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 alternate, 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 pyrollidine ring found in previously characterized eudistomins G, H, I, and P, 20-23-

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APPROVAL

of a thesis submitted by

Ken Kinzer

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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Date _Oct 17 '86_

DEDICATED TO:

Connie,

for being her,

for being there.

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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 pyrollidine 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, coelentrazine, 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 B-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 ^{1}H 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 ^{1}H 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 ^{1}H 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
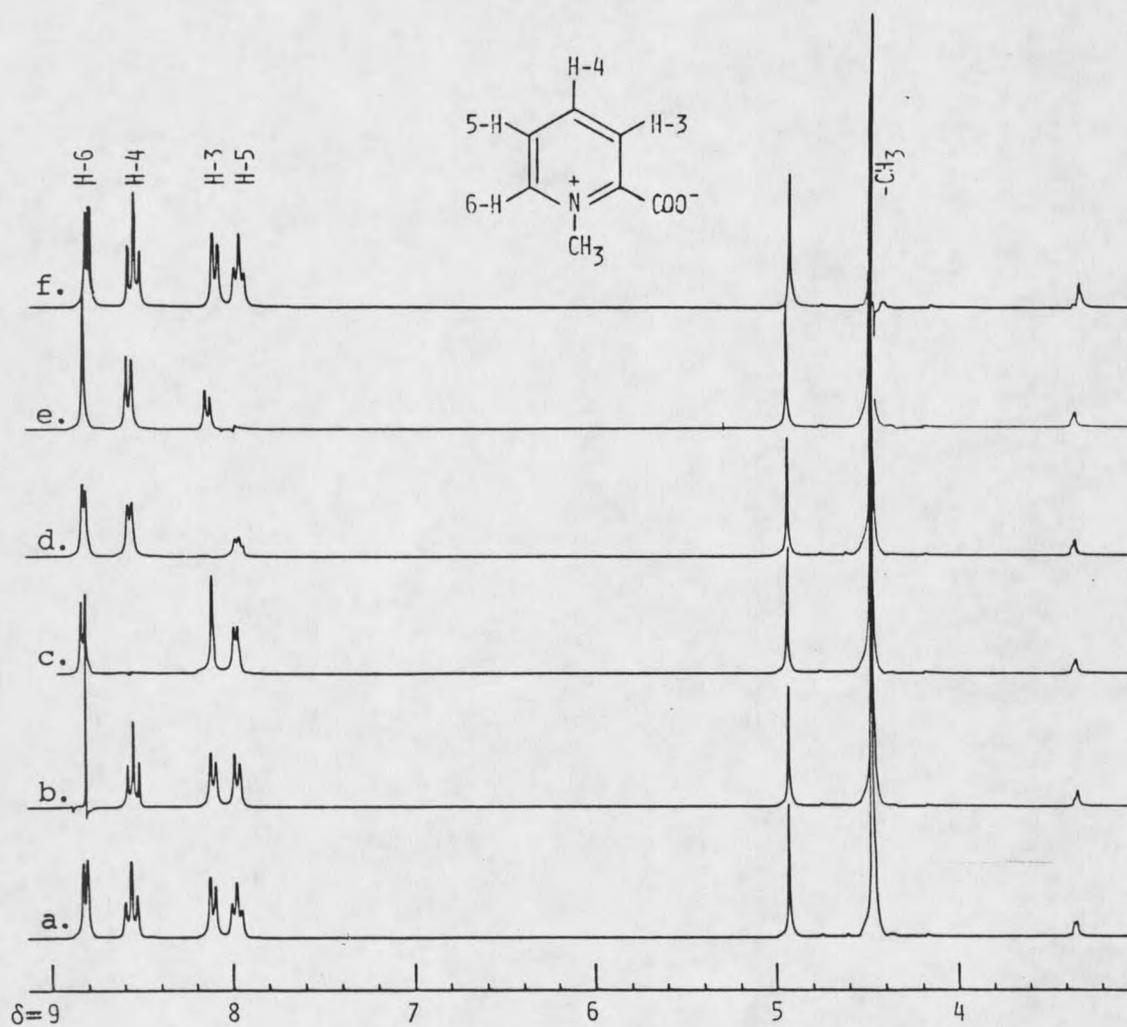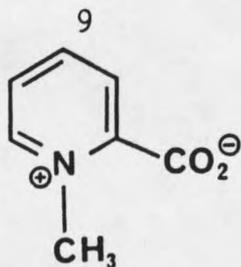to correlate well with the observed polarity of this compound.

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

$$9$$

1

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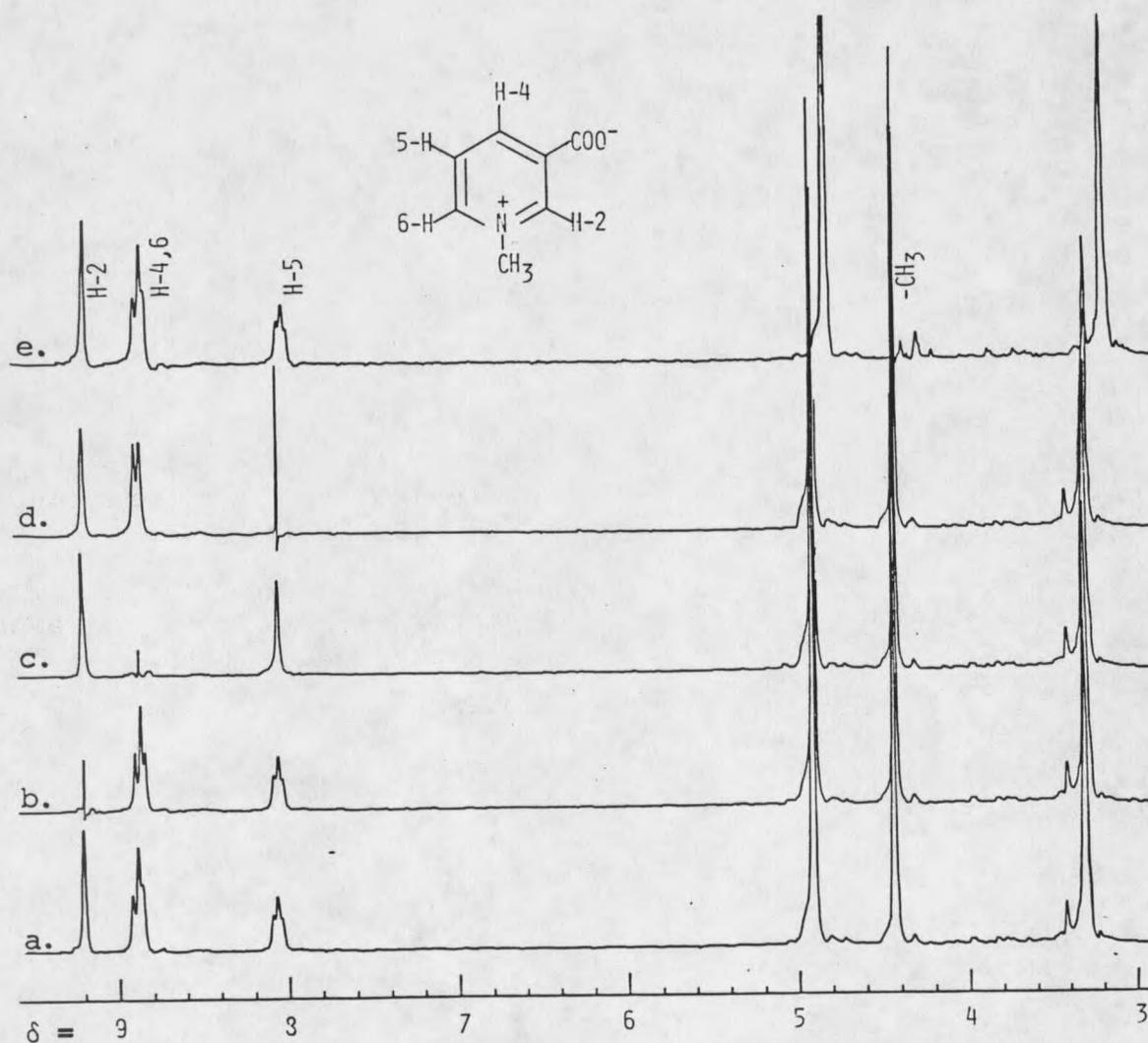
2
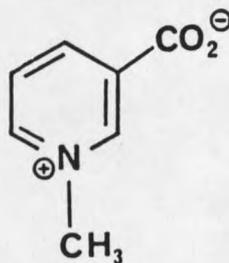
Figure 2. ^{1}H NMR spectrum of trigonelline, coupled (a) and decoupled (b-e).

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 role 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

Rx. 1: homarine + $NH_4^+ \longrightarrow (CH_3)_nNH_{4-n}$ + picolinic acid

Rx. 2: homarine + $^+NH_2CH_2OH \longrightarrow (CH_3)_3^+NCH_2CH_2OH$

(ethanolamine)   (choline)

[SAM]   [O]

picolinic acid   + $(CH_3)_3^+NCH_2COO^-$

(glycine betaine)

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