report of these compounds in other homarine containing organisms. This apparent lack of N-methyl picolinic acid precursors may be due to very small quantities present, or facile chemical modification including methylation occurring on these compounds in the organism.

Ecological functions of trigonelline. Trigonelline was isolated by E. Jahns (32), from the plant Trigonella foenum-graecum about 48 years before the characterization of homarine. This N-methyl nicotinic acid betaine has been the subject of far more research than homarine. This is due primarily to its far greater prevalence in both the phytological as well as zoological realms. Trigonelline has been reported to be in several gymnosperms and monocotyledons as well as many dicotyledonous plants (33), a number of marine organisms (17, 34), and its presence has been noted or inferred in a large number of microorganisms and higher animals (35). The biosynthesis of this cognate compound from nicotinic acid, Figure 4, hints at its major biological function. Plants produce this molecule via the Preiss-Handler pathway and it is

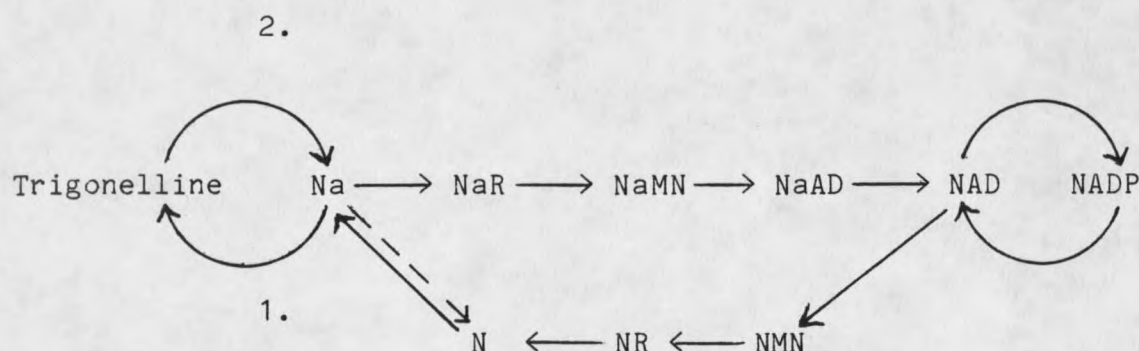


Figure 4. The formation of trigonelline as a side reaction in the synthesis of NAD, the Preiss-Handler pathway. Methylation of nicotinic acid by S-adenosylmethionine (1) yields trigonelline. Demethylation by demethylating enzyme (2) reverses the first former reaction (42).

Na = nicotinic acid, R = riboside, MN = mononucleotide
AD = adenine dinucleotide, N = nicotinamide

found in the urine of rats given normal diets as well as those given doses of nicotinic acid (36), as a major metabolite of the Tryptophan-NAD pathway. These data point toward trigonelline as being a non-toxic nicotinic acid transport and storage molecule. Because nicotinic acid may be used as an antifouling agent, it would seem plausible that *P. gurneyi* would store this molecule in the trigonelline, or methylated, form. Interestingly, there has recently been a spurt in trigonelline research since it has been found to have important growth regulatory functions in leguminous plants (37, 38, 39, 40), but no hormonal role for this compound has been found in animals.

Bioassay results of homarine and related compounds.

Homarine and trigonelline, as well as their biogenic precursors and product metabolites, picolinic and nicotinic acid, were run through a series of in-house bioassay systems. A summary of these assays is found in Table 1. The most interesting of the results were obtained from the seed germination assay. The seed germination/growth regulation assays were performed by application of 10^{-4} to 10^{-10} M concentrations of the target compounds to lettuce seeds and observing growth of roots and cotyledons in 48 hours. The results of this assay showed that nicotinic acid at 10^{-7} M concentration reduced root growth by 37% while picolinic acid at 10^{-4} M caused a 73% reduction in root growth. This data pointed toward a general toxicity of these compounds toward plants at least during the early developmental stages. On the other hand, the two subject molecules of our research enhanced plant growth. At 10^{-7} M to 10^{-9} M values, homarine increased root growth over controls by 25%. The 3-carboxy isomer increased root growth at the 10^{-4} to 10^{-6} M concentrations by 14 to 19%; and also at the 10^{-4} M level, increased coleoptile growth by 18%. Further, synergistic effects of the 2 and 3-carboxy homologs became evident in this assay. Here, the combination of the two increased root growth by 20 to 40% in the 10^{-4} M to 10^{-10} M ranges respectively. Also, coleoptile growth was enhanced by 20% in the 10^{-9} M concentration.

BioassayCompound

	Homarine	Trigonelline	Picolinic acid	Nicotinic acid
Antimicrobial	0	0	1-2 mm ^{a,b}	0
Seed germination/ growth regulation	+26% (10^{-7} M)	+19% (10^{-6} M)	-63% (10^{-4} M)	-37% (10^{-7} M) ^b
Phytotoxicity	0	0	0	0
Brine Shrimp ^c	0	0	0	0

Table 1. Pharmacological assays of target methylated aromatics and their precursor and/or product demethylated homologs.

^a this is the zone of inhibition of microbial growth on a culture plate.

^b(-) values indicate biological inhibition, while (+) values show enhancement growth.

^ctoxicity toward brine shrimp.

The only other bioassay to show any activity was in the antimicrobial test where picolinic acid inhibited Rhizoctonia solani fungal growth as well as Escherichia coli and Corynebacterium michiganensis bacterial growth by 5-10%. None of the four compounds showed any phytotoxicity toward adult Johnson grass, Sorghum halepense, or leafy spurge, Euphorbia esula, at 100 or 250 ppm. Nor did any of these compounds show any toxicity toward 48 hr. old brine shrimp larvae at 0.25 mg/ml.

Conclusions. The literature as well as our bioassay work point toward homarine and trigonelline being rather benign compounds while their demethylated counterparts show some toxicity from a pharmacological standpoint. The results of the seed germination/growth regulation bioassay essentially summarize the previous work done by a number of other investigators. The main point is that homarine and trigonelline derive their importance in being methylated derivatives of homologous demethylated precursors. In the case of homarine, it is this molecule that seems to be important as a methyl reservoir and donator for other molecules, especially osmoregulatory amino acids, in P. gurneyi and other marine invertebrates. On the other hand, trigonelline, notwithstanding its hormonal implications in legumes, appears important as a non-toxic reservoir for an important biomolecule, nicotinic acid, in a wide variety of organisms. At any rate,

the implication of a direct role for these two methyl isomers in a defense pheromonal capacity in P. gurneyi appear rather meager. Rather, their functions are related to the more general biological applications of molecular synthesis in the case of homarine and molecular storage in the case of trigonelline.

Siphonaria alternata

The target siphonarin compounds in this part of the study are not of totally unknown genealogy. These compounds are members of a large class of secondary metabolites, the polyketides. While a majority of the ketides are built up of acetate units via acetyl-CoA, the siphonarins are based on propionate units. Polyketides based on the C-3 unit include a number of macrocyclic compounds including erythromycin A (42).

The general source of the precursor polypropionate ketals in this study is to be found in a genus of gastropod mollusks, the Siphonaria. These are air breathing organisms resembling limpets that live in a number of areas of the world (43). Recent chemical studies involving the structure elucidations of a number of novel secondary metabolites includes species collected from Australia, Chile, and Florida (44). All of the Siphonaria examined thus far, including S. diemenensis, S. pectinata, and S. lessoni contained polypropionate components (45), some of which proved to have antibiotic activity (46).

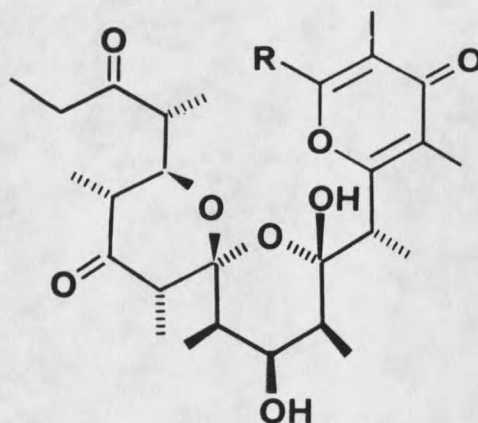
It has been known for some time that Siphonaria species possess "repugnatorial glands" that secrete defensive chemical

substances, and are a trail following organism (47). Our work centered on the possible involvement of the γ -pyrone polypropionate metabolites of S. alternata in trail following and/or defensive or stress pheromone activity. Previous work done by the Cardellina group showed that the polypropionate siphonarins A and B, 3 and 4, as well as an open chain form now called siphonarin F, 14, were present in the Bermudian S. alternata (11). Contemporaneous work by the Faulkner group (10) on S. zelandica and S. atra of Australia and S. normalis of Hawaii showed the same siphonarins to be present in these species. Therefore, the combined results of these two groups showed the widespread occurrence of these secondary metabolites with the implication that these compounds must have some generalized physiological function, possibly in keeping with the demonstrated trailing and secretion capabilities of this genus.

S. alternata were collected in two groups. The first consisted of 50 individuals which were hand picked and instantly placed in acetone. This procedure was designed to act as a control in that these rapidly killed organisms should contain only these compounds associated with their normal placid lifestyle. At the same time, a second group consisting of ten times as many organisms was collected but allowed to remain together, alive in the collection container for several hours before slowly freezing them. In effect, this was the experimental group. Any changes in the polypropionate composition of this slow killed group would then be assumed to

3 $R = CH_3$

4 $R = C_2H_5$



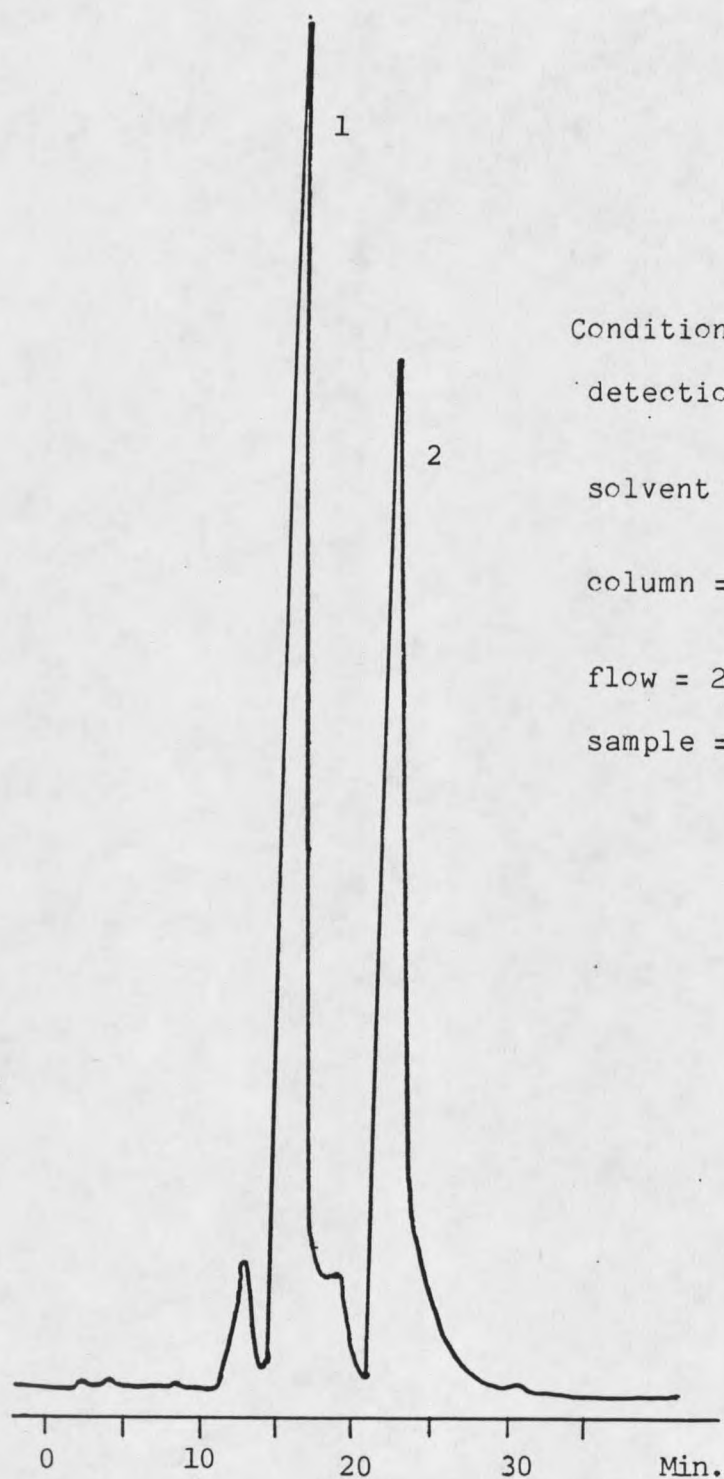
be due to the stressful environmental conditions that they underwent. Polypropionate compositions were then to be compared between the control versus the experimental group on a milligram per limpet basis.

A kind of qualitative difference between the organic chemical composition of the two groups was observed as early as the first stage in the molecular separation process via the Sephadex LH-20 column. The extracted organics of the fast killed group eluted in six colorless or light brown bands as detected by the UV monitor set at 254 nm. On the other hand, using the same procedure, the organics of the slow killed group came off in nine fractions. Nearly all these fractions were associated with but did not necessarily coincide with brightly colored orange, yellow, and pink bands.

The polypropionate containing fractions were then chromatographed on a BioBeads S-X4 column followed by a second Sephadex LH-20 column using a more polar solvent than the previous LH-20 system. At this point, structural differences in the chemical composition of the two groups began to be observed.

All of the polypropionates from the fast killed group separated from the other components on the third column. And when these metabolites were placed on the reverse phase ODS HPLC, they cleanly separated into two polypropionate compounds. Comparison of the ^1H NMR spectra of the fractions corresponding to the two major UV absorbance peaks from the HPLC elution, Figure 5, to the data from previous structure elucidation of siphonarins A and B by the Cardellina and Faulkner groups showed that the first polypropionate eluted was siphonarin A, the second was siphonarin B, the ethyl homolog of siphonarin A.

On the other hand, the fast killed group gave two siphonarin containing fractions from the same third column. A test run of a sample from the first major siphonarin band on the ODS HPLC showed this fraction contained many compounds based upon the large number of UV absorbance peaks observed. The second, minor siphonarin containing fraction separated on this ODS HPLC system yielding two polypropionates whose ^1H NMR did not correspond to either siphonarins A or B. At this point, attention was directed toward the separation and isolation of siphonarins A and B from the large LH-20 fraction. To accomplish this, the fraction was run through a reverse phase ODS gravity flow column using 100% acetonitrile as the solvent. This 84 mg fraction eluted in 7 fractions where the second and fifth fractions, each near 35 mg, proved to contain polypropionates, based upon analysis of their ^1H NMR spectra. The fifth fraction was then chromatographed on the reverse phase



Conditions:

detection = Beckman 160
set at 254nm

solvent = acetonitrile:water
(3:2)

column = Beckman Altex, prep.
ODS, 1.0 x 25 cm

flow = 2.6 ml/min

sample = 1.1 mg/15 μ l injection

Figure 5. UV absorbance trace of final separation of siphonarin A (1) from siphonarin B (2) via reverse phase ODS HPLC.

ODS HPLC system under the same conditions as those for the siphonarins containing fraction from the fast killed group giving a separation similar to that noted in Figure 5. However, in this case, the total siphonarins A and B per limpet was only about half that of the fast killed control group. This caused us to look closely at other polypropionate containing fractions that up to that point had been largely ignored.

Isolation and structure determination of siphonarins C through J. Like siphonarins A and B, the structure of siphonarins D had been previously determined by the Cardellina group (11); therefore, the isolation of this compound was ^1H NMR guided based on previous spectra.

The comparison of the structures of siphonarins A and B and their retention time from the reverse phase ODS gravity column and the ODS HPLC system to the retention time of the other siphonarins, gave a good preliminary indication that the structures of these compounds were even more closely allied to the parent siphonarins A and B than were those of the K and L compounds to be discussed later. This whole group of siphonarins eluted from the second fraction of the gravity flow ODS column as opposed to the A and B compounds which came off in the fifth fraction. But the compounds in this fraction had to be more closely related to the parent A and B compounds than siphonarins K and L because the K and L homologs had eluted separate from siphonarins A and B on the polar LH-20 column

while the novel siphonarins had coeluted with the A and B compounds on this column. Further structural relationships can be assigned based on the elution of these siphonarins from the reverse phase ODS HPLC system. A total of nine fractions were collected. Siphonarins G and I came off in the second fraction while siphonarins C, E, H, and J eluted in the fourth fraction. Finally, siphonarins D and F eluted in the seventh fraction. Each of these fractions was subsequently separated into their pure components by normal phase silica HPLC.

Based on ^1H NMR spectra, siphonarins C, E, G, and I, were shown to be the methyl homologs of siphonarins D, F, H, and J respectively. Again, as was the case with the A B, structures, the methyl component of each pair eluted before its ethyl counterpart. Therefore, based on this information, it can be inferred that siphonarins C and D are closely related to the E and F compounds; while G and H are related to siphonarins I and J. But it can be seen that with four siphonarins coming off in the middle ODS HPLC fraction, there is an overlap of the pairing; therefore, all four pairs are closely related. Further support for this idea comes from a comparison of infrared spectra in Figures 6, 7, and 8, which represent the parent siphonarin B compound to two distinct, representative congeners, siphonarins F and J. Here the spectra are for all practical purposes identical. This points to the fact that all these compounds have very similar or identical functionality. Further, the UV spectra for all siphonarins isolated with the

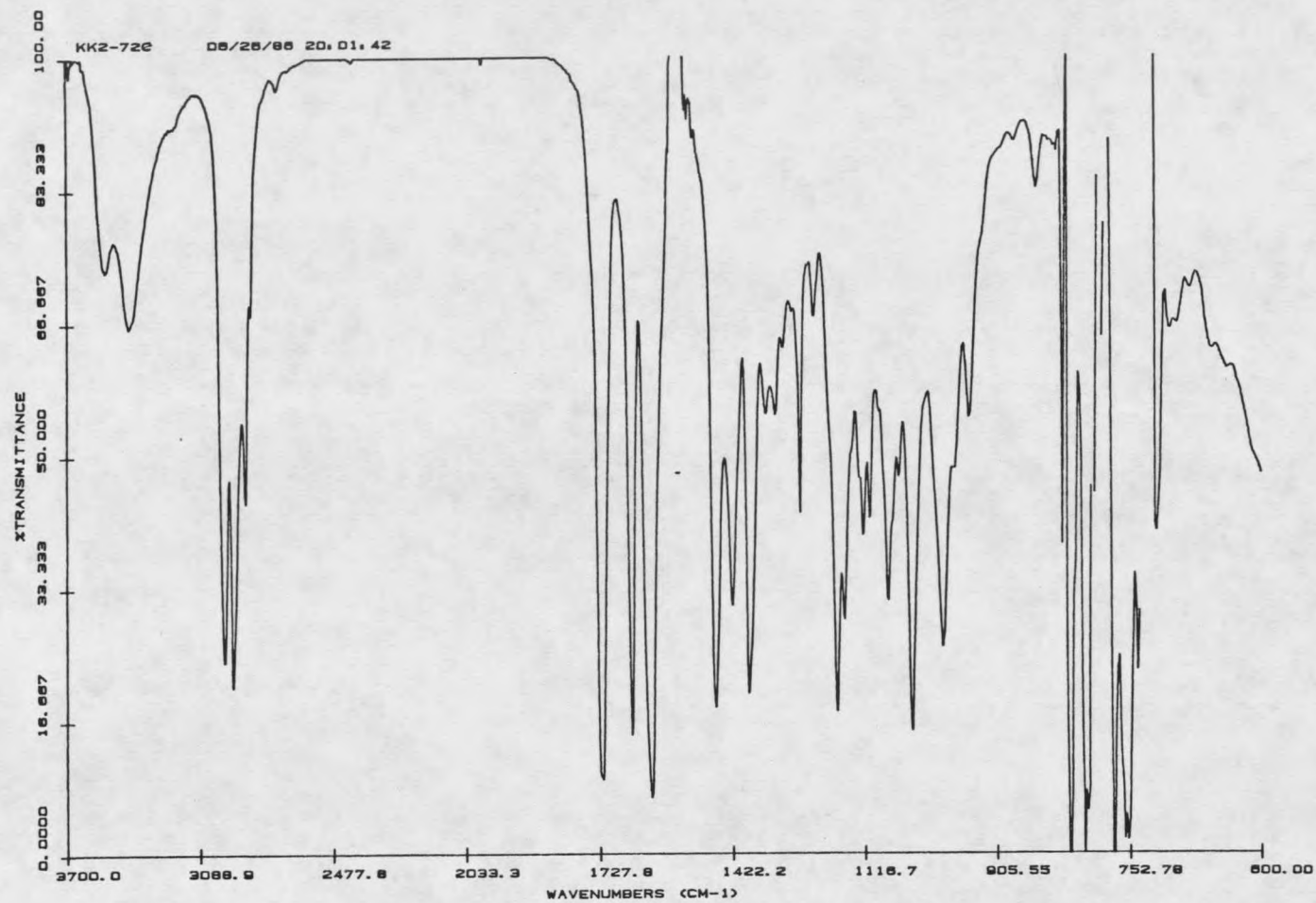


Figure 6. IR spectrum of siphonarin B

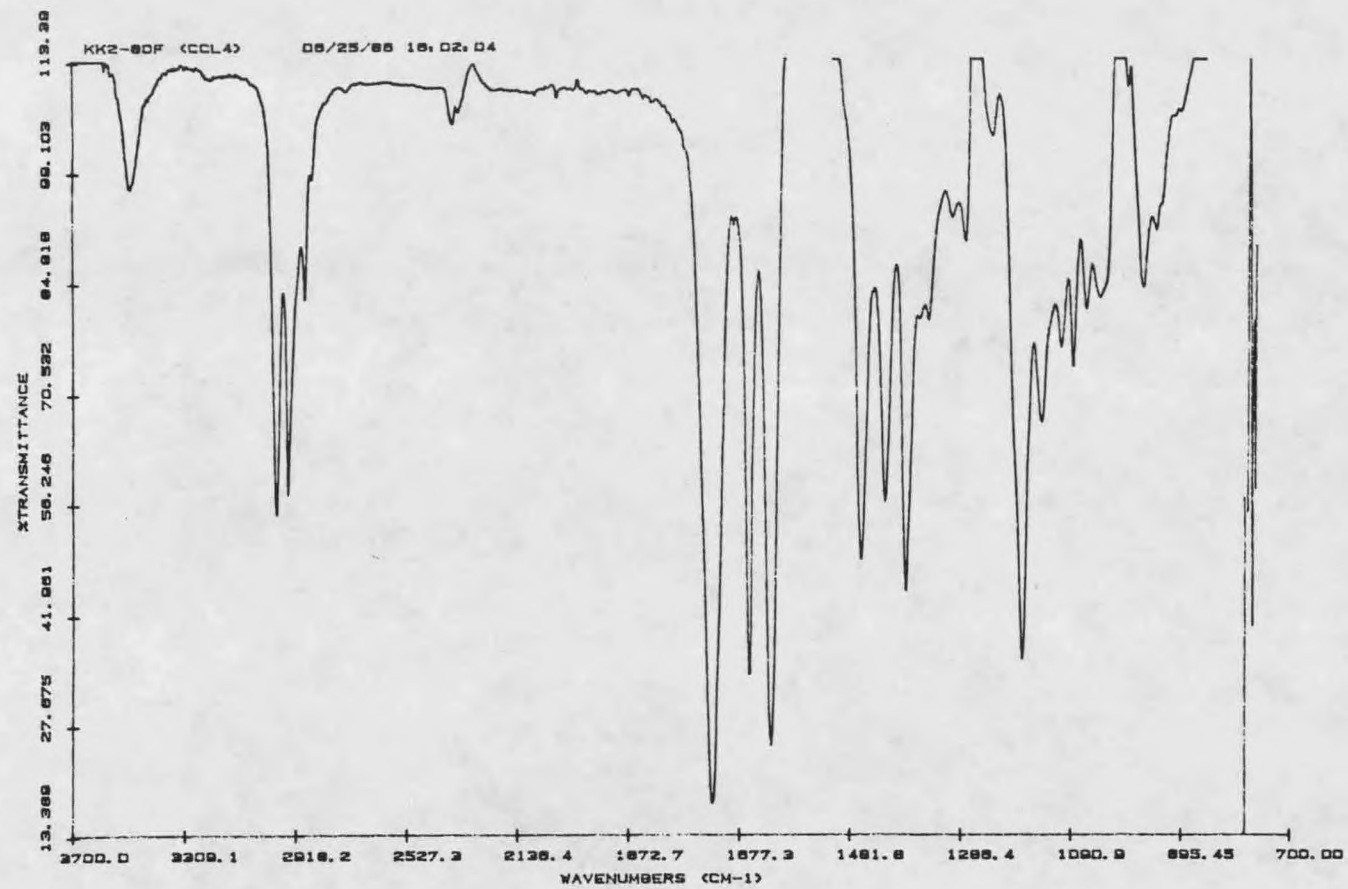


Figure 7. IR spectrum of siphonarin F.

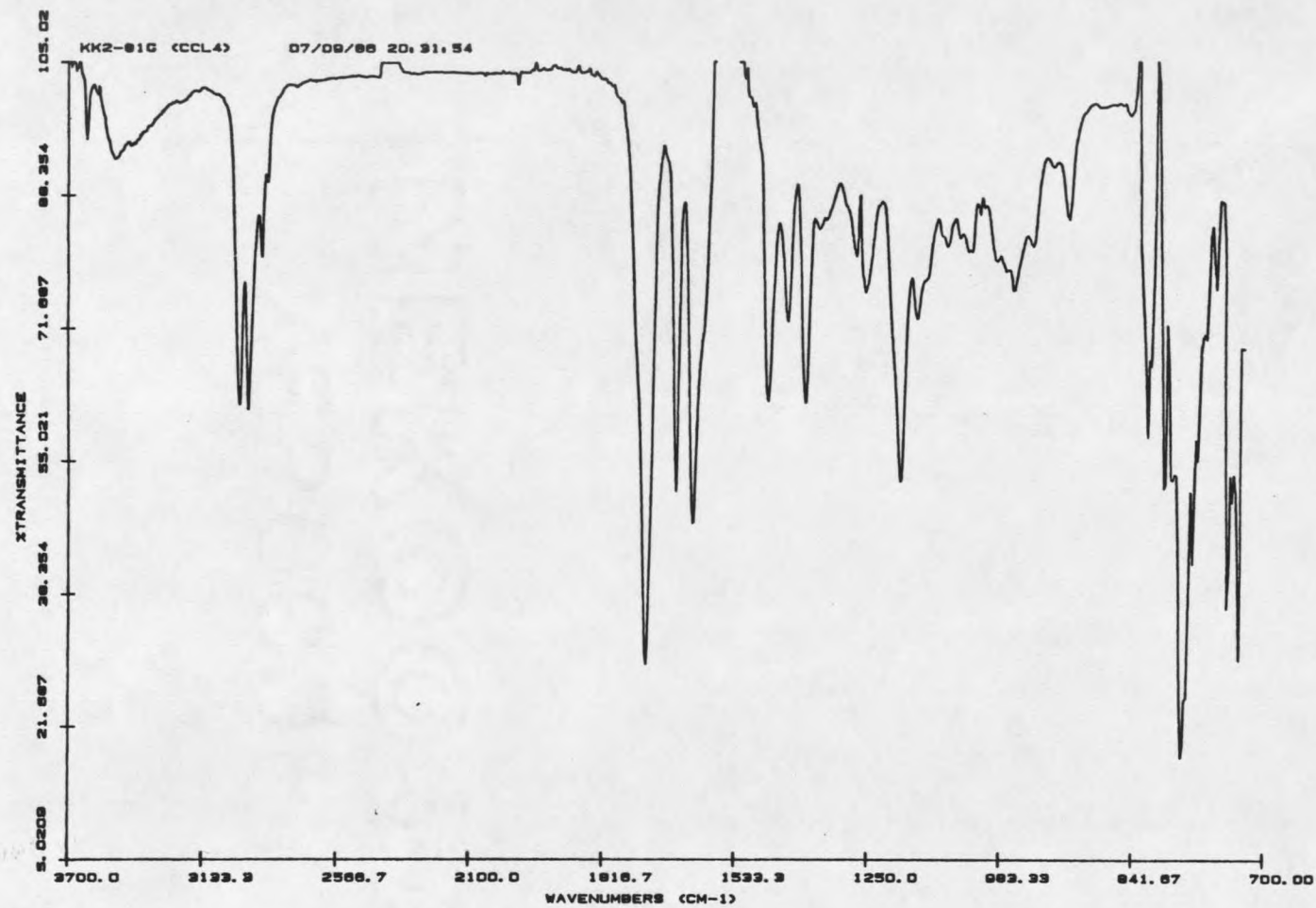


Figure 8. IR spectrum of siphonarin J.

exception of the K and L compounds have λ_{max} of 260 nm, and extinction coefficients between 7,500 and 14,300. Also, high resolution MS further confirmed the similarity of the compounds by assigning a molecular weight of 506.29 to all the methyl compounds and all the ethyl homologs had a weight of 520.30.

Proton NMR pointed out further similarities and differences in the eight structures. This is illustrated in Figures 9 and 10 where the ^1H NMR spectra of siphonarins E and I, which are representative of the two compound groups, are reproduced. In all cases, the γ -pyrone ring, 5a, with the attached C-14 methine and C-25 methyl groups were identical to the siphonarin A and B precursors. Further, based on decoupling experiments, each polypropionate chain contained two hydroxide or ether functionalities on methines, each flanked by methine bearing methyl carbons, one at near 3.6 ppm and the other at 5.5 or 5.0 ppm, 5b, depending on the structure.

The ^1H NMR spectra also showed some subtle differences so that these four siphonarin pairs could again be placed in two, two pair groupings. In the first group are siphonarins C and D with E and F; in the second are siphonarins G and H plus I and J. Interestingly, this supports the groupings already established based on the order of elution from the ODS HPLC column. Based on decoupling and the following data, the C group siphonarins were given partial structure 5c for their C-1 through C-4 groups. The key data here were two ^1H doublet of quartets between 2.3 to 2.5 ppm. Siphonarins G through J were assigned

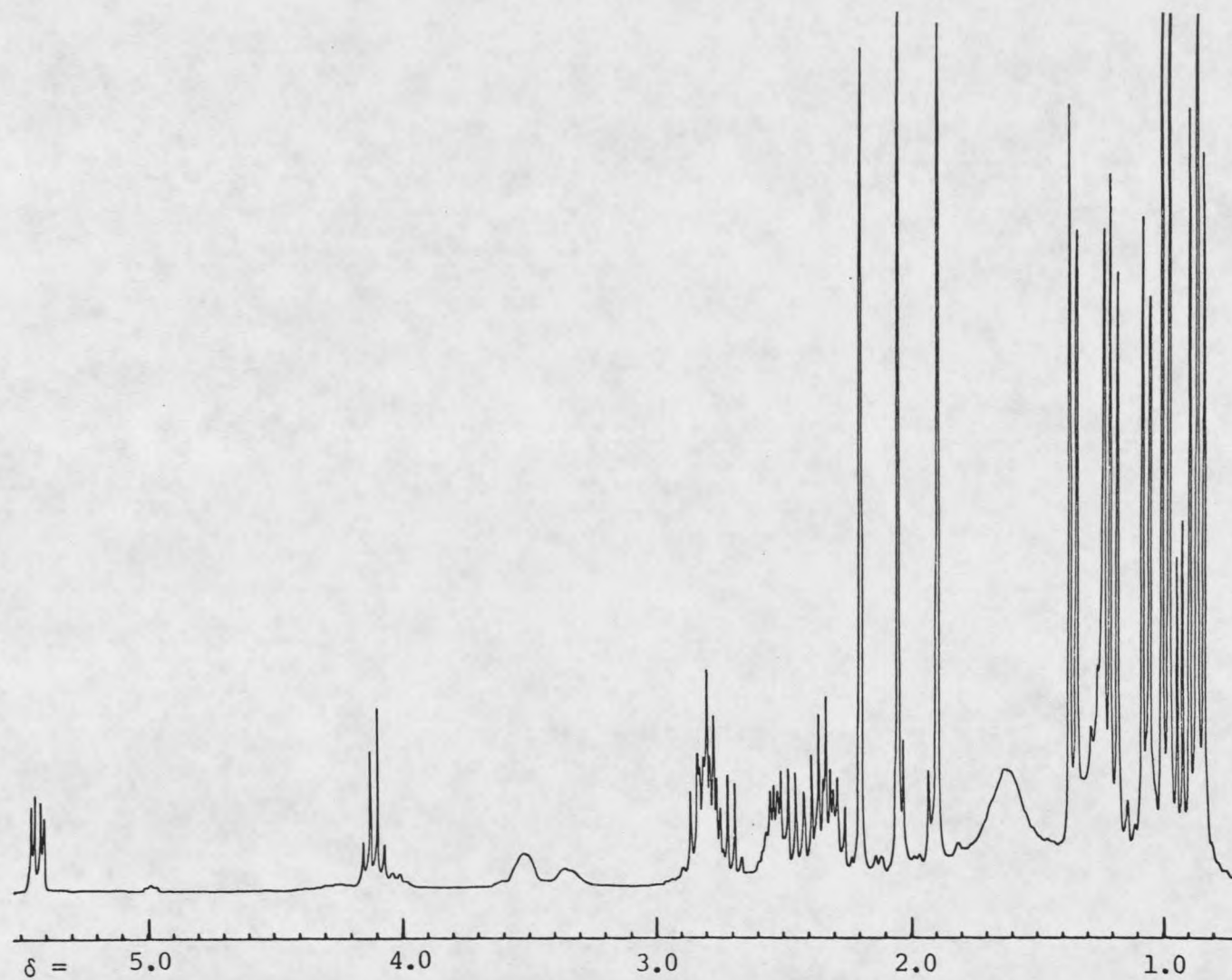


Figure 9: ^1H NMR spectrum of siphonarin E.

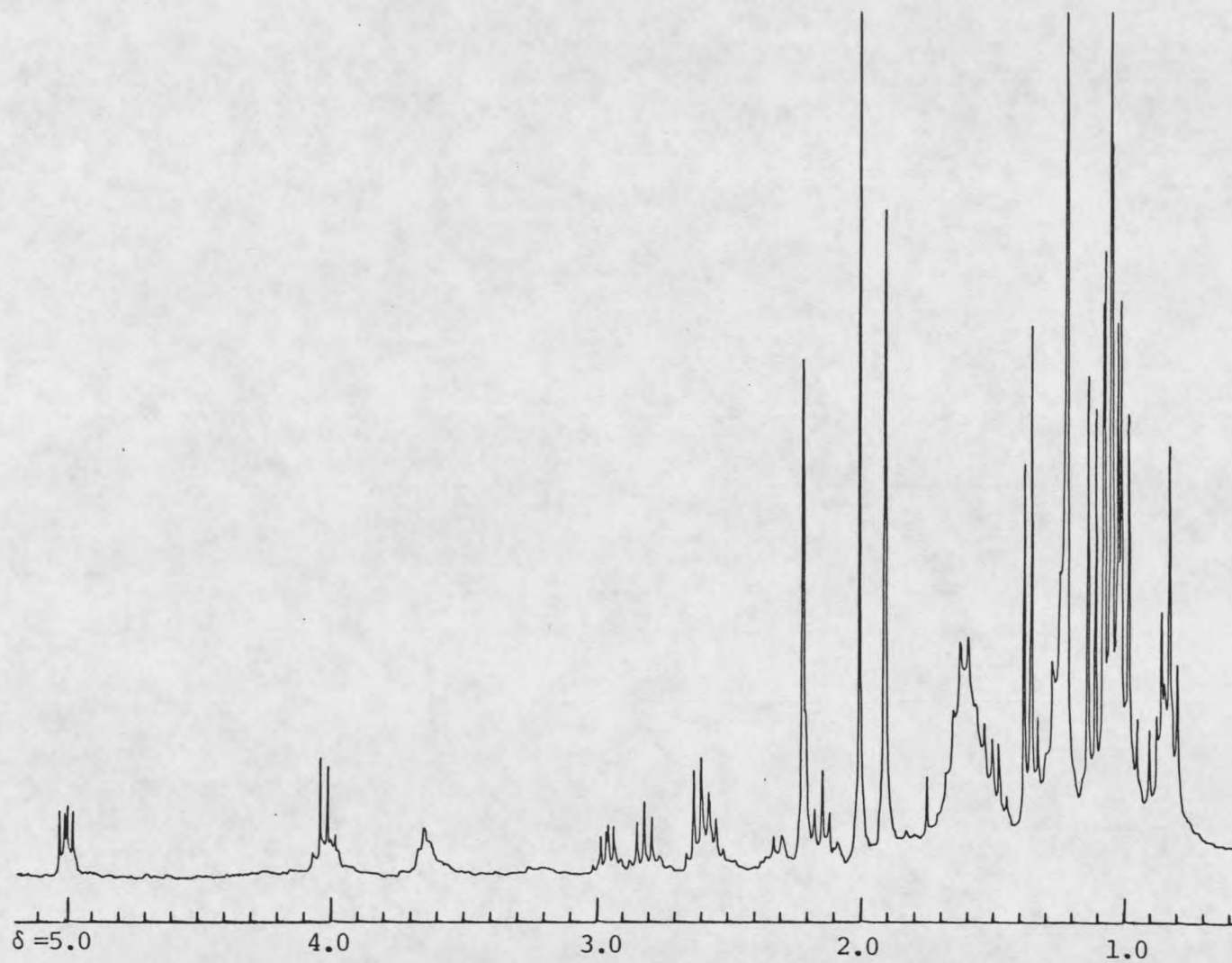
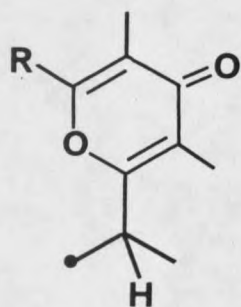


Figure 10. ^1H NMR spectrum of siphonarin I.

part structure **5d** primarily based on the fact that their two ^1H doublet of quartets were found at 1.45 to 1.65 ppm. Precedence for the assignment of the open chain carbonyl structure, **5c**, for siphonarins C through F and the hemi-ketal, **5d**, for the other four siphonarins is based on work reported by Faulkner and Ireland (10). They reported the ^2H shift at C-2 as being 2.4 ppm in siphonarins A and B; and 1.6 ppm for dihydrosiphonarins A and B. Each of these pairs had C-3 functionalities analogous to that for siphonarins C through F and G through J respectively.

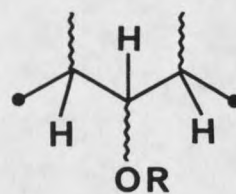
The argument as to whether C-3 or any other carbon is in fact a hydroxyl, ketal, or carbonyl functionality could be far more clearly answered by the use of ^{13}C NMR data. Unfortunately, with the exception of siphonarin F which had its structure previously determined, none of these compounds was present in excess of 1.9 mg. Therefore, these and all other siphonarin structure determinations had to be made placing especially heavy reliance on ^1H NMR data.

Based on the fact that siphonarin F had been previously isolated and its structure determined (11), it seemed logical to assign structures to the remaining seven compounds consistent with that of the F structure. Based on the previously known data for siphonarin F and the fact that its methyl homolog, as well as siphonarins C and D were all shown to have the same part structure **5d**, these four compounds were assigned the general structure **6**. Siphonarins G through J, due to their relatively different proton shift values for the C-2 end of the molecule



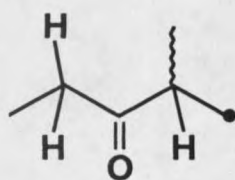
5a

$R = \text{CH}_3 \text{ or } \text{C}_2\text{H}_5$

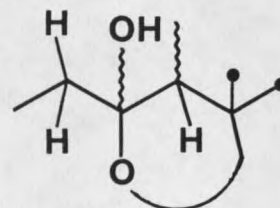


5b

$R = \text{H or ALKYL}$



5c



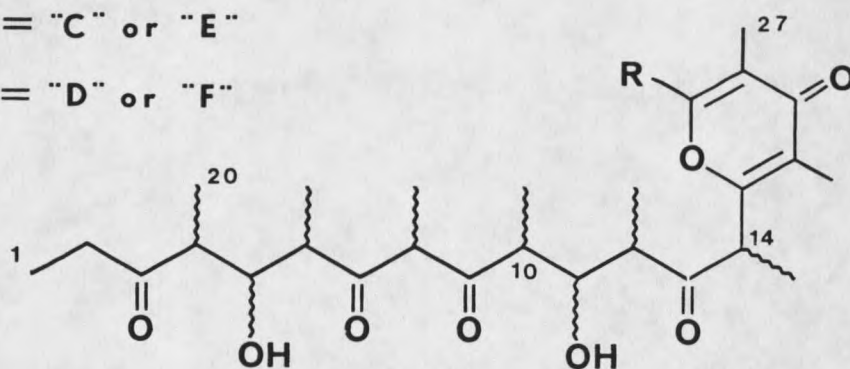
5d

had to be assigned a structure that could account for the formation of a hemi-ketal functionality. Further, the compound must be plausible from a biosynthetic point of view as well as bear a discernable relationship with the other siphonarins, including the A and B structures. Structure 7 represents this compound. The biosynthesis of this molecule in all likelihood proceeded from a cyclization of the C-11 hydroxyl to the C-7 carbonyl to form the ketal ring. This was followed by a spontaneous cyclization of the now O⁻ group at C-7 to the carbonyl at C-3 to form the hemi-ketal. Points to be made in favor of this compound and its synthesis include the fact that this is likely to be similar to the route followed for the formation of siphonarins A and B. Except that in the case of these two compounds, the cyclization process started at the C-4 end as opposed to the C-11 end in the novel cyclic compounds. Further, the ketal formation reaction is very facile, especially in the formation of a six membered ring. Another possibility is the formation of a hemi-ketal functionality between the C-11 carbonyl directly to the C-3 functionality. In this case a ten membered ring would be formed. While a macrocyclic structure such as this is possible, it is not as likely as structure 8 based on the fact that the entropy to be overcome in this system is far greater than in the six membered ring.

Mass spectral data seem to confirm the ketal versus open chain structures assigned to the two siphonarin groups. The ring structures showed losses of C₈H₁₃O₂ and C₁₄H₂₃O₄. In both

$R = CH_3 = \text{"C" or "E"}$

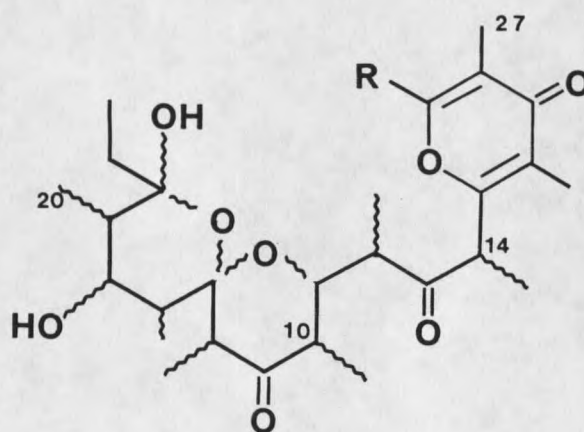
$R = C_2H_5 = \text{"D" or "F"}$



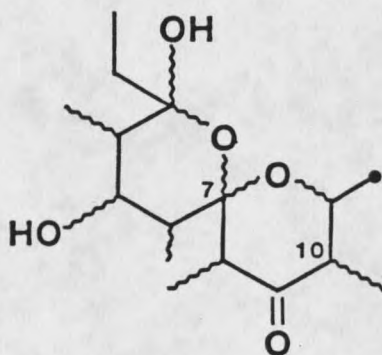
6

$R = CH_3 = \text{"G" or "I"}$

$R = C_2H_5 = \text{"H" or "J"}$



7



8

cases, these losses are due to ketal ring fissions at the C-7 and C-10 positions respectively. On the other hand, while the open chain forms showed these fragments, the $C_{14}H_{23}O_4$ loss in these compounds was two to four times less intense than the same fragment in the ring forms. Further, all the open chain compounds showed a loss of $C_8H_{13}O$ not found in any of the cyclic forms. This loss of only one oxygen can be explained by a cyclization of the chain form back on itself where either the C-5 or C-3 oxygen is transferred to a point closer to the pyrone ring culminating in the loss of the C_8 particle. Because siphonarins G through J are already cyclized, a mechanism to allow for a $C_8H_{13}O$ loss from the hemi-ketal ring is even less likely to occur than this loss from the chain form.

The next problem was to determine where to put the two oxygen bearing methine structures. Decoupling experiments had shown that the methine bearing ether proton at 5.00 ppm in the siphonarin G group was coupled to a methine bearing methyl proton at 2.1 ppm and to a second methine bearing methyl proton

near 2.94 ppm. Because the proton shifts at 1.45-1.65, 2.1, and 5.00 ppm were unique to only these four compounds, it seemed logical that the functionalities represented by these shifts should be placed in proximity to each other at C-2, C-4, and C-5 respectively; thereby placing the other hydroxyl with neighboring methyl methines at C-10 through C-12. The only problem here was that the 3.6 ppm proton was shown to be coupled to a hydroxyl functionality in all eight compounds. In the ketal structure envisioned for siphonarins G through J, 7, the methine at C-11 is no longer on a hydroxyl bearing carbon but on an ether carbon. Therefore, the 3.6 ppm hydroxyl methine with its flanking methyl methines cannot be at C-10 through C-12 but must go to the front of the chain; and the methine at 5.00 ppm which never shows OH broadening, must go to the C-11 position. Another problem to explain is the presence of one of the methyl methines attached to the 5.0 ppm hydroxyl methine proton at 2.1 ppm as opposed to 2.8 ppm shifts found in this proton in the siphonarin C group. This 0.7 ppm shift can only be accounted for by placement of this proton at the C-12 position where the proton can experience shielding by the underside of the γ -pyrone ring. The ring is held in this position by hydrogen bonding interactions by the hemi-ketal OH at C-3 and the carbonyl at C-13. One problem still to be resolved is, why does the methine at 5.00 or 5.50 ppm never show OH broadening, even in the open chain form?

At this point in the structure elucidation of the siphonarins, it became apparent that the remainder of the ^1H NMR spectra for all these compounds were nearly identical with the exception of the methyl group at C-4. The significance of this will become apparent below. But it can be stated that this one shift was indicative of the point of overlap between these compounds observed earlier during the ODS HPLC order of elution.

Now was the time to invoke stereochemical arguments to differentiate between the two chain structures as well as the two cyclic ketals. A good bit of these data came from optical rotation studies as summarized in Table 2. Here it can be seen that the siphonarin E, F, and G, H pairs have negative rotations while the other two pairs have positive values. This suggests a structural as well as biogenic relationship between compounds E, F versus G, H and I, J versus C, D. Further, compounds A and B also show a positive rotation suggesting they are related to the C, D and I, J pairs. Therefore, it was felt that these four compounds be given the same stereochemistry about C-4 as compounds A and B while the E, F and G, H pairs were given the opposite stereochemistry at this site. As a disclaimer, it must be noted that the use of optical rotation values to assign stereochemical structures rests on the assumption that changes in rotational values are in fact directly related to changes in the structures being proposed. The use of rotational values to assign relative structures is supported by the ^1H NMR shift observed for the methyl attached to C-4. Both the C, D and G, H

Compound	optical rotation [α] _D ²⁰
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Siphonarin A	+12.1°
B	+10.0
C	+38.2
D	+29.5
E	-52.4
F	-58.7
G	-65.6
H	-58.1
I	+28.2
J	+35.0
K	-8.6
L	-6.9

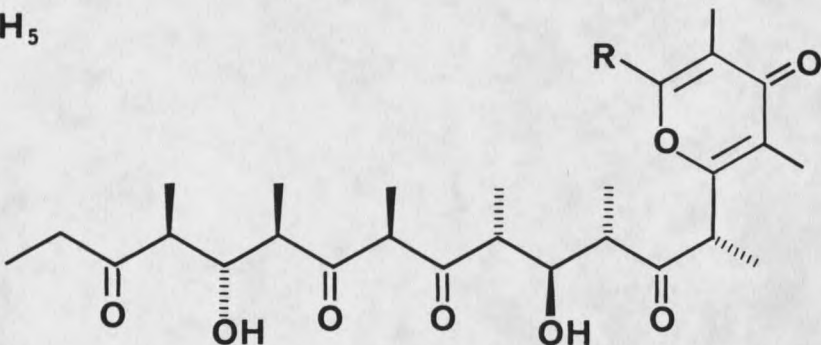
Table 2. Optical rotation comparisons for all isolated siphonarins. All compounds done in CHCl₃.

pairs had this proton at 1.20 and 1.27 ppm respectively; while these same protons were found at 1.08 ppm in the other two pairs. Based on these arguments, which still leave room for doubt, but are the best possible given the 1 and 2 mg quantities of these compounds available, structures 9 through 16 have been assigned for siphonarins C, D, I, J, E, F, G, and H respectively.

Structure elucidation of siphonarins K and L. These two polypropionates were initially isolated together and separated from the other compounds, as noted earlier, on the third separation column while working with metabolites of the slow killed limpet group. Separation of these two siphonarin homologs was subsequently achieved on the same HPLC system used to isolate siphonarins A and B. This fact in itself indicated that these two compounds were closely related to siphonarins A and B. This was further substantiated by a comparison of the infrared spectra of siphonarin B, Figure 6, to those of the novel siphonarins. All IR spectra showed absorptions at 2981 and 2938 cm^{-1} , indicative of CH_2 and CH_3 C-H stretches. A further absorption at 1724 cm^{-1} indicated the presence of non-conjugated ketones; and peaks at 1657 and 1621 cm^{-1} gave evidence for a γ -pyrone ring. But there were two major differences in the IR spectra of the new siphonarins compared to those of the A and B types. First, in the two novel siphonarins, there was a total lack of absorbance above 3000

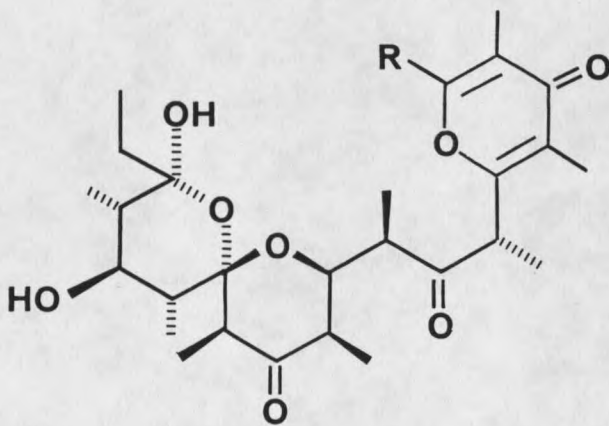
9 R = CH₃

10 $R = C_2H_5$



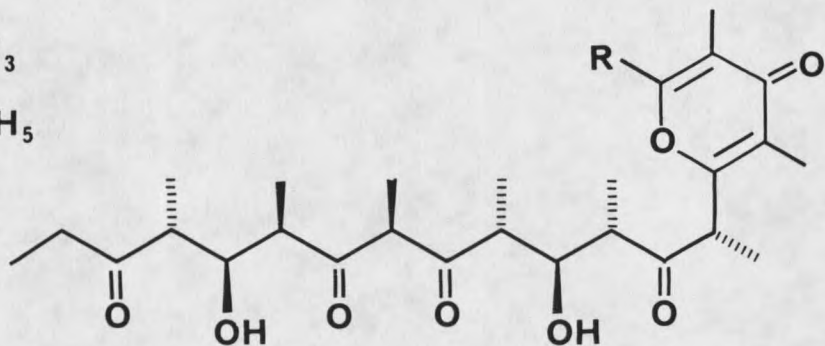
11 **R = CH₃**

12 $R = C_2H_5$



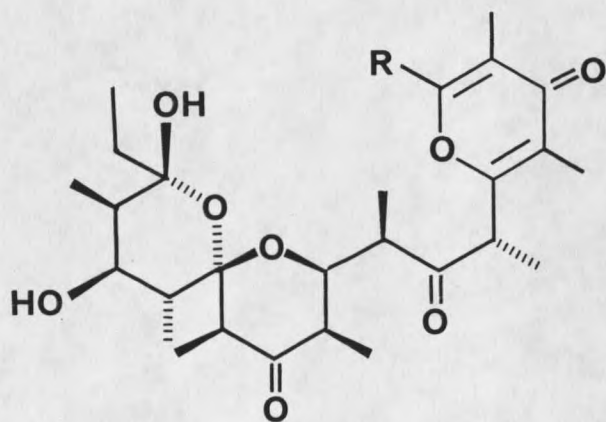
13 $R = \text{CH}_3$

14 $R = \text{C}_2\text{H}_5$

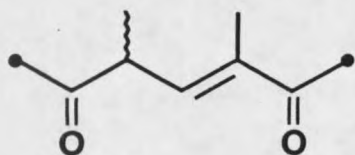
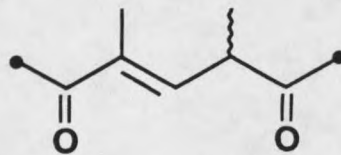


15 $R = \text{CH}_3$

16 $R = \text{C}_2\text{H}_5$



cm^{-1} , thereby indicating a lack of -OH functionality; further, a peak at 1679 cm^{-1} was present, pointing toward the existence of unsaturated ketones in these two molecules. ^1H NMR further confirmed the γ -pyrone nature of these molecules based on the presence of two methyl singlet signals at 1.90 ppm and one more at 2.20 ppm for the methyl analog and two methyl signals at 1.91 ppm as well as a methylene quartet at 2.53 ppm with a methyl triplet at 1.12 ppm for the ethyl homolog. Their general polypropionate nature was further confirmed by the presence of four more methyl signals in the 0.9 to 1.2 ppm range. Key data indicating double bonds in siphonarins K and L were based on two pairs of one hydrogen doublets at 6.49 and 6.40 ppm with their attached methyl groups α to the double bonds at 1.98 and 1.84 ppm respectively. Further, UV spectra gave λ_{max} of 240 nm and 238 nm for the methyl and ethyl homologs respectively. Calculated λ_{max} for an α,β -unsaturated acyclic ketone with alkyl groups at the α and β positions is 237 nm (48), which correlates well with the experimental values. These data, along with the knowledge that their likely parent molecules, siphonarins A and B, had two hydroxyl groups while these molecules had none, led us to believe that siphonarins K and L were in fact dehydration products of their biogenic precursors. From this reasoning, part structures **17a** or **17b** were possible for the two alkene groups on the polypropionate chain. To differentiate between them, mass spectroscopy came into play. The ultimate choice of the position of the double bond was based

**17 a****17 b**

on the fact that a cleavage β to a double bond and α to a carbonyl is more likely than cleavages α to a double bond as well as a carbonyl. And either of these breaks is far more likely than a cleavage through the double bond. The results of 12 ev and 70 ev high resolution as well as negative and positive chemical ionization mass spectrometry, as illustrated in Figure 11, favored the 4,10-diene forms for siphonarins K and L, **18** and **19**, as opposed to any of the other three isomeric forms. Key MS fragmentations included the presence of the mass 125 fragment at several times the intensity of the mass 57 or 96 fragments. The same argument was true of the 193 mass fragment relative to the 262 and 222 mass particles. Also, at no time was a mass 235 fragment observed, which is to be expected where the double bond is at the C-10 position.

The structures for these two dehydrated compounds fit well with the previous discussion on the variable stereochemistry about C-5 and C-5 in siphonarins C through J. Here it can be argued that both optical rotation types, siphonarins C and D as well as E and F, form the olefinic products K and L. Along with the data presented in the last section, the position of the

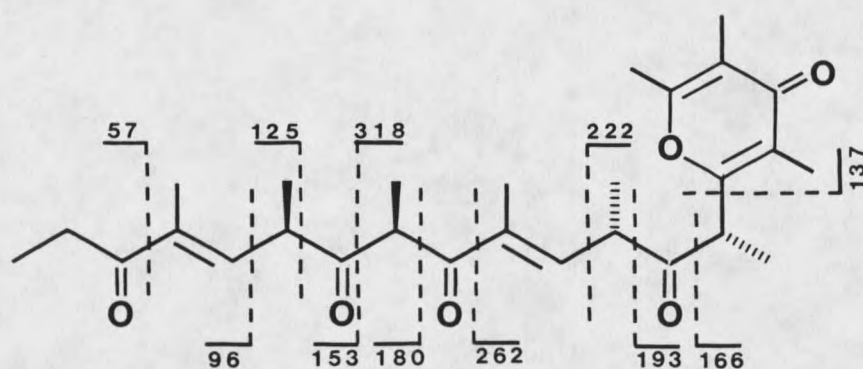
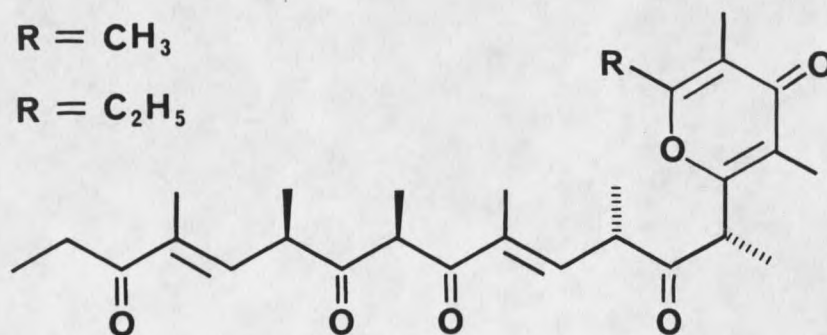


Figure 11. The mass spectral fragmentation pattern of siphonarin K. Siphonarin L, the ethyl γ -pyrone homolog gave analogous fragmentations.

18 $R = \text{CH}_3$

19 $R = \text{C}_2\text{H}_5$



double bond at C-4 lends support for the argument in favor of epimerization of the methyl about C-4.

Ecological implications of siphonarins in stressed vs. unstressed individuals. Reflecting back upon the original hypothesis under which this project was initiated, the results are rather surprising but not altogether unexpected. Originally it was felt that the quantity of siphonarins A and B per limpet would change relative to the two S. alternata groups; and that the stressed group would in all likelihood show an increase in one or both of these compounds. Inspection of the first two data lines for each of the limpet groups in Table 3 shows that in fact, the opposite occurred, at least for these two compounds.

The fact is that the control and experimental groups produced nearly equal total siphonarin quantities. Based on the data in Table 3, for purified siphonarins, the slow killed, or experimental group, limpets produced 88% of the total compound quantity compared to the fast killed control group. In reality, the two groups produced more nearly identical quantities for two reasons. First, the A and B siphonarins of the control group were run through a total of four separation columns, while the experimental group siphonarins A, B, K and L eluted through five columns and the remaining polypropionates went through six columns. The results being that losses of compound due to technique, adsorption onto packing materials, and decomposition,

Compound	Homolog	Fast killed	slow killed
		($\times 10^{-2}$ mg/limpet) $\pm 5\%$	($\times 10^{-2}$ mg/limpet) $\pm 10\%$
Siphonarin A	methyl	4.60	2.86
	B ethyl	4.80	1.60
	C methyl	----	0.28
	D ethyl	----	0.46
	E methyl	----	0.39
	F ethyl	----	0.77
	G methyl	----	0.15
	H ethyl	----	0.21
	I methyl	----	0.17
	J ethyl	----	0.38
	K methyl	----	0.26
	L ethyl	----	0.55
	Total methyls	4.60	4.12
	Total ethyls	4.80	3.96
	Total siphonarins	9.40	8.24

Table 3. A comparison of the quantity and quality of siphonarins present in the fast versus slow killed S. alternata groups.

increased by nearly 50% for the slow killed limpet compound separations as opposed to the fast killed group. That is, based on recovery data, there was an overall loss of 1.3% of the mass of organic soluble extracts through all four steps in the separation, while overall losses during separation of the experimental group organic soluble separations was near 2.5%. In both cases, nearly all these losses occurred during HPLC separation. The second reason for suspecting that the slow killed limpets contained a greater quantity of siphonarins than that noted in Table 3 is based on the observations that the fifth and eighth fractions of the ODS HPLC contained approximately 3 mg or 0.6×10^{-2} mg/limpet of inseparable polypropionate compounds. This quantity alone represents an additional 6% compound relative to the control group.

In addition to the fact that the total quantity of siphonarins in both mollusk groups are roughly equal, it can also be stated that the total amounts of methyl versus ethyl homologs of the siphonarin compounds are not only equal, within experimental error within a limpet group, but, more importantly, are equal between the two groups. Based on how this equality in quantity is maintained by the stressed limpet group lies an intriguing observation.

If one compares the quantities of the likely precursor molecules, siphonarins A and B, in the two mollusk groups; it becomes quickly apparent that the slow killed group has only 47%, or roughly half of these compounds as the fast killed,

unstressed group. Why? It appears that while the unstressed group has put, or more aptly, left all its 'eggs in one basket', the stressed group has greatly diversified this relatively homogenous quantity of polypropionates into a plethora of closely related but structurally distinct compounds under the influence of relatively long term environmental stress.

Further, it has been found that siphonarin B is the preferred substrate for modification. This is evidenced by the fact that in the experimental group the quantity of siphonarin B is only 56% of its methyl homolog; in the control group both homologs are present in nearly equal amounts. The explanation for the apparent lack of this ethyl ketal is found by a comparison of methyl to ethyl compounds in each of the daughter, cognate siphonarin pairs. In every pair, there is more of the ethyl compared to its methyl cogener. This varies by as little as a 1.4:1 ratio for the siphonarin G:H pair to as much as a 2.2:1 ratio for the siphonarin I to J ratio. In general the ratio is near 2:1 for the ethyl to methyl homologs resulting in a near 1:1 ratio of total ethyl siphonarins to their total methyl counterparts in the experimental group.

One question that might be raised concerning these results is that only 10% as many of the control individuals were collected as compared to the experimental group. Therefore, if other siphonarin compounds were present in the smaller control group, they may not be observable in the ^1H NMR spectra. But in

defense of the collection techniques of the Cardellina group, it must be pointed out that while 10% of one or three mg may not be visible in the NMR, the collective sum of nearly 35 mg of "product daughter" siphonarins should have become very apparent when the purified siphonarin fraction from the LH-20 column was placed on the ODS HPLC. First, the UV absorbance trace should have shown a number of peaks if there were a near 1:1 ratio of A and B compounds versus daughter metabolites, the trace in Figure 5 shows this not to be the case. Further, the ^1H NMR spectra of these HPLC fractions should have shown a number of spurious signals, none were found. Finally, the near jigsaw accuracy with which the siphonarin quantities complement each other would seem to preclude this type of gross error. A final question that has some validity is that these novel metabolites may be merely artifacts of the collection process. As has been stated earlier, the ketal functionality is very reactive; therefore, it may be argued that during the several hours of confinement, the limpets were exuding various acidic and basic waste products which may have acted as catalysts to open and recyclize the original siphonarin A and B compounds. While this possibility cannot be discounted, a major problem with this hypothesis is why should a purely non-biological reaction as a simple acid or base catalysis favor the ethyl homolog by nearly 2:1 over the methyl? It must be noted that the ethyl versus methyl groups are located far from any reactive carbonyl or hydroxyl centers

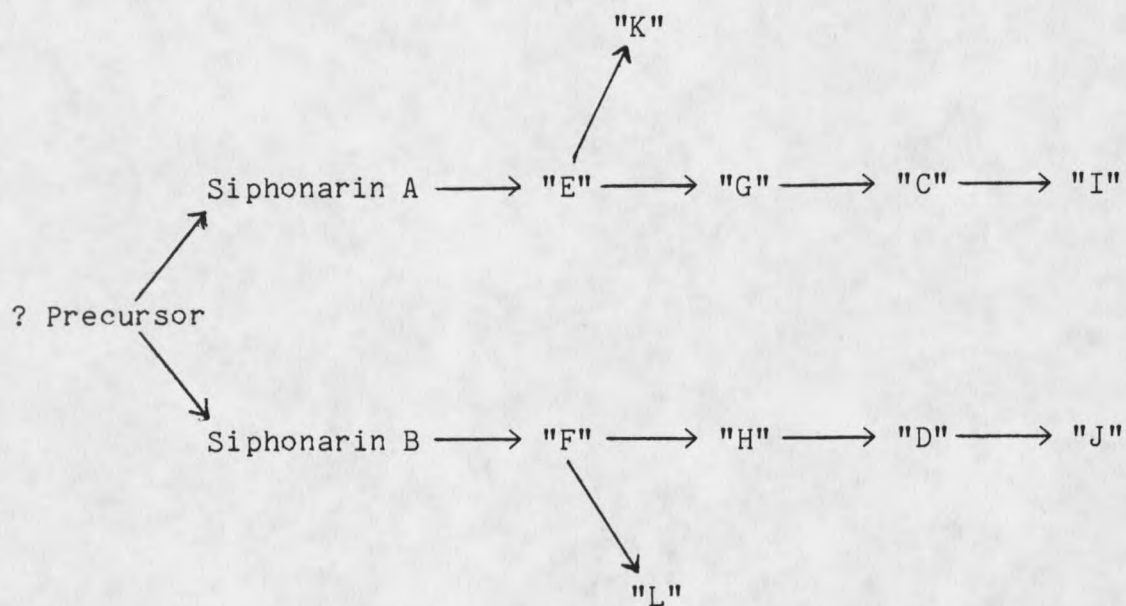
organic reaction in the two parent compounds. On the other hand, the ethyl versus methyl functionalities may function as "markers" for enzymatic activity and thus influence the rate of a biosynthetic reaction.

What are the biological or environmental inferences to be derived from the data? From a biogenic standpoint, there appear to be three possible relationships between these molecules. In all cases, siphonarins A and B are the direct precursors to all the other siphonarins isolated in this study. In the first case, as related by Scheme 1, siphonarins A and B undergo a ring opening with a change in stereochemical configuration about C-4 and C-5 from (+) to (-) as seen in Table 2, to yield siphonarins E and F respectively. Each of these compounds may then undergo a dehydration reaction to yield the K and L compounds respectively; or undergo a recyclization about the -OH of C-11 to the carbonyl C-7 with a further spontaneous cyclization of the now -O⁻ of C-7 to the carbonyl C-3 giving structures G and H respectively. Each of these compounds may then undergo a ring opening with a change in the stereochemistry at C-4 to yield siphonarins C and D which are stereoisomers of their precursor E and F forms respectively. Finally, these may then undergo a recyclization with retention of configuration to yield structures I and J which are stereoisomers of their precursor G and H compounds respectively. The main evidence for this scheme is based on the concept that siphonarins E and F are present in the greatest amounts as seen in Table 3; and therefore should

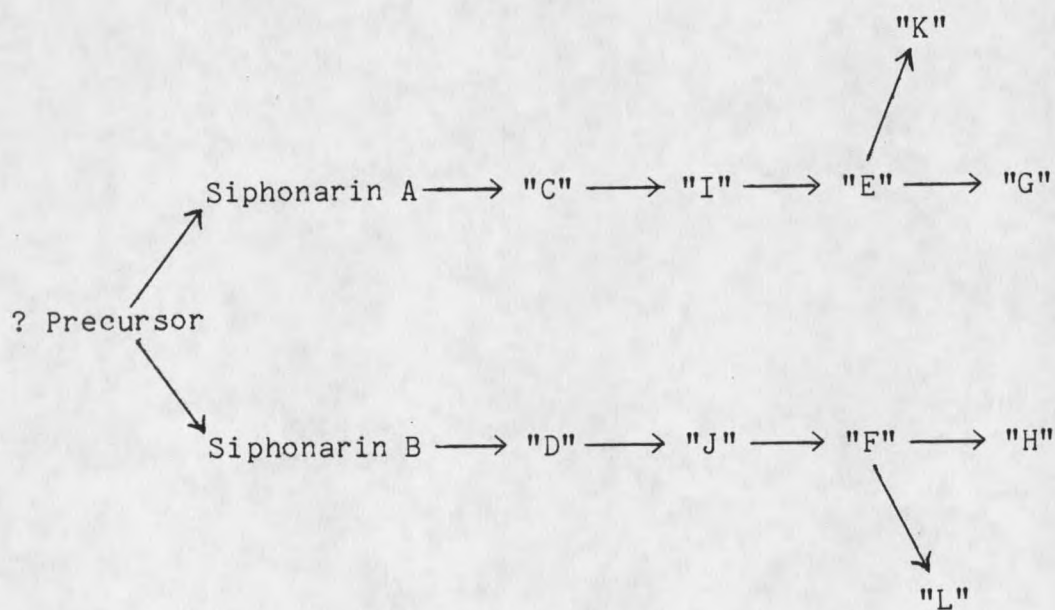
logically be put near the beginning of the metabolic sequence near the most abundant compounds siphonarins A and B.

Scheme 2 illustrates the second possible sequence of events. Here siphonarins A and B open with retention of configuration about C-4 and C-5 to give the C and D forms respectively. The subsequent ring closures and openings in this sequence are analogous to those noted for Scheme 1. The main argument in favor of this metabolic sequence is based not on the abundance of compounds present as in the former case, but on an economy of stereochemical transformations. In this case there is essentially one change in stereochemistry going from the A structure to the end of the sequence; which occurs during the siphonarin I to E transformation. On the other hand, in the former scheme, there are two transformation. In the latter case it might be argued that the E and F forms are present in rather large quantities due to the metabolic buildup of this compound near the end of the chain.

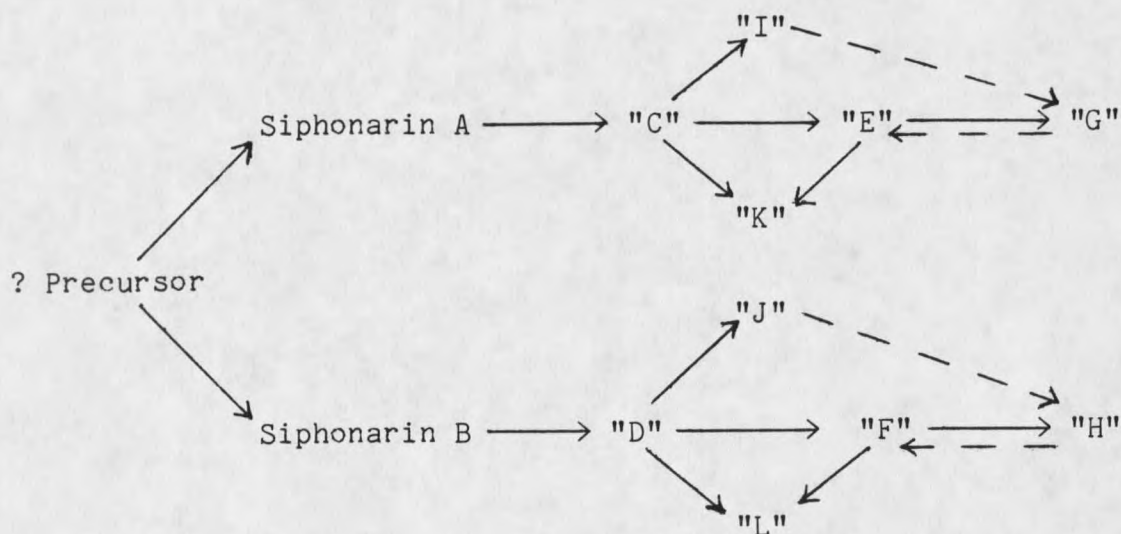
A third and more involved series of events is illustrated in Scheme 3. In this case, siphonarin A opens with retention of configuration to siphonarin C. At this point a number of possibilities arise. This compound may undergo epimerization of the methyl about C-4 allowing inversion of stereochemistry resulting in the formation of structure E. This results in the methyl going from an axial to an equatorial position. Or compound C may undergo recyclization with retention of stereochemistry to form structure I. Finally, C may dehydrate



Scheme 1. Possible biosynthetic sequence based on abundance of each compound.



Scheme 2. Possible biosynthetic sequence based on conservation of stereochemistry.



Scheme 3. Possible biosynthetic pathway based on conservation of stereochemistry and ease of formation of dehydrated forms.

to form the dialkene, siphonarin K which no longer has a chiral center at C-4. Going further along this sequence of events, the cyclic compound I may undergo epimerization at C-4 and invert its stereochemistry but still remain in the cyclic but siphonarin G form. Or siphonarin E may cyclize with retention of stereochemistry to form compound G or vice versa. Finally, open chain form E may undergo dehydration to form compound K. Like Scheme 2, this sequence only requires one change in stereochemistry to go through the chain of events; but this scheme has two additional features. First, it allows for either of the open chain compounds to become dehydrated to form K. Contrary to what Schemes 1 and 2 may imply, there is no data to suggest that only siphonarin E may go to form K. Further, this

scheme may be the most realistic from an ecological point of view. This system allows for several routes along which some of the compounds may be synthesized thereby facilitating rapid molecular interconversion which may be required under environmental stress. A possible method for testing between these three schemes might be found in the analysis of two groups of slowly terminated limpets in which one group is terminated more slowly than the other. In the first case, the more slowly terminated group should give relatively more of the C and I forms while the latter case would predict a buildup of the E and K compounds.

From an ecological view it appears that siphonarins C through L are in fact alarm pheromones or, at the very least, stress metabolites. The fact that siphonarins A and B were present in the control limpet group suggests that these two compounds have a more sedate function, possibly as trail marking pheromones (9). But comparison of the amounts of these compounds in the unstressed versus stressed study groups suggests a second function. That being as a ready source of precursor molecules to be synthesized into alarm or stress signals as environmental conditions warrant.

As noted earlier, it was thought that the limpet response toward stress would be an increase in the amount of siphonarins A and B. But at this point we must conclude that rather than an increase in quantity, it was an increase in quality, as

evidenced by a change in composition, of the metabolites produced by the limpets under stress. From a practical standpoint, this makes sense, because logically it would not be helpful for an organism in a stressed state merely to make more of a compound that it already had in a presumably unstressed state. Another explanation for the increase in kind rather than amount of molecules produced may be found in chemistry and the element of time. If the production of a chemical stress communication is to elicit a protective response on the part of that organism or a neighboring one, then that response must be made rather rapidly. Siphonarins A and B, with their relatively complex polypropionate structures would require a relatively long time period to elapse from DNA translation through the actual production of the required siphonarins. With these requirements and mechanism in mind, it would be far more timely to use pre-existing molecules as the basis for new congeners in which a relatively small number of enzymes would be required to effect such relatively simple changes as ring openings, dehydrations, or stereochemical transformations.

Further, one must realize that in this case the slow killed limpets were under a variety of negative stimuli which included removal from their normal habitat, handling by "alien organisms," and subjugation to hypothermic conditions, only alleviated by final chemical extermination, all occurring under extremely crowded conditions in a closed foreign container. The point is that these organisms were under a number of less than

normal stimuli. If these conditions can be coupled to the theory that a number of isolated siphonarins from this experimental group are in fact end products of a biochemical pathway, and that nature does not make molecules in a haphazard manner, then it can be argued that each of these compounds, especially structures C through L, is a finely tuned phrase of chemical communication. That is, one of the compounds may be interpreted by the limpet as "it is cold" or another as "I'm being bothered," etc.

While that last statement may be stretching the interpretative bounds of the data, the major conclusion remains, that being, under stress S. alternata produces a variety of secondary metabolites having siphonarins A and B as their biogenic precursors.

Eudistoma olivaceum

The β -carboline s comprise a large molecular subgroup from the widespread alkaloid family. The β -carboline s themselves have been found in a wide variety of organisms as well as functions (49, 50). Two forms of β -carboline s related to our research, the piperidine and pyridoindole s, have been found in organisms ranging from plants to mammals. These two forms have equally diverse functions, including inhibition of cancer DNA synthesis by a group of pyridinium β -carboline s (51); and cAMP phosphodiesterase inhibition by the pyridoindole s (52). Also,

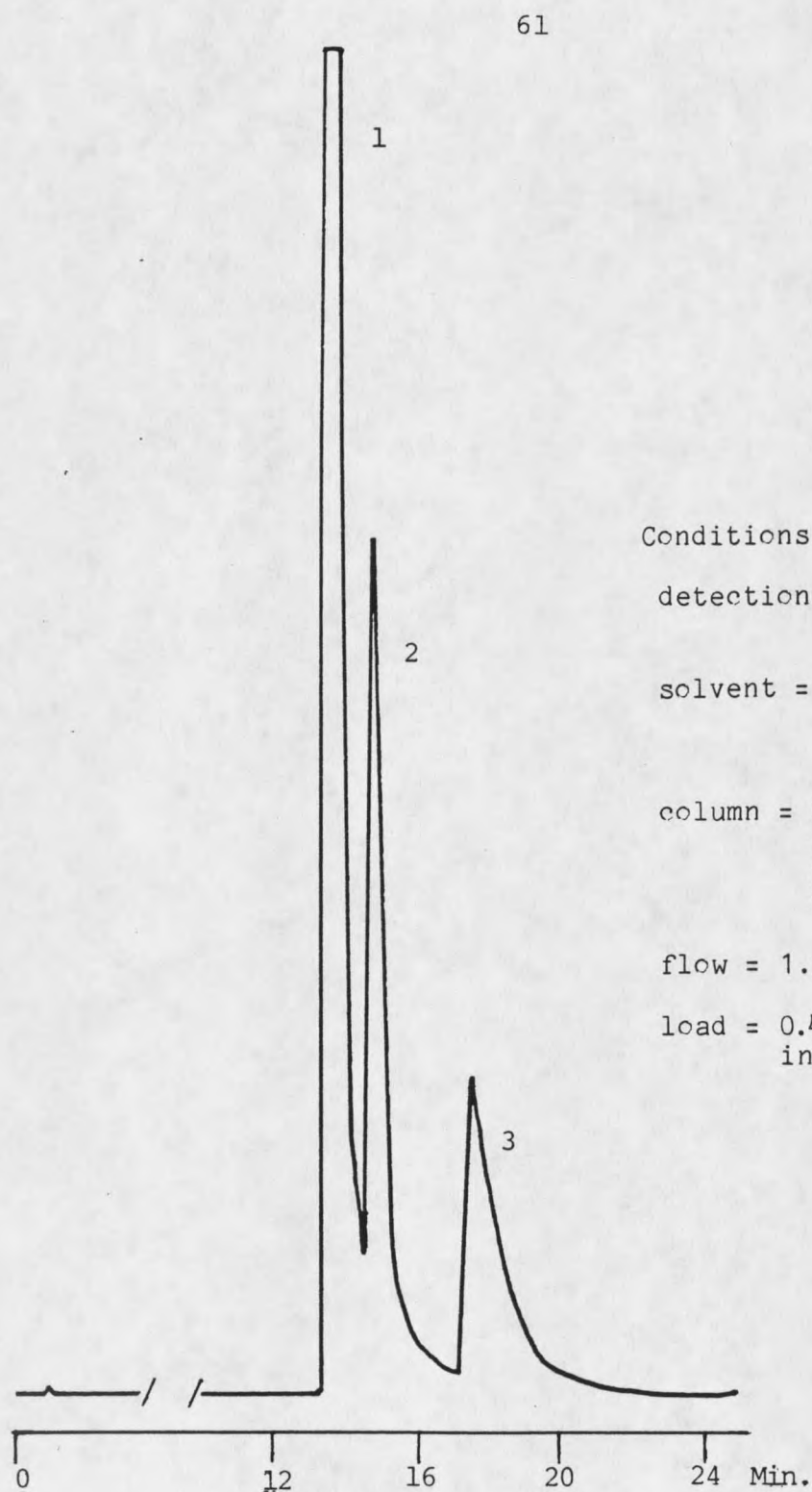
the piperidine and pyridocarboline Harmala alkaloids owe their nerve depressant (53), hallucinogenic, and cytotoxic activity to their ability to act as reversible inhibitors of monoamine oxidase (54). The carboline containing extracts from the marine chordate, E. olivaceum, are essentially pyridoindole in nature, and it is these compounds that have been the focus of our attention in this portion of the project.

The genus Eudistoma consists of rock encrusting tunicates that inhabit shallow marine waters. Within the last several years, these organisms have been the source of a number of novel, biologically active β -carboline derivatives (12, 13, 55, 56). These secondary metabolites, collectively known as eudistomins or eudistomidins, contain an indole moiety with possible Br or OH substitution to the benzene ring, while a piperidine or pyridine ring is fused to the pyrrole ring. Further, many of the eudistomins exist as tetracycles in which the one and two positions of the piperidine are fused to an oxathiazepine ring (12), or a pyrrole or pyrrolidine ring may be attached to the pyridine heterocycle (13). The possible utility of these compounds for human use has been demonstrated by Rinehart, who has recently patented two of the pyrrolyl compounds, eudistomins A and M, as antiviral agents (15).

Isolation of Eudistomins G, H, I, and P. The emphasis of our research was to define a method to separate cleanly the various β -carbolines found in E. olivaceum. The methylene

chloride solubles of the extracted ascidian had been previously reduced to β -carboline enriched fractions through a series of gravity flow columns by the Cardellina group (57). Because the ODS conditions used by Rinehart (13) did not work on our fractions for the final separation procedure, the search for an alternative procedure was pursued. It was found that a normal phase propylamine HPLC system would afford a reasonable separation of the various eudistomin isomers. Most of the gravity column fractions contained mixtures of eudistomins G, 20, H, 21, and I, 22. Figure 12 is representative of the separation obtained by this system. Ultimately it was determined that the total of these three compounds resulted in a respective ratio of 1:11:4.

The isolation of eudistomin P was due as much to a stroke of serendipity as to any skill in separation technique. Three β -carboline containing fractions were put through the normal phase column. One of these contained some eudistomin H, another contained two previously undefined carbolines; while a third eluted only small amounts of UV absorbing compounds. After these 90:10 methylene chloride:hexane runs, the column was given a routine 100% acetonitrile flush. It was at this time that close to two milligrams of an intensely UV absorbing compound came off the column. Upon rerunning this fraction in a 100% acetonitrile propylamine mode, a quantity of compound equivalent to one-third that of eudistomin G was obtained. That compound proved to be eudistomin P, 23.



Conditions:

detection = Beckman 160
set at 254nm

solvent = methylene
chloride:hexane
(9:1)

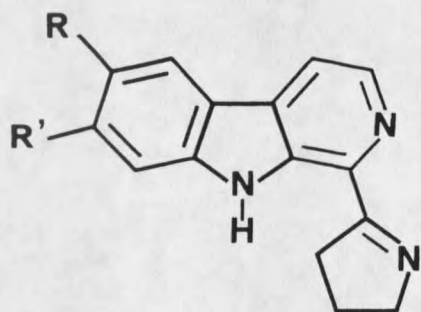
column = Chemcosorb,
analytical
propylamine 0.46
x 25 cm

flow = 1.3 ml/min

load = 0.4 mg/15 μ l
injection

Fig. 12 UV absorbance trace for normal phase HPLC separation of Eudistomins H (1), G (2), and I (3).

Because Cardellina had previously isolated and elucidated the structure of eudistomin H (55), and all of these compounds had been isolated by the Rinehart group (13), the identification of these four compounds was guided largely by the comparison of spectral data, especially ^1H NMR data as can be seen in Table 4.



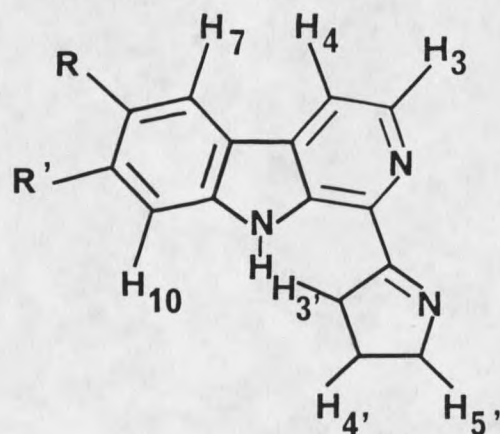
20 $\text{R} = \text{H}, \text{R}' = \text{Br}$

21 $\text{R} = \text{Br}, \text{R}' = \text{H}$

22 $\text{R} = \text{R}' = \text{H}$

23 $\text{R} = \text{OH}, \text{R}' = \text{Br}$

Structure Determination of Eudistomins R, S, and T. Proton NMR spectra were taken on what were thought were routine β -carboline eluent fractions from the normal phase propylamine HPLC system. One glance at the NMR spectrum Figure 13, proved otherwise. The second and third fractions of a five fraction run contained compounds that showed pyridoindole carboline signals in the 7.4 to 8.5 ppm range roughly corresponding to those of the four known eudistomins. But while the other eudistomins had sets of signals at 2.1, 3.3, and 4.3 ppm corresponding to their pyrrolidine ring systems, these three compounds had no proton signals above 4.7 ppm. This meant that these three substances had to be almost completely aromatic or heteroaromatic in nature. The intense signal at 4.72 ppm was



Proton(s)	Eudistomin			
	G	H	I	P
H ₃	8.51,d (5.2)	8.51,d (5.3)	8.49,d (5.2)	8.47,d (5.2)
H ₄	7.96,d (5.0)	7.95,d (5.2)	7.99,d (5.2)	7.93,d (5.2)
H ₇	7.98,d (8.3)	8.26,d (1.8)	8.13,d (7.6)	7.74,s
R	7.39,dd (8.5,1.8)	Br	7.28,dd (8.2,2.8)	OH
R'	Br	7.63,dd (8.6,1.8)	7.55,dd (2.8,2.1)	Br
H ₁₀	7.73,d (1.6)	7.46,d (8.6)	7.55,d (2.8)	7.74,s
N-H	9.08,s	9.02,s	9.10,s	9.10,s
H _{3'}	3.28,m	3.33,m	3.32,m	3.34,m
H _{4'}	2.08,m	2.09,m	2.07,m	2.09,m
H _{5'}	4.26,m	4.26,m	4.26,m	4.26,m

Table 4. ¹H NMR data for eudistomins. Units as δ ppm, multiplicity, (J=Hz) in CDCl₃.

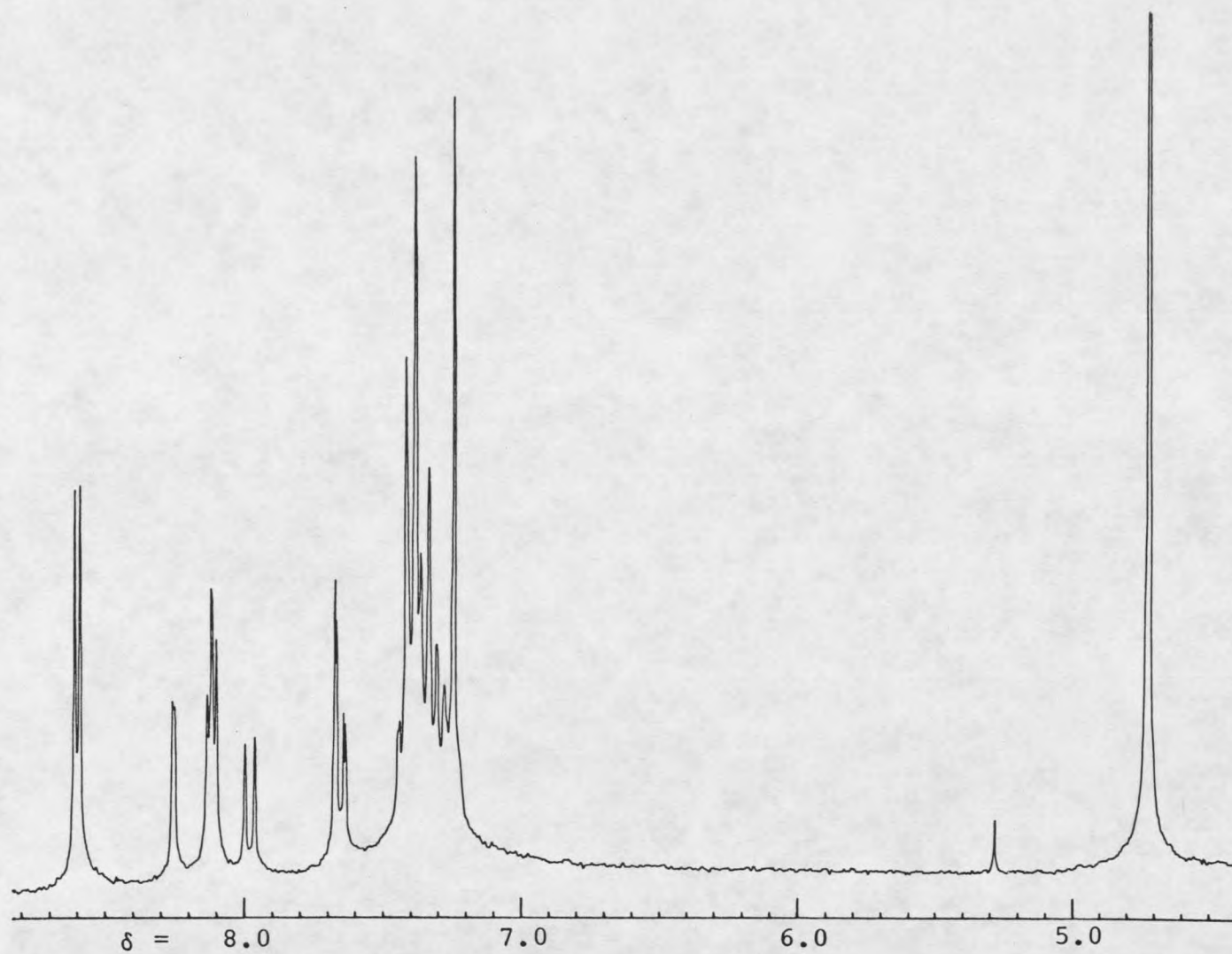


Figure 13. ^1H NMR of eudistomins R and S mixture in CDCl_3 .

initially assigned to a pyridinium methyl group. This assignment was based on our previous experience with the homarine project as well as the rather widespread occurrence of pyridinium β -carbolines in nature (58). But because the number of protons actually represented in the 4.72 ppm peak was suspect doubt was cast upon this structure. Further, these three compounds eluted from a 90:10 methylene chloride:hexane solvent system like eudistomins G, H, and I, but the more polar, OH containing, eudistomin P did not. Because an ionic pyridinium group should have acted more like eudistomin P than like the others, the pyridinium hypothesis became rather improbable. Further, while eudistomin H, the most rapidly eluting compound up to this point, came off the propylamine system in 13 minutes, the first "new" eudistomin containing fraction eluted in 5 minutes. This indicated a major difference in functionality of these new compounds compared to the known eudistomins. At this point, work concentrated on the low field ^1H NMR signals which were analyzed in conjunction with mass spectral data.

It was found that running the ^1H NMR sample of these novel eudistomins in 90:10 $\text{CDCl}_3:\text{CD}_3\text{OD}$ greatly aided the elucidation process by the spreading out of several overlapping signals, Figure 14. Decoupling experiments showed that these compounds did indeed have the basic pyridoindole skeleton common to the other known eudistomins. These experiments as well as an apparent inconsistency in the ^1H NMR integration values led us to suspect that the first, major eudistomin containing fraction

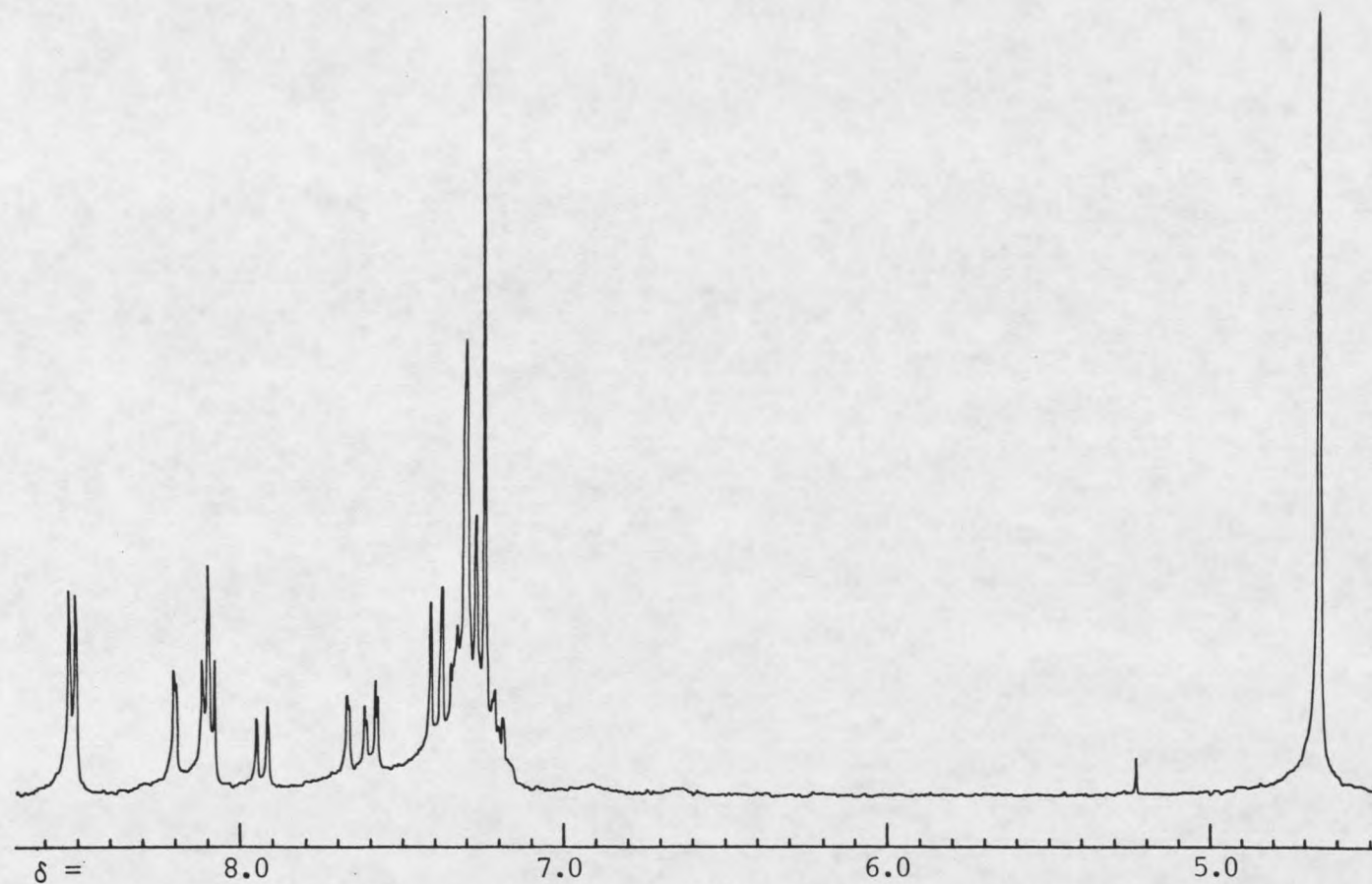


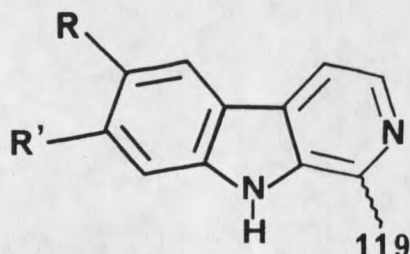
Figure 14. ^1H NMR of eudistomins R and S mixture in 90:10 $\text{CDCl}_3:\text{CD}_3\text{OD}$.

did not contain one but had two carboline compounds of a near 1:1 ratio. As an example, the doublet at 8.6 ppm, Figure 13, had an intensity value two times that of the signals at 8.2 and 8.0 ppm; therefore, the protons corresponding to these signals must be in a 2:1:1 ratio. With these data, the signal at 8.6 ppm can be interpreted as two similar protons on the same compound or two similar protons on different compounds. Knowing that these protons were on the pyridine group of the basic pyridoindole skeleton, made the former possibility very unlikely. Finally, comparison of the proton NMR spectrum for this fraction done in CDCl_3 to those of eudistomins G and H showed that this fraction was in fact a near 1:1 mixture of eudistomin G and H analogs. At this point, mass spectral data played a key role in the structure determination of these three compounds. The two compounds from the first β -carboline containing fraction were indistinguishable by MS(EI). This was to be expected, since the mass spectra of eudistomin G versus H were essentially the same. Here compounds R and S had a molecular ion m/z of 364/366 at 100% and 96% of base height respectively. This indicated the presence of one Br in these compounds and therefore lent further evidence toward the eudistomin G and H similarity. Ultimate proof for the pyridoindole molecular portion was found in the 245/247 fragment. This corresponded to a loss of a 119 mw particle from the molecular ion resulting in the fragment given in part structures 24a and 24b. Location of the Br on C-9 versus C-8 in

24 a $R = H, R' = Br$

24 b $R = Br, R' = H$

24 c $R = R' = H$

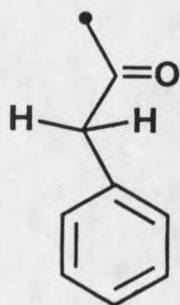


eudistomins R and S respectively was based 1H NMR decoupling as well as spectral comparisons to eudistomins G and H.

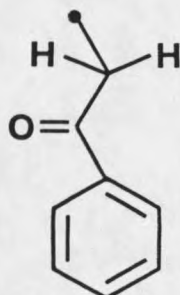
Based on GC-MS, the second carboline containing fraction was shown to contain roughly a 4:1 mixture of eudistomin T to a combination of eudistomins R and S. Eudistomin T had a molecular weight of 286. This corresponds to structure 24c which is like its R and S homologs but without the bromine. Further, this compound showed a major fragment at 167, the base peak. This corresponded to the 245/247 fragment of eudistomins R and S in which all lost the somewhat less stable 119 mw particle.

With the basic pyridoindole nature of these compounds established, attention focused on the common 119 molecular weight fragment. Based on HRMS(EI), this fragment correlated to a molecular formula of C_8H_7O . This along with the fact that all but two of the protons associated with this fragment were found as overlapping multiplets in the 7.45 and 7.27 ppm range gave strong indications of a benzene type moiety. A final piece of

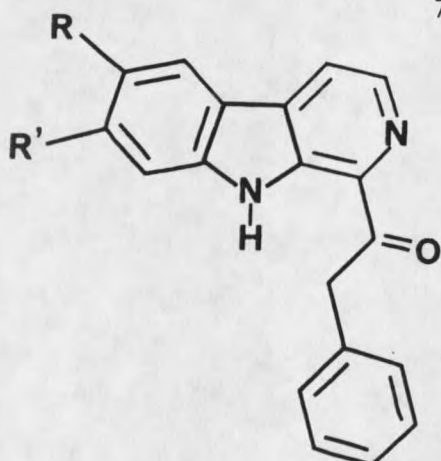
the puzzle was given by IR. While the IR spectra of these compounds was nearly identical to those of the other eudistomins, these compounds showed a major peak at 1669 cm^{-1} which was absent from all the other spectra. This shift is near the range for an aryl ketone. With this information, it became apparent that the proton NMR shift at 4.72 could be assigned to a methylene group adjacent to an aryl as well as a carbonyl functionality giving rise to part structures 25a or 25b. The carbonyl shift is $10\text{--}15\text{ cm}^{-1}$ below the shift expected of a typical aryl ketone. Further, HRMS showed a major fragment with a weight of 91. This corresponds to a tropylium ion. Both the IR and MS data pointed toward the placement of the carbonyl group adjacent to the pyridoindole group, therefore placing the methylene group nearer the benzene ring giving rise to structures 26 through 28 for eudistomins R, S, and T respectively.



25a



25b



26 $R = H, R' = Br$

27 $R = Br, R' = H$

28 $R = R' = H$

The fact that these structures exist is not surprising based upon their relatively simple mode of biogenesis starting from two common amino acids. The indole portion is merely a tryptophan while the 119 mw moiety was a phenylalanine residue. In all likelihood these two molecules were combined along the shikimic acid pathway where phenylpyruvic acid, a phenylalanine precursor was combined via a Mannich reaction involving condensation with tryptamine, a decarboxylated tryptophan (59) to yield a α -toluidine pyridoindole structure assigned to the three novel eudistomins. The pyrrolidine eudistomines are also synthesized via the same route but in this case the pyrrolidine group was most likely obtained via the diamino acid ornithine in an initial sequence similar to that involved in the synthesis of the tropane alkaloids (60). So, from E. olivaceum, a number of compounds have been isolated and characterized by various groups, including our own. Further, the biogenesis of these compounds is at least nominally understood. But one major question remains in this project: of what ecological value are these compounds to this organism?

We have come full circle. At the beginning of this thesis it was pointed out that the major emphasis of this project was to determine methods of separation as well as the ecological implications of secondary metabolites from several marine organisms. To an extent these objectives have been accomplished. In the P. gurneyi project, homarine and trigonelline were isolated and characterized. The ecological value of these isolated aromatic betaines was reviewed largely based on previous work done by a number of other investigators over the years as well as some pharmacological screens run in our laboratory. The ecological value of the siphonarin compounds isolated in the S. alternata part of the research was obtained almost entirely via inferences based upon chemical structures and their most likely scheme of biological synthesis. To wrap up our project, a number of known as well as novel compounds were isolated from E. olivaceum. Like the other studies preceding it, we were successful in developing a method of isolation for closely related compounds. But unlike the other work, there were no answers, at least as far as the question of ecology was concerned. But even this lack of information adds more evidence to an already established fact. That point being, especially in the world of natural products, the important answers one thinks he/she has are merely one small part in understanding the ultimate question.

What is life all about?

SUMMARY

During work on this thesis a number of objectives have been accomplished. These include development of separation techniques, identification of known compounds, as well as structure elucidation of novel compounds. Also the ecological significance of these compounds was assessed using both previously reported data and conclusions as well as inferences based on our data.

In the P. gurneyi project, two previously identified compounds, homarine and trigonelline were isolated, identified, and their possible biological significance reviewed. These two compounds were separated from other water soluble compounds through the use of a C₁₈ reverse phase system followed by Sephadex LH-20. Homarine and trigonelline were separated from each other through the use of a reverse phase, propylamine HPLC system. This separation scheme, especially the use of the propylamine column, has not been cited in the literature. Based on a search of the literature as well as our bioassay results, it was concluded that these two compounds apparently act as storage molecules. Demethylation of homarine and trigonelline gives rise to picolinic acid and nicotinic acid respectively. Both of these products are known to be toxic, at least in plants; therefore, these products may be used by the sea pen as

antifouling agents. Further, work by other groups has shown homarine to be a methylating agent which includes the formation of betaine. Because glycine betaine has been shown to have an osmoregulatory role, it has been surmised that homarine may therefore have an indirect function in osmoregulation.

In the project involving Siphonaria alternata, the results were far greater in scope than in the work on the sea pen both in terms of number compounds isolated and identified as well as determination of the ecological significance of these compounds to their mollusk progenitors. In this phase of our work, twelve related polypropionate compounds were isolated and identified. Three of these, siphonarins A, B, and F had been identified by our or other groups. Of the remaining nine novel compounds, siphonarins C, D, and E were shown to be open chain isomers of compounds A and B, while siphonarins G through J were found to be rearranged cyclic ketals based on an opposite ring closure from open chain compounds relative to the A and B forms. The final two compounds, siphonarins K and L, were determined to be dialkenes and therefore dehydration products, most likely from the open chain forms. In these compounds, structure determination and stereochemistry were based primarily on ^1H NMR, MS and optical rotation spectral data. The methods used to separate these compounds included a series of gel permeation systems followed by HPLC. It was a reverse phase, ODS HPLC system that allowed a clean separation of siphonarins A and B. Final separation of siphonarins C through J was done on a silica

HPLC system. Based on a comparison of masses of siphonarins isolated from control versus experimental limpet groups it was determined that siphonarins A and B act as ready precursors to siphonarins C through L when a stressful environmental condition arises. In other words, evidence has been accumulated to show that under stress stimuli, S. alternata will produce a number of metabolites which apparently have siphonarins A and B as their biogenic precursors.

In the final phase of our work, a total of seven compounds were isolated from the tunicate Eudistoma olivaceum. The separation of these compounds was effected on a normal phase, propylamine HPLC system which proved to be an improvement over procedures developed by other groups to separate these eudistomin compounds. In this project, four of the compounds isolated, eudistomins G, H, I and P, were previously identified by our or the Rinehart group. But three of the compounds, eudistomins R, S, and T proved to be novel. In these compounds, the pyrrolidine ring has been replaced by an α -toluidone group.

While a number of problems have been solved and structures identified, numerous questions remain or have been posed based on the new data obtained. Some of these questions include: if homarine and trigonelline are acting as storage forms for picolinic and nicotinic acids in the sea pen, then why haven't these acids been found in this organism? In the Siphonaria project, a number of questions remain. One question centers

around the stereochemical configuration of siphonarins C through J. Here, the problem is, can a change in optical rotation be translated to a change in a specific site of chirality? In the problems dealing with the ecology of the Siphonaria a major question revolves about the fact that we do not really understand which kind of stimulus causes the production of which new siphonarin product. In dealing with E. olivaceum, one basic question that might be asked is what are the functions of all these β -carboline in this organism? The bottom line in this discussion is that this thesis, like all works in science, has attempted to answer some questions; but it also has caused new ones to be asked. The ultimate value of this work can only be measured by how many of these questions will be used to start future projects by other investigators.

EXPERIMENTAL

Ptilosarcus gurneyi

Materials.

Three different columns were used for the separation and isolation of homarine and trigonelline. First used was a 2.5 x 41 cm reverse phase flash column with octadecylsilane packing by J.T. Baker Chemical Co. Next used was a 1.7 x 125 cm Sephadex LH-20 column with a particle size of 25-100 from Pharmacia Inc. propylamine, 7-CH (NH₂), column by Chemco Scientific Co. Ltd. The first two systems were monitored by an ISCO V₄ absorbance detector operating at 254 nm. The last column was used with an HPLC apparatus which included a Beckman 112 solvent delivery system and a Beckman 160 absorbance detector set at 254 nm.

Characterization was done on a number of systems. UV spectra were recorded on a Carey 14 Spectrophotometer; and data reported in nm. The IR spectra were measured on a Nicolet Analytical Instruments FT IR; data were reported in cm⁻¹. Mass spectra were obtained through use of VG Instruments 7070E-HF spectrometer. NMR spectra were obtained on a Bruker WM-250 FT NMR using CD₃OD as solvent and internal standard; data are reported as δ units (ppm) relative to TMS ($\delta = 0$).

Collection and extraction of P. gurneyi. This coelenterate was collected on the Pacific coast near Sidney, British

Columbia, Canada, on a sandy bottom, in a strong current, at a depth of 7m in March 1985. At the time of collection, the specimens appeared to be free of any overgrowth or macroscopic diatomaceous fouling.

P. gurneyi individuals (114.7g dry weight) were placed in acetone for 4 days, at which time the acetone was removed by filtration. This was followed by grinding with methanol in a Waring blender. After pulverizing, the methanol was removed by suction filtration and combined with the acetone fraction. This solution was then reduced in volume under vacuum to an aqueous suspension. The remaining solids were steeped in dichloromethane two times for 16 and 4 hr. periods, respectively. Then the aqueous and dichloromethane extracts were combined and partitioned. The organic phase was evaporated under vacuum to yield 8.78 g (7.7% dry weight) while the aqueous phase was lyophilized to give 18.38 g (16% dry weight). Later, the aqueous portion underwent a desalting procedure which consisted of two 150 ml filtration extractions with 4:1 methanol:acetonitrile. The resultant filtrate was reduced to dryness under vacuum to yield 7.61 g (6.6% dry weight). The remaining solids of this procedure were shown to be salts and a small amount of small, saturated organic molecules indicated by the presence of few, weak proton signals in the ^1H NMR spectrum in general and the lack of hydrogen signals below 4 ppm.

Isolation. The desalted, lyophilized, filtrate was chromatographed on a series of three columns. A summary of

these columns may be found in Table 5. The material was first chromatographed in a series of three to five 150 mg runs on an ODS reverse phase column removing 75-90% of the inorganic salts and small organics. The combined contents from the three to five previous runs on the C₁₈ column were then chromatographed on an LH-20 column removing several aromatic, possibly amino acid, type compounds. Finally, the third fraction from the LH-20 column was chromatographed on a propylamine reverse phase HPLC system. Figure 15 gives evidence that homarine and trigonelline were separated in pure form.

Characterization.

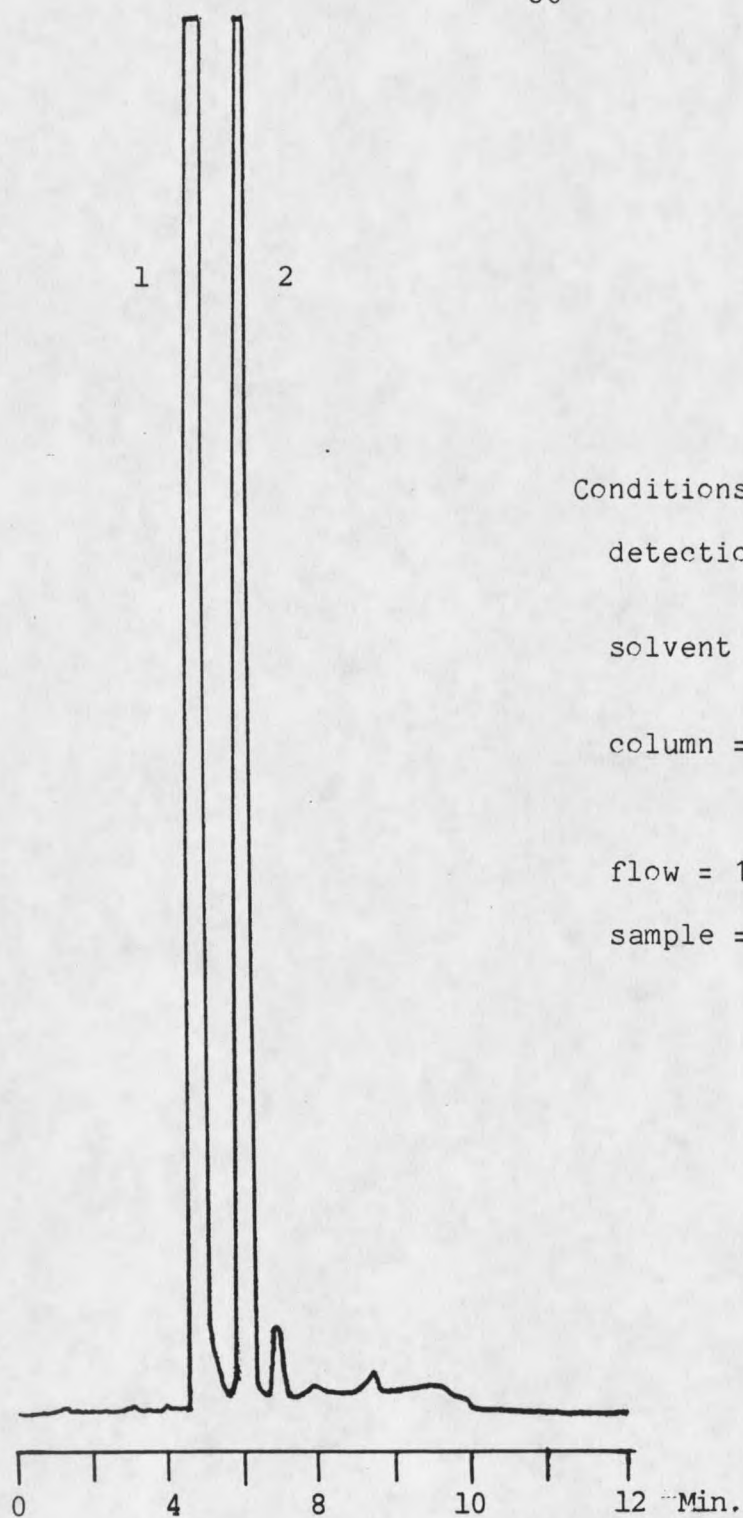
Homarine: A colorless crystalline glass, UV: $\lambda_{\max}^{\text{CH}_3\text{OH}}$, 273nm, ($\epsilon=5900$), $\lambda_{\min}$ 239nm; IR: $\nu_{\max}$ 3394, 1665, 1636, 1495, 1453, 1358, 1155, cm^{-1} ; MS (FAB): m/z 138 (MH^+), 94; ^1H NMR (CD_3OD): $\delta=4.44$ (3H, s) 7.94 (1H; dd, $J=6.5, 7.3$) 8.08 (1H, d, $J=7.1$) 8.52 (1H, dd, $J=7.4, 8.3$) 8.76 (1H, d, $H=6.1$); ^{13}C NMR (D_2O): $\delta=46.54$ (q), 124.83 (d), 127.08 (d), 145.44 (d), 146.28 (d), 152.10 (s), 165.78 (s).

Trigonelline: A colorless crystalline glass, UV: $\lambda_{\max}^{\text{CH}_3\text{OH}}$, 265nm ($\epsilon=3200$), 271nm (shoulder), $\lambda_{\min}^{\text{CH}_3\text{OH}}$, 240nm; IR: $\nu_{\max}^{\text{CH}_2\text{Cl}_2}$, 3045, 2973, 1652, 1421, 1346, 1249, 1018 cm^{-1} ; MS (FAB): m/z 138 (MH^+) 94; ^1H NMR (CD_3OD): $\delta=4.43$ (3H, s) 8.05 (1H, dd, $J=6.7, 7.0$) 8.87 (two 1H overlapping doublets) 9.20 (1H, s); ^{13}C NMR (D_2O): $\delta=48.66$ (q), 128.07 (d), 137.41 (s), 145.19 (d), 146.38 (2C, d), 168.12 (s).

Column	Eluent	Total frac. from column	Frac. containing structures 1 and 2	% of initial ^a dry weight
1 st : ODS rev. phase flash	CH ₃ OH:H ₂ O (85:15)	3	2	0.8
2 nd : Sephadex LH-20	CH ₃ OH:CH ₃ CN (4:1)	6	3	0.5
3 rd : Propylamine rev. phase HPLC	CH ₃ CN:H ₂ O (7:3)	7	2 (homarine) 4 (trigonelline)	0.4 0.04

Table 5. Isolation sequence of homarine and trigonelline.

^aTotal yield of fraction containing target molecules relative to the initial 114.7 g dry weight of P. gurneyi.



Conditions:

detection = Beckman 160
set at 254 nm

solvent = acetonitrile:water
(7:3)

column = Chemco, analytical,
propylamine,
.46 x 25 cm

flow = 1.3 ml/min

sample = 2.0 mg/20 μ l
injection

Figure 15. Uv absorbance trace for final HPLC separation of homarine (1) from trigonelline (2)

Preparation of homarine: Picolinic acid, 200 mg in 6 ml 1:1 methanol:acetonitrile was treated with 0.25 ml iodomethane at 50°C and refluxed for 24 hrs. Then the reaction mixture was brought to pH 7 with several drops 10% Na₂CO₃. This mixture was reduced to dryness under vacuum and chromatographed on a Sephadex LH-20 (1.7 x 139 cm) column, eluting with methanol:acetonitrile (4:1) giving six fractions. The second fraction was chromatographed on a polyacrylamide, Bio Gel P-2 flash (2.5 x 30 cm) column. Based on ¹H NMR, UV, and MS comparisons, fraction 3 (46 mg) proved to be homarine (21%).

Preparation of trigonelline: This was done by essentially the same procedure as that for the production of homarine. But here 300 mg of nicotinic acid was reacted with 0.40 ml iodomethane at 60°C for 18 hours resulting in 302 mg of trigonelline (89% yield).

Siphonaria alternata

Materials:

The isolation of the target polypropionate metabolites was achieved on a series of chromatographic columns. The first was a 1.8 x 131 cm column with Sephadex LH-20 packing of a 25-100 particle size from Pharmacia Inc. Next used was a BioBeads S-X4, 4.1 x 90 cm column with a particle size of 200-400 mesh from Bio Rad Laboratories. The third column used was another with Sephadex LH-20 packing, but the column size was 2.4 x 184 cm. A

final gravity flow column used on only the extract of the slow terminated limpets was a 2.5 x 144 cm reverse phase column utilizing octadecysilyl packing from J.T. Baker Chemical Co. The first HPLC column used on both extracts was a preparative 1.0 x 25 cm Beckman Altex octadecylsilyl column. A final HPLC column used on several fractions from the slow killed limpets was a 0.46 x 1.0 cm Alltech analytical silica column.

The gravity flow columns were monitored by an ISCO-V₄ absorbance detector operating at 254 nm. The HPLC columns were run with a Beckman 112 HPLC solvent delivery system and a Beckman 160 absorbance detector set at 254 nm.

Characterization was done by UV, IR, MS, and NMR using the same instruments as were used in the P. gurneyi portion of this project. In addition, optical rotations were taken using a Perkin-Elmer 241 MC Polarimeter.

Collection and Extraction: As was the case with P. gurneyi, the collection and extraction of S. alternata was done by other members of our group.

All specimens were collected from Devanshire Bay, Bermuda on July 16, 1984. Collection was done in two groups. The first group consisted of 50 limpets with a fresh weight of 24.43 g. This group was rapidly terminated by immediate placement in acetone upon collection. The second group consisted of 524 individuals with a fresh weight of 348.7 g. This group was slowly terminated by allowing them to remain together in the

collection container for several hours, then slowly frozen, after which they were steeped in acetone. Both sets of mollusks remained in acetone, at below 0°C for approximately eight weeks. At this time, both groups were filtered whole, and washed two additional times in acetone. The acetone was then evaporated to a water suspension which was partitioned with methylene chloride. The organic soluble fractions were evaporated under vacuum to dryness yielding a total of 78.16 mg or 1.56 mg per limpet for the fast killed group; while the slow killed group gave a total of 617.20 mg or 1.18 mg organics per limpet. The water soluble fractions were lypholyzed giving 6.28 mg per limpet for a total of 314.00 mg of this material from the quick killed group; while 11.64 mg per organism was obtained from the slow killed limpets yielding a total of 6.098 g of aqueous soluble material from this group.

Of the 617.20 mg organic soluble extract from the slow killed group, 34.0 mg or 5.50% was removed for assays thereby effectively reducing the amount of organic extracts actually chromatographed by 5.50%. Therefore, the number of individuals in the slow killed group whose constituents were actually separated was reduced by 28.8 organisms leaving the actual number of limpets chromatographed at 495. It is this adjusted S. alternata count for the experimental group that was used later for siphonarin yield per limpet calculations.

Isolation of siphonarins A and B. Because these compounds had been previously isolated and identified by our group, all isolation procedures were guided by ^1H NMR spectra using the known data from following these compounds through the separation process.

The organic soluble extracts of both limpet groups underwent most of the same separation procedures resulting in the isolation of a number of polypropionate compounds. Both organic extracts were chromatographed on Sephadex LH-20, followed by BioBeads S-X4 which in turn was followed by chromatography on another LH-20 column using a more polar solvent than the first LH-20 system. For solvent and compound elution details see Table 6. While the siphonarin containing fraction of the fast killed control group from this third separation step was relatively pure the analogous fraction from the slow killed experimental group had an unacceptably high amount of other compounds mixed in with the target siphonarins A and B. Therefore, the 83.7 mg second fraction from the LH-20 column was subjected to chromatography on an ODS reverse phase, gravity flow column. The result was a reduction in this original fraction to 34.0 mg of a reasonably pure siphonarin A and B mixture which eluted as the fifth and sixth fractions.

The final step in the separation of the polypropionate A and B compounds from each other as well as non-target impurity compounds was achieved by the use of an ODS reverse phase HPLC system. See Figure 5 for details. The result was the isolation

Column	Solvent	Pure siphonarins isolated
Sephadex LH-20	methylene chloride:methyl-t- ether:isopropanol (1:1:1)	----
Biobeads S-X4	hexane:methylene chloride: ethyl-acetate (4:3:1)	----
Sephadex LH-20	methanol:methylene chloride (1:1)	----
ODS rev phase	acetonitrile (100%)	----
ODS rev. phase-HPLC	acetonitrile:water (3:2)	A,B K,L
Silica HPLC	methyl-t-butyl ether;methylene chloride (3:1)	C,D E,F G,H I,J

Table 6. The systems used, in order, to separate the various siphonarin compounds.

of 14.15 mg of siphonarin A and 7.90 mg of siphonarin B from the slow killed group; while the ten times small group accounted for 2.30 mg and 2.40 mg of siphonarins A and B respectively.

Isolation of siphonarins C through J: These metabolites coeluted with siphonarins A and B on the first Sephadex LH-20 column, the BioBeads S-X4 column, and the second LH-20 column. Separation of these open chain and cyclized polypropionates from the two parent cyclic ketal forms occurred on the gravity flow ODS reverse phase column where siphonarins C through J eluted in the second fraction. This fraction was then run on an ODS reverse phase HPLC system where siphonarins G and I coeluted in the second fraction. The C, F, H, and J compounds came off in the fourth fraction; while the D and E structures coeluted in the eighth of nine fractions. Each of these fractions was then separated to individual compounds via normal phase silica HPLC. Refer to Tables 3 and 5 for solvent details and yields.

Isolation of siphonarins K and L: The isolation process involved with siphonarins K and L is essentially the same as that employed on their siphonarin A and B congeners from the experimental group. All four of these polypropionates coeluted on the first two columns. The separation of the parent compounds from the open chain, dehydrated forms occurred during elution from the third column, a Sephadex LH-20 column, where siphonarins A and B eluted in the second fraction, while siphonarins K and L eluted as a third fraction. This fraction

was essentially a shoulder on the UV absorption trace for the elution of the siphonarin A and B band. Later, this third fraction was chromatographed on the same ODS HPLC system used on the A and B compounds, yielding 1.1 mg of siphonarin K and 2.0 mg of siphonarin L.

Characterization. For all optical rotation values see Table 2.

Siphonarins A and B: essentially the same as that reported by the Faulkner group (10) with the exception of the UV data.

Siphonarin A: UV: $\lambda_{\text{max}}^{\text{CH}_3\text{OH}}$, 259nm ($\epsilon=12,700$).

Siphonarin B: UV: $\lambda_{\text{max}}^{\text{CH}_3\text{OH}}$, 259nm ($\epsilon=10,400$).

Siphonarin C: UV: $\lambda_{\text{max}}^{\text{CH}_3\text{OH}}$, 260nm ($\epsilon=10,400$); IR: $\nu_{\text{max}}^{\text{CCl}_4}$, 3505, 2932, 2882, 1658, 1618, 1455, 1381 cm^{-1} ; HRMS(EI): obs m/z 506.2886, $\text{C}_{28}\text{H}_{42}\text{O}_8$ calc. 506.2880; ^1H NMR (CDCl_3): δ = 5.46 (1H, dd, $J=9.0$, 3.6) 4.15, (1H, q, $J=7.1$) 3.61 (1H, dd, $J=7.7$, 4.4) 3.17 (2H, brs) 2.89 (2 1H, m) 2.85 (1H, dq, $J=7.1$, 3.3) 2.73 (1H, q, $J=7.1$, 2.52 (1H, dq, $J=4.5$, 2.9) 2.3-2.45 (2H, m) 2.23 (3H, s) 2.01 (3H, s) 1.90 (3H, s) 1.42 (3H, d, $J=7.1$) 1.09 (3H, d, $J=7.2$) 1.08 (3H, d, $J=7.1$) 1.04 (3H, d, $J=7.4$) 1.00 (3H, d, $J=7.3$) 0.99 (3H, d, $J=6.9$) 0.97 (3H, t, $J=7.0$).

Siphonarin D: UV: $\lambda_{\text{max}}^{\text{CH}_3\text{OH}}$, 261nm ($\epsilon=10,600$); IR: $\nu_{\text{max}}^{\text{CCl}_4}$, 3502, 2938, 2880, 1656, 1618, 1456, 1379 cm^{-1} ; HRMS(EI):

obs. m/z 520.3038, $C_{29}H_{44}O_8$ calc. 520.3036; NMR ($CDCl_3$): δ =
 5.46 (1H, dd, J=8.8, 3.6) 4.19 (1H, q, J=7.1) 3.63 (1H, brdd)
 3.14 (2H brs) 2.90 (2 1H, m) 2.82 (1H, dq, J=6.9, 3.3) 2.74 (1H,
 q, J=7.3) 2.57 (2H, q, J=7.7) 2.50 (1H, dq, J=7.3, 4.4) 2.30-
 2.44 (2H m) 2.01 (3H, s) 1.92 (3H, s) 1.43 (3H, d, J=7.2) 1.17
 (3H, t, J=7.5) 1.09 (3H, d, J=7.2) 1.07 (3H, d, J=6.9) 1.04 (3H,
 d, J=7.2) 1.02 (3H, d, J=7.1) 1.00 (3H, d J=7.1) 0.99 (3H, t,
 J=7.1).

Siphonarin E: UV: $\lambda_{max}^{CH_3OH}$, 259nm ($\epsilon=7,900$); IR: $\nu_{max}^{CCl_4}$,
 3494, 2938, 1723, 1660, 1619, 1559, 1380 cm^{-1} ; HRMS(EI): obs.
 m/z $C_{28}H_{42}O_8$ calc. 506.2880; 1H NMR ($CDCl_3$): δ =5.43 (1H, dd,
 J= 9.2, 3.5) 4.11 (1H, q, J=6.9) 3.53 (1H, brdd) 3.36 (2H, brs)
 2.83 (2 1H, dq, J=7.0, 2.0) 2.78, (1H, m) 2.69 (1H, q, J=7.2)
 2.52 (1H, dq, J=7.2, 3.2) 2.3-2.5 (2H, m) 2.20 (3H, s) 2.05 (3H,
 s) 1.89 (3H, s) 1.35 (3H, d, J=7.1) 1.19 (3H, J=7.2) 1.06 (3H,
 d, J=7.1) 0.99 (2, 1H, d, J=7.2) 0.89 (3H, t, J=7.2) 0.85 (3H,
 d, J=6.7).

Siphonarin F: UV: $\lambda_{max}^{CH_3OH}$, 260nm ($\epsilon=12,550$); IR: $\nu_{max}^{CCl_3}$,
 3500, 2939, 2880, 1722, 1656, 1617, 1458, 1379 cm^{-1} ; HRMS(EI):
 obs. m/z 520.3042, $C_{29}H_{42}O_8$ calc. 520.3036; 1H NMR ($CDCl_3$): δ =
 5.44 (1H, dd, J= 9.1, 3.5) 4.13 (1H, q, J= 6.9) 3.53 (1H, dd, J=
 9.9, 3.3) 3.35-3.40 (2H brs) 2.84 (1H, dq, J= 7.1, 2.4) 2.83 (1H,
 dq, J=7.1, 2.4) 2.80 (1H, dq, J= 7.1, 2.0) 2.72 (1H, q, J=7.2)
 2.54 (2h, q, J=7.1) 2.53 (1H, m) 2.3-2.5 (2H, m) 2.07 (3H, s)

1.90 (3H, s) 1.36 (3H, d, $J = 6.8$) 1.20 (3H, d, $J = 7.1$) 1.13 (3H, t, $J = 7.7$) 1.06 (3H, d, $J = 7.2$) 0.99 (3H, d, $J = 6.7$) 0.88 (3H, t, $J = 7.3$) 0.83 (3H, d, $J = 6.8$).

Siphonarin G: UV: $\lambda_{\max}^{\text{CH}_3\text{OH}}$, 259nm ($\epsilon = 11,250$); IR: $\nu_{\max}^{\text{CCl}_4}$, 3505, 2937, 2802, 1723, 1660, 1621, 1458, 1381, cm^{-1} ; HRMS (EI): obs. m/z 506.2890, $\text{C}_{28}\text{H}_{42}\text{O}_8$ calc. 506.2880; ^1H NMR (CDCl_3): $\delta =$ 4.99 (1H, dd, $J = 6.4, 1.5$) 3.99 (1H, q, $J = 7.1$) 3.58 (1H, dd, $J = 8.9, 3.2$) 3.30 (2H brs) 2.93 (1H, dq, $J = 7.2, 2.4$) 2.76 (1H, dq, $J = 8.7, 1.8$) 2.63 (1H, dq, $J = 5.2, 1.7$) 2.57, (1H, q, $J = 7.0$) 2.21 (3H, s) 2.11 (1H, dq, $J = 6.8, 2.8$) 2.01 (3H, s) 1.92 (3H, s) 1.5–1.7 (2H, ddq, $J = 10.8, 7.6, 4.5$) 1.35 (3H, d, $J = 7.1$) 1.27 (3H, d, $J = 7.3$) 1.05 (3H, d, $J = 7.0$) 1.04 (3H, d, $J = 6.8$) 0.98 (3H, d, $J = 7.1$) 0.90 (3H, d, $J = 7.0$) 0.81 (3H, t, $J = 7.4$).

Siphonarin H: UV: $\lambda_{\max}^{\text{CH}_3\text{OH}}$, 259nm ($\epsilon = 14,100$); IR: $\nu_{\max}^{\text{CCl}_4}$, 3503, 2938, 2878, 1722, 1660, 1620, 1457, 1380 cm^{-1} ; HRMS(EI): obs. m/z 520.3038, $\text{C}_{29}\text{H}_{44}\text{O}_8$ calc. 520.3036; ^1H NMR (CDCl_3): $\delta =$ 4.98 (1H, dd, $J = 6.15, 1.8$) 4.05 (1H, q, $J = 6.8$) 3.60 (1H, dd, $J = 5.6, 2$) 3.35 (2H, brs) 2.93 (1H, dq, $J = 6.9, 2.0$) 2.77 (1H, dq, $J = 7.1, 1.8$) 2.62 (1H, m) 2.58 (1H, m) 2.55 (2H, q, $J = 7.4$) 2.11 (1H, dq, $J = 6.7, 1$) 2.03, (3H, s) 1.93 (3H, s) 1.5–1.7 (2H, ddq, $J = 9.7, 7.6, 6.5$) 1.36 (3H, d, $J = 6.9$) 1.27 (3H, d, $J = 7.3$)

1.14 (3H, t, J=7.4) 1.06 (3H, d J=6.6) 1.04 (3H, d, J=7.1) 0.98 (3H, d, J=7.1) 0.89 (3H, d, J=6.7) 0.86 (3H, t, J=7.7).

Siphonarin I: UV: $\lambda_{\max}^{\text{CH}_3\text{OH}}$, 259nm ($\epsilon=14,300$); IR: $\nu_{\max}^{\text{CCl}_4}$, 3505, 3414, 2932, 2882, 1722, 1659, 1622, 1458, 1381 cm^{-1} ; HRMS(EI): obs. m/z 506.2854, $\text{C}_{28}\text{H}_{42}\text{O}_8$ calc. 506.2880; ^1H NMR (CDCl_3): δ = 5.00 (1H, dd, J=7.4, 2.4) 4.03, (1H, q, J=7.0) 3.64 (1H, dd, J=8.4, 1) 3.20 (2H, brs) 2.97 (1H, dq, J=7.2, 2.1) 2.82 (1H, dq, J=7.0, 0.5) 2.61 (1H, q, J=6.9) 2.57 (1H, dq, J=6.9, 1.1) 2.22 (3H, s) 2.15 (1H, dq, J=7.1, 2.3) 2.01 (3H, s) 1.92 (3H, s) 1.5-1.7 (2H, ddq, J=10.2, 7.1, 2) 1.38 (3H, d, J=7.1) 1.13 (3H, d, J=7.1) 1.08 (2 3H, d, J=6.8) 1.05 (3H, d, J=6.8) 1.01 (3H, d, J=7.0) 0.84 (3H, t, J=7.4).

Siphonarin J: UV: $\lambda_{\max}^{\text{CH}_3\text{OH}}$, 260nm ($\epsilon=7,400$); IR: $\nu_{\max}^{\text{CCl}_4}$, 3502, 3415, 2939, 2881, 1721, 1656, 1620, 1459, 1378, cm^{-1} ; HRMS (EI): m/z obs 520.3028, $\text{C}_{29}\text{H}_{44}\text{O}_8$ calc. 520.3036; ^1H NMR (CDCl_3): δ = 5.00 (1H, dd, J=7.4, 2.7) 4.06 (1H, q, J=7.0) 3.67 (1H, brdd), 3.20 (2H, brs) 2.96 (1H, dq, J=7.2, 2.1) 2.84 (1H, dq, J=6.9, 0.5) 2.59 (1H, m) 2.58 (1H, q, J=7.7) 2.55 (2H, q, J=7.8) 2.15 (1H, dq, J=6.9, 0.5) 2.02 (3H, s) 1.93 (3H, s) 1.5-1.7 (2H, ddq, J=7.5, 6.5) 1.39 (3H, d, J=7.2) 1.02 (3H, d, J=7.4) 1.00 (3H, d, J=6.7) 0.84 (3H, t, J=7.4).

Siphonarin K: UV: $\lambda_{\max}^{\text{CH}_3\text{OH}}$, 240nm ($\epsilon=6,200$), shoulder 258nm ($\epsilon=5070$); IR; $\nu_{\max}^{\text{CHCl}_3}$, 2981, 2937, 1723, 1660, 1621, 1415,

1381 cm^{-1} ; MS(EI): no observed parent ion, see Figure 11 for fragmentations; ^1H NMR (CDCl_3): δ = 6.47 (1H, d, J = 8.9) 6.42 (1H, d, J = 9.2) 4.39 (1H, q, J = 6.5) 3.94 (1H, q, J = 7.0) 3.61 (1H, dq, J = 9.1, 2.6) 3.50 (1H, dq, J = 10.0, 2.9) 2.21 (2H, ddq, J = 7.4, 6.4) 2.20 (3H, s) 2.01 (3H, s) 1.94 (3H, s) 1.89 (3H, s) 1.83 (3H, s) 1.40 (3H, d, J = 2.1) 1.37 (3H, d, J = 7.1) 1.22 (3H, d, J = 7.5) 1.19 (3H, d, J = 6.3) 0.91 (3H, t, J = 7.6).

Siphonarin L: UV: $\lambda_{\text{max}}^{\text{CH}_3\text{OH}}$, 238 nm (ϵ = 3,480), shoulder 260nm (ϵ = 2850); IR: $\nu_{\text{max}}^{\text{CHCl}_3}$, 2937, 2981, 1723, 1660, 1621, 1552, 1414, 1381 cm^{-1} ; MS(EI): no observed parent ion, see Figure 11 for fragmentations; ^1H NMR (CDCl_3): δ = 6.50 (1H, d, J = 9.4) 6.39 (1H, d, J = 9.0) 4.40 (1H, q, J = 6.6) 3.98 (1H, q, J = 6.2) 3.61 (1H, dq, J = 9.7, 3.2) 3.49 (1H, dq, J = 9.4, 3.2) 2.53 (2H, q, J = 7.1) 2.22 (2H, ddq, J = 7.8, 2.6) 2.02 (3H, s) 1.97 (3H, s) 1.90 (3H, s) 1.84 (3H, s) 1.39 (3H, d, J = 7.4) 1.38 (3H, d, J = 6.8) 1.21 (3H, d, J = 6.7) 1.17 (3H, d, J = 7.1) 1.11 (3H, t, J = 7.4) 0.88 (3H, t, J = 7.4).

Eudistoma olivaceum

Materials:

All final separation and purification of the eudistomins was done on one column, a 0.46 x 25 cm analytical Chemcosorb propylamine column from Dychrom Co. This column was part of an HPLC system which included a Beckman 112 solvent delivery system and a Beckman 160 absorbance detector set at 254 nm.

Characterization by UV, IR, NMR and MS was done using the same instruments utilized in the homarine investigation.

Collection, Extraction, and Initial Separation.

Individuals of the ascidian Eudistoma olivaceum (Polycitoridae) were collected in 1979, and 1981 by J.H. Cardellina II. These tunicates were obtained from the shallow waters in Tucker's Town Harbor, and Walsingham Pond, Bermuda. The Cardellina group then extracted these organisms and subjected the 1.90 grams of dichloromethane soluble extract to chromatographic separation. The initial separation of the crude extract was done on a Sephadex LH-20 column utilizing the step gradient elution technique (57). One of the hexane-dichloromethane fractions was then continued through a series of gravity flow, gel permeation columns by our group resulting in a number of relatively clean mixtures of eudistomins.

Isolation of Eudistomins: As can be seen in Figure 12, eudistomins H, G, and I, respectively, eluted from a reverse phase propylamine HPLC system in relatively pure form. Even though the injections were kept to a relatively small 0.4 mg/run there still was tailing due in all likelihood to the low solubility of these compounds in the 90:10 dichloromethane:hexane solvent system used during the elution of this column. The tailing was the cause of the coelution of eudistomin G with the residual of the previous eudistomin H. Therefore, the HPLC fractions containing the second eudistomin were recycled using

the same column, but with 100% dichloromethane as eluent. The quantities of eudistomins H, G, and I isolated were 31.3, 3.0, and 10.9 mg respectively.

The isolation of eudistomin P was carried out on the propylamine column; but because the previous solvent system would not elute this compound, a more polar, 100% acetonitrile system was used in which 1.1 mg of this more polar OH bearing compound was obtained in pure form.

Eudistomins R, S, and T came from a crude gravity flow column fraction that did not contain the first three pyridoindoles. Eudistomins R and S eluted together on the normal phase propylamine system yielding approximately 0.8 mg for each. Eudistomin T eluted in the following fraction in approximately 80% pure form resulting in about 0.3 mg of this compound.

Characterization: All ^1H NMR data for eudistomins G, H, I, and P are reported in Table 4.

Eudistomin G: UV: $\lambda_{\text{max}}^{\text{CH}_2\text{Cl}_2}$, 227nm ($\epsilon=16,600$), 285nm ($\epsilon=10,200$) 365nm ($\epsilon=5,000$); IR: $\nu_{\text{max}}^{\text{CCl}_4}$, 3359, 3080, 2927, 1604, 1473 cm^{-1} , MS(EI): m/z (rel int) 313/315 (100/96), 286 (10), 271/273 (14/15), 233 (11), 103 (14).

Eudistomin H: UV: $\lambda_{\text{max}}^{\text{CH}_2\text{Cl}_2}$, 227nm ($\epsilon=12,100$), 288nm ($\epsilon=6,100$) 363nm ($\epsilon=1,900$); IR: $\nu_{\text{max}}^{\text{CCl}_4}$, 3355, 3065, 2925,

1604, 1473 cm^{-1} ; MS(EI): m/z (rel int) 313/315 (100/96), 286 (10), 271/272 (15/14), 233 (13), 103 (14).

Eudistomin I: UV: $\lambda_{\text{max}}^{\text{CH}_2\text{Cl}_2}$, 224nm ($\epsilon=14,400$), 282nm, ($\epsilon=11,300$), 368nm, ($\epsilon=5,700$); IR: $\nu_{\text{max}}^{\text{CCl}_4}$, 3369, 3064, 2960, 2928, 2517, 1630, 1607, 1433 cm^{-1} ; MS(EI): m/z (rel int) 235 (100), 206 (14), 193 (21), 140 (9), 103 (11).

Eudistomin P: UV: $\lambda_{\text{max}}^{\text{CH}_2\text{Cl}_2}$, 222nm ($\epsilon=12,500$), 303nm, ($\epsilon=5,700$), 370nm ($\epsilon=1,500$); IR: $\nu_{\text{max}}^{\text{CCl}_4}$, 3540, 3361, 2928, 1607, 1482 cm^{-1} ; MS(EI): m/z (rel int) 329/331 (100/99), 302 (9), 287/289 (14/15), 249 (7).

Eudistomins R + S: UV: $\lambda_{\text{max}}^{\text{CH}_2\text{Cl}_2}$, 227nm ($\epsilon=39,900$), 292nm, ($\epsilon=21,400$), 379nm ($\epsilon=7,600$); IR: $\nu_{\text{max}}^{\text{CCl}_4}$, 3437, 3065, 3032, 2928, 2565, 1669, 1623, 1585 cm^{-1} ; MS(EI): (rel int) 364/366 (100/96), 336 (12), 273/275 (13/13), 245/247 (87/89), 166 (55), 139 (19), 91 (24); HRMS(EI): obs 364.0201, $\text{C}_{19}\text{H}_{13}\text{N}_2\text{OBr}$, calc 364.0252.

Eudistomin R: ^1H NMR: (CDCl_3) $\delta=8.60$ (1H, d, $J=5.0$), 8.12 (1H, d, $J=3.4$), 7.97 (1H, d, $J=8.3$), 7.67 (1H, d, 1.4), 7.40 (1H, d, $J=8.6$), 7.45-7.27 (5H, overlapping m's), 4.72 (2H, s).

Eudistomin S: ^1H NMR: (CDCl_3) $\delta=8.60$ (1H, d, $J=5.0$), 8.25 (1H, d, $J=1.7$), 8.11 (1H, d, $J=4.8$), 7.65 (1H, d, $J=8.7$),

7.40 (1H, d, J=8.6), 7.45-7.27 (5H, overlapping m's), 4.72 (2h, s).

Eudistomin T: MS(EI): m/z (rel int) 286 (77), 258 (9), 195 (13), 167 (100), 140 (24), 91 (9), HRMS(EI): obs. 286.1059, $C_{19}H_{14}N_2O$, calc. 286.1084.

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