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**Isonitrile biosynthesis in a marine sponge**

Hagadone, Mark Raymond, Ph.D.

University of Hawaii, 1991

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ISONITRILE BIOSYNTHESIS IN A MARINE SPONGE

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE  
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT  
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DOCTOR OF PHILOSOPHY

IN CHEMISTRY

MAY 1991

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In the memory of my Father, Roger Lee Hagadone.

## ACKNOWLEDGMENT

My deepest regards to Dr. Arne Holm whose brilliant synthetic ability provided this work with the majority of its success; to Dr. Deborah Roll, who never ceased to help me with the actual underwater field work at a point in her life when she had very little time to spare; and to Fred Hertlein III, my partner in business and life, for his understanding and financial support throughout this undertaking.

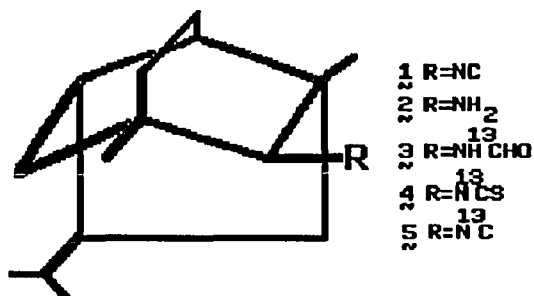
It is difficult to acknowledge adequately the enormous degree of support, guidance, and care with which my professor and lifelong friend, Dr. Paul Scheuer, has provided me. This work, my education in chemistry, and indeed, my livelihood, has been molded and infinitely promoted by his influence.

It is also with appreciation and gratitude that I acknowledge the support provided to me by my mother Ellen, Rebecca, once my wife, Malia, my daughter, and Dustin, my son, all of whom missed those vanishing opportunities to play in the park because I was "in the lab."

## ABSTRACT

Ever since the discovery in 1957 of a naturally occurring isocyano metabolite in a terrestrial microorganism, the biogenesis of the isocyano group has been an intriguing unsolved scientific puzzle. The first marine-derived isocyano compounds were isolated from sponges in 1973, when the question of their biogenesis arose. While these questions are superficially identical they differ fundamentally in that all known terrestrial isocyanides are derivatives of amino acids, while all known marine isocyanides are of terpenoid origin. Another characteristic feature of the marine metabolites is their frequent co-occurrence with formamido and isothiocyanato derivatives sharing the same carbon skeleton.

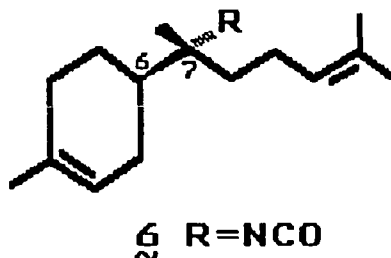
The first biogenetic hypothesis dealt with the interrelationship of these three functions. Because N-formyl compounds are more frequently encountered than isocyano derivatives, the relationship of these two functions became the first objective, which was extended to encompass the origin of the isothiocyanato function.



Natural 2-isocyanopupukeanane (1, R = NC) was degraded to the 2-amino (2, R = NH<sub>2</sub>) compound, from which <sup>13</sup>C labeled formamide (3, R=NH<sup>13</sup>CHO), isothiocyanato (4, R = N<sup>13</sup>CS), and isocyano (5, R = N<sup>13</sup>C) were synthesized.

The synthetic experiments were carried out by a newly developed technique on living sponges, *Ciocalypta* sp., on the north shore of O'ahu. Semisynthetic precursors were enclosed in gelatin capsules and surgically embedded in the animals by researchers equipped with SCUBA. After incubation times varying from 2 to 4 weeks the animals were removed from their habitat and analyzed by gas chromatographic / mass spectrometric techniques.

It was conclusively demonstrated that 2-isocyanopupukeanane (1) is the precursor of the formamido and isothiocyanato functions and that the reverse reaction does not take place.



In the course of this work the first naturally occurring isocyanato compound, 2- isocyanatobisabolene (6) was isolated from the sponge, *Ciocalypta* sp.

Some of the results of this work have been published.<sup>1,2</sup>

---

<sup>1</sup> Hagadone, M. R.; Scheuer, P. J.; Holm, A. J. *Am. Chem. Soc.* **1984**, *106*, 2447-2448.

<sup>2</sup> Gulavita, N. K.; Dilip de Silva, E.; Hagadone, M. R.; Karuso, P.; Scheuer P. J. *J. Org. Chem.* **1986**, *51*, 5136-5139.

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## I. INTRODUCTION

### Background of the Isocyano Functionality

In contrast to its relatively recent discovery in natural products, the isocyano function has been known to synthetic chemists for approximately 125 years. Lieke in 1859 reacted allyl iodide and silver cyanide to obtain a reasonable yield of a liquid with a penetrating odor to which he assigned the structure of allyl cyanide.<sup>1</sup> However, he was surprised to obtain only formic acid upon acidic hydrolysis of the presumed allyl cyanide. Lieke's experiments were then discontinued due to "continuing complaints in the neighborhood about the vile odor."

Apparently Lieke carried out all of his experiments outdoors because "opening a vessel containing the nitrile (sic) is sufficient to taint the air in a room for days."<sup>1</sup>

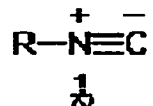
Several years later Hofmann developed a synthesis of isonitriles, which eventually evolved into a useful qualitative reaction for the detection of primary amines, the so-called carbylamine reaction.<sup>2</sup> This involves the reaction of primary amines with chloroform and a strong base such as ethanolic potassium hydroxide solution, solid alkali hydroxides, or potassium t-butoxide, which leads to the production of the corresponding isonitrile and its characteristic odor. It was not until after the extensive work by Gautier,<sup>3</sup> which dealt with the peculiar bonding

relationships in this new class of compounds, that isonitriles were recognized as configurational isomers of nitriles. Gautier envisioned isonitriles as "true homologs of hydrocyanic acid" and believed that, like the acid, "they have the greatest of deleterious effects on an organism."<sup>3</sup> However, in reality, isonitriles with few exceptions exhibit no toxicity to mammals; mice apparently can withstand oral and subcutaneous doses of 500 to 5000 mg/kg of most isonitriles.<sup>3</sup> One notable exception is 1,4-diisocyanobutane, which is rather toxic with an LD<sub>50</sub> in mice of less than 10 mg/kg.<sup>3</sup>

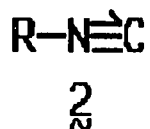
Once again the vile odor of isonitriles was confirmed by Gautier's observation that methyl and ethyl isonitrile were characterized by "detestable odors which at the same time were reminiscent of artichokes and phosphorus."<sup>3</sup>

### Constitution

In 1930, several decades after Gautier's research, Lindemann and Wiegrebe proposed the correct polar structure for the isonitrile function (1), based on the structure of carbon monoxide as postulated by Langmuir.<sup>4</sup>



Two decades after that work, extensive microwave studies confirmed the linearity of the C--N--C bond system and led to the current modern notation (2).<sup>5</sup>



Isonitriles are perhaps best characterized by their sharp, intense absorption in the 2120 to 2250  $\text{cm}^{-1}$  region of the infrared spectrum. They show little if any uv absorption, usually below 200 nm, which is thus of little diagnostic value.

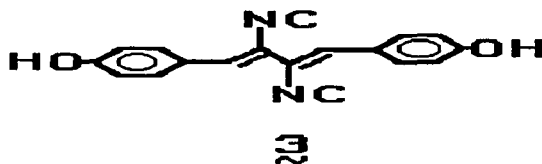
The mass spectra of isonitriles are similar to those of nitriles in that  $\alpha$ -bond fragmentation predominates, presumably via a cyclic intermediate. However,  $\beta$ -bond cleavage occurs more often in the isonitrile than in the nitrile, thus demonstrating the relative weakness of the isocyano R - N bond. In aromatic isonitriles the predominant fragmentation observed is loss of hydrogen cyanide, in analogy with the behavior of the corresponding nitriles.<sup>6</sup>

#### Occurrence

The phylum Porifera (sponges) represents a diverse and chemically interesting group of widely distributed invertebrates, predominantly marine.

These organisms have been found to elaborate a broad spectrum of secondary metabolites that possess varied biological activity and often uncommon molecular structural features.

Notable among the fascinating structural features found within constituents of Porifera and Mollusca is the isocyano function, which outside of Porifera is known only from a few terrestrial microorganisms and/or from laboratory synthesis. Naturally occurring isonitriles are relatively rare and by 1973 only one, the metabolite xanthocillin (3)\* isolated from *Penicillium notatum*, had been described in the literature.<sup>7</sup>

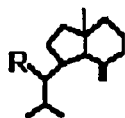


Because of its unusual chemical properties and structure, the question of its biosynthetic origin was raised almost immediately after its initial discovery. Interest in the biosynthetic origin of the isonitrile increased substantially in 1973 with the discovery of axisonitrile-1 (4), and subsequently its formamide and isothiocyanate, by Fattorusso

---

\* It is interesting to note that recently another structurally related fungal metabolite, MK4588, was isolated from the culture broth of the microorganism *Leptosphaeria* sp.<sup>8</sup> This after the passage of almost 35 years from the original discovery of xanthocillin by Hagedorn and Tönjes!

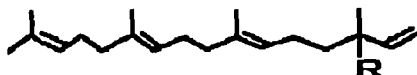
and co-workers in the sponge *Axinella cannabina*.<sup>9</sup>



R=NC, NHCHO, NCS

4

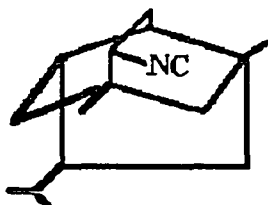
Two years later Scheuer and co-workers<sup>10</sup> isolated a series of isocyanide-formamide pairs (5) from a sponge, *Halichondria* sp. Also present were their isothiocyanates, presumably derived from the respective isocyano precursors.



R=NC, NHCHO

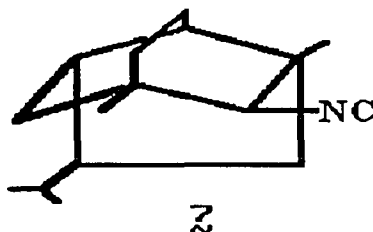
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In 1976 a naturally occurring isocyanosquiterpene was discovered in a nudibranch, *Phyllidia varicosa*.<sup>11</sup> This mollusk uses 9-isocyanopupukeanane (6) for protection against predators and sequesters it from its prey, a species of the sponge genus *Ciocalypta* (initially believed to be *Hymeniacidon* sp.).



6

*Ciocalypta* sp., which may be found in caves on the rocky north shore of O'ahu, also elaborates an isomeric isonitrile, 2-isocyanopupukeanane (2),<sup>12</sup> both of which have been found in secretions isolated from *Phyllidia varicosa*.



By the end of 1989, nearly 200 naturally occurring isonitriles were isolated and reported in the literature,<sup>13</sup> including several fungal or microbiological metabolites from species such as *Trichoderma*, as well as a variety from the phylum Porifera.

The initial investigations gave rise to a hypothesis that formamide may be the biogenetic precursor of the isonitrile, which in turn is transformed into the isothiocyanate. It is this hypothesis which gave rise to our earliest biosynthetic investigations.

#### Previous Biosynthetic Research

Achenbach and Grisebach proposed in 1965 that a one-carbon donor provides the isocyano carbon and the amino acid tyrosine the nitrogen in xanthocillin (3), a mold metabolite from *Penicillium notatum*.<sup>14</sup> While tyrosine was incorporated, neither formate nor methionine provided the

carbon of the isonitrile.

In 1979, Sodano et al.<sup>15</sup> attempted the incorporation of <sup>14</sup>C- labeled axamide-1 (8) into marine sponge *Axinella cannabina*, in accord with the postulate that axamide-1 would be transformed *in vivo* to axisonitrile-1 (9) (Figure 1).

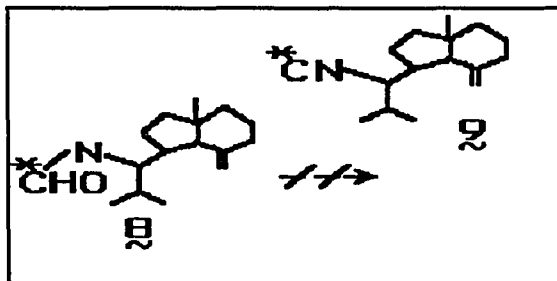


Figure 1. Attempted biotransformation of axamide-1 (8) into axisonitrile-1 (9).

In this experiment, <sup>14</sup>C-labeled axamide-1, dissolved in ethanol, was administered to *Axinella cannabina*, which was maintained in well-aerated sea water aquaria in the laboratory. After five days, the sponge was removed and the metabolites isolated by a standard chemical purification scheme. The authors were able to show that the sponge had taken up and metabolized some of the radiolabeled precursor by the consistent amount of radioactivity (0.1% of administered radioactivity) found in the free fatty acid fraction of the sponge metabolites. However, no radioactivity was found in the axisonitrile-1 fraction, thus negating the original hypothesis. In conclusion, the authors stated that false negative results could arise from biosynthesis which was occurring at a very slow rate.

I succeeded in demonstrating that the formamide (10) and isothiocyanate (11) metabolites of 2-isocyanopupukeanane (12) are derived from the isonitrile (Figure 2). These results have been published.<sup>16</sup>

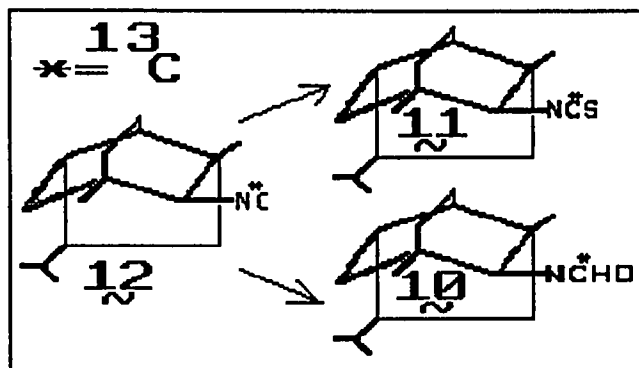
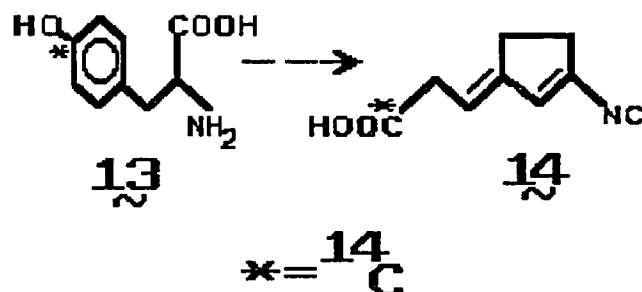


Figure 2. Biotransformation of 2-isocyanopupukeanane to the corresponding isothiocyanate and formamide in *Ciocalypta* sp.

Perry and Phuoc Buu<sup>17</sup> conducted biosynthetic studies with the isonitrile acid (14) from the fungus *Trichoderma hamatum*.



In that work, administration of <sup>14</sup>C-labeled [U-<sup>14</sup>C]-L-tyrosine (13) to the culture medium yielded the radioactive acid after two days.

Although this demonstrates the intermediacy of tyrosine in the biosynthesis of (13), it did not shed any light on the origin of the carbon in the isonitrile functionality.

Many problems have been encountered with the incorporation of  $^{14}\text{C}$ -labeled substrates into marine sponges. In particular, studies by Walton and Pennock<sup>18</sup> have encountered erratic incorporation of  $^{14}\text{C}$ -labeled precursors in the sponges *Grantia compressa* and *Suberites domuncula*. It was apparently very difficult to determine whether the sponges survived the incubation period, let alone assess the viability of their normal metabolism during the course of the incorporation experiments. An example of poor incorporation leading to erroneous conclusions came to light in the biosynthetic investigation into the origin of the brominated phenols and quinones isolated from *Aplysina* (*Verongia*) species.<sup>19</sup> These compounds were originally proposed to be derived from mono or dibromo tyrosines. However, efforts by Minale et al. who attempted incorporation of radiolabeled tyrosine into *Verongia aerophobia* were negative.<sup>20</sup> In this work, no evidence was obtained for the biogenetic hypothesis.

Rinehart and Tymiak<sup>21</sup> modified Minale's incorporation techniques by encapsulating labeled precursors in a phosphatidylcholine-cholesterol film.

These two components are normally found in sponge tissue, and since the sponge is known to be a filter feeder, encapsulation of the isotopically labeled precursors with subsequent ingestion by the sponge was expected to facilitate incorporation. This was indeed the case, and Tymiak and Rinehart were able to demonstrate the conversion of phenylalanine and tyrosine (15) to the diene (16) and to the rearranged metabolite dibromo-homogentisamide (17), thereby supporting the proposed biogenetic scheme (Figure 3).

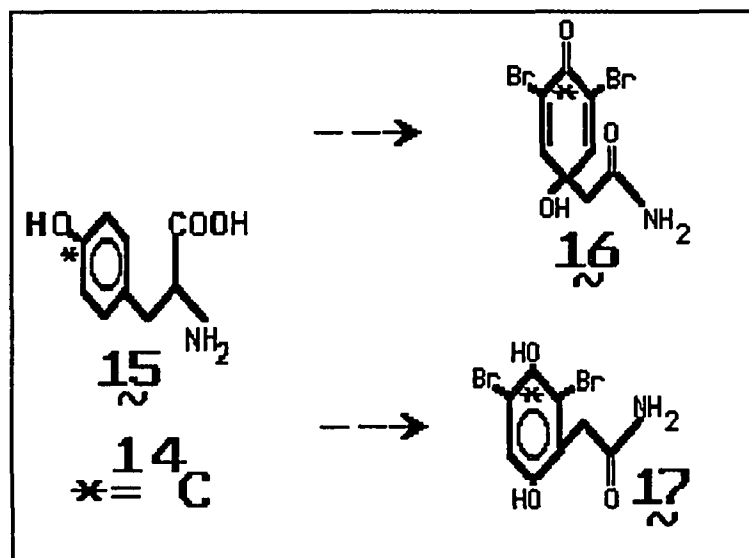


Figure 3. Incorporation of labeled tyrosine and phenylalanine into the dienone (16) isolated from the sponge *Aplysina fistularis*.

#### Role of Stable Isotopes in Biosynthetic Studies

The first toxicological studies using deuterium oxide were conducted within one year after the initial published

report of the discovery of deuterium.<sup>22</sup> Not long after this milestone in metabolism studies, employing the stable isotope deuterium,  $^{13}\text{C}$  was employed as a tracer in the bacteriological fixation of carbon dioxide.<sup>23</sup> As a by-product of developing the atomic bomb during World War II, the first shipments of radioactive carbon-14 had become available in 1945. This created a tremendous amount of excitement in the field of isotope chemistry. An historic meeting in 1947 entitled, "The Use of Isotopes In Biology and Medicine", was considered by many to be the watershed of scientific isotope usage.<sup>24</sup>

At that meeting the potential for radioactive tracers, although relatively recently introduced, was clearly apparent. In the following twenty years applications of radioactive tracers completely overshadowed the use of deuterium and carbon-13. With the advent of relatively inexpensive quadrupole mass spectrometers interfaced with gas chromatographs, and the explosive growth of Fourier transform nuclear magnetic resonance and infrared spectrophotometric methods, research with stable isotopes once again flourished.

Important reasons for this resurgence were an increased awareness of the health hazards of radioactivity as well as a greater availability of stable isotopes. A few of the more important stable isotopes which have found increasing application in biology and chemistry are shown in Table I.

**Table I. Important Stable Isotopes With Widespread Application in Chemistry**

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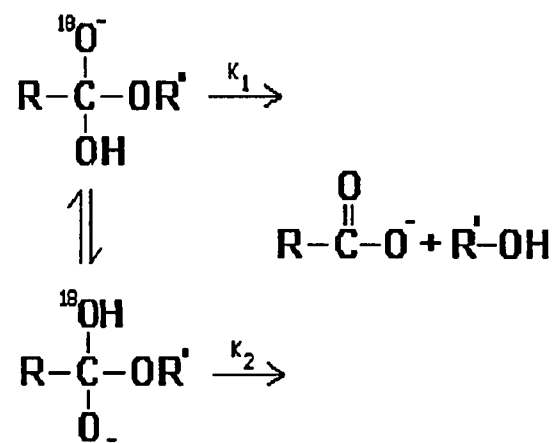
Element	Stable Isotope Mass	% Natural Abundance
H	2	0.015
C	13	1.11
N	15	0.37
O	17	0.037
	18	0.204
S	33	0.76
	34	4.22
Fe	54	5.82
	57	2.19
	58	0.33
Zn	66	27.81
	67	4.11
	68	18.57
	70	0.62

---

Carbon-13 has become important in mechanistic studies involving theoretical organic chemistry as well as in biochemistry and medicine. For example, in mechanistic organic chemistry, both carbon-13 and oxygen-18 have been extensively employed for the critical study of mechanism. Both isotopes suffer far less from exaggerated rate effects normally associated with deuterium.

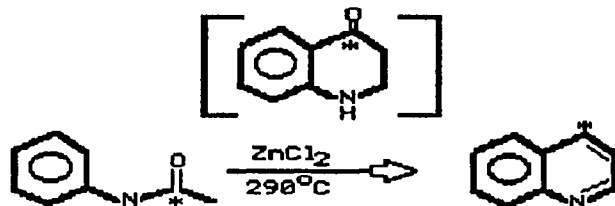
Quantitation is simplified because such forward and reverse rate constants are typically within a few percent of one another for both labeled and unlabeled substrates. This results in relaxed mechanistic assumptions involving verification of first or second order reaction rates.

Perhaps the most widely cited application of the use of oxygen-18 in the study of mechanism is that of the simple alkaline hydrolysis of ethyl benzoate in aqueous solution.<sup>25</sup> In this work Bender studied both reaction rates and isotopic label distribution between products and reactants. He and others<sup>26</sup> were able to show that the mechanism in Scheme 1 accounted for both observed oxygen exchange, and reaction rates of exchange under both low and high pH conditions. This mechanism accounts for the occurrence of oxygen exchange and for the observation of first-order hydrolysis in hydroxide ion and ester concentrations. It involves the reversible formation of a tetrahedral addition intermediate in low concentration. Oxygen exchange then occurs because the intermediate can collapse back to reactants in two ways, only one of which results in the loss of <sup>18</sup>O label.



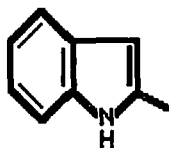
**Scheme 1**

Analogously, carbon-13 has been employed<sup>27</sup> to study the synthesis of quinoline, involving the pyrolysis of *N*-methylacetanilide with zinc chloride at 290°C (Scheme 2).<sup>28</sup>



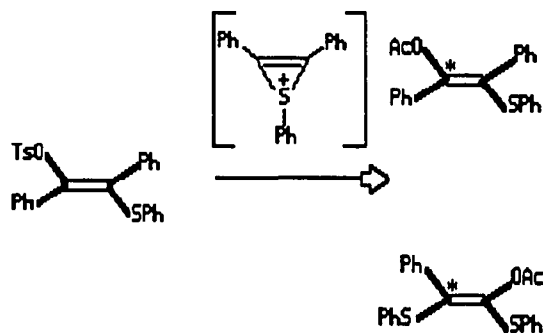
Scheme 2

This result contradicted a previously proposed mechanism involving 2-methylindole (17) as an intermediate.<sup>29</sup>



17

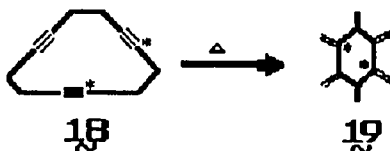
$^{13}\text{C}$  has also been employed in the study of the mechanism of the solvolysis of trans-1,2-diphenyl-2-(phenylthio)vinyl-1- $^{13}\text{C}$  tosylate in acetic acid (Scheme 3).<sup>30</sup>



**Scheme 3**

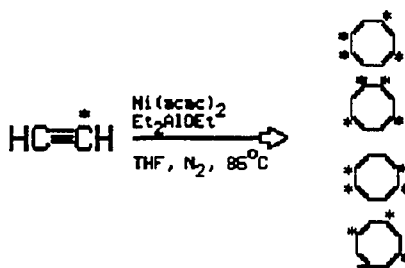
Since equal amounts of both products were produced, the existence of a thiirenium ion was postulated as an intermediate in the solvolysis.

Vollhardt used carbon-13 to study both the mechanism of the conversion of 1,5,9-cyclodecatriyne (**18**) to hexaradialene (**19**) (Scheme 4),<sup>31</sup>



**Scheme 4**

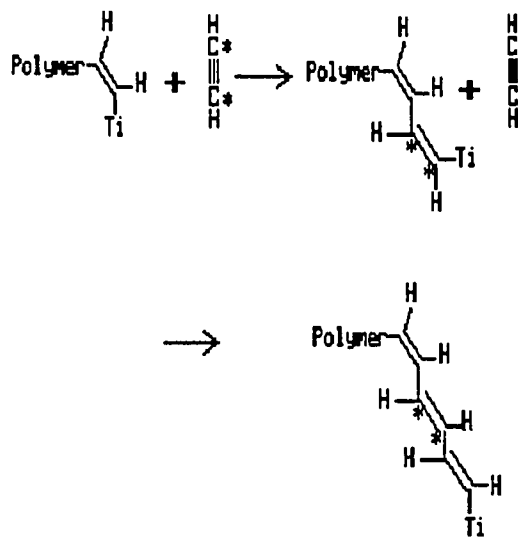
and the mechanism for the formation of 1,3,5,7-cyclooctatetraene from acetylene (Scheme 5).<sup>32</sup>



**Scheme 5**

In that case, the pattern of labeling indicated the polymerization proceeds via simple coordination of acetylene about the nickel catalyst.

In an interesting application involving the use of  $^{13}\text{C}$  to precisely measure internuclear distances to within one percent accuracy in amorphous solids, homonuclear dipole splitting between two contiguous  $^{13}\text{C}$  atoms was determined with the use of NMR techniques.<sup>33</sup> In this application, the mechanism of the Ziegler-Natta polymerization of acetylene was explored.<sup>34</sup> Because the resultant polymer exhibited contiguous  $^{13}\text{C}$  atoms 1.37 Å apart, a mechanism favoring direct insertion of acetylene into the titanium-polymer bond as indicated below, was postulated (Scheme 6) as the mechanism for Ziegler-Natta polymerization of acetylene.

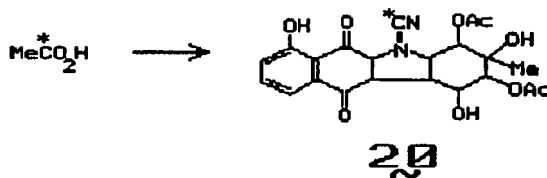


**Scheme 6**

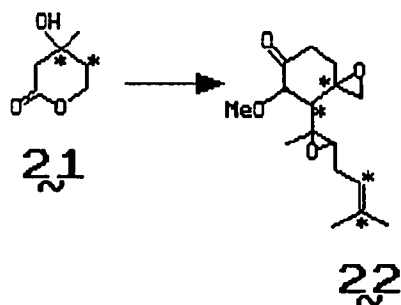
In the fields of biochemistry and medicine, the use of stable isotopes, in particular carbon-13 and deuterium, have expanded dramatically within the last 15 years.

A number of studies have been successfully completed over the years involving methodology employing a combination of different analytical techniques. These include numerous examples of the use of  $^{13}\text{C}$  in the elucidation of biosynthetic pathways in plants, and microorganisms.

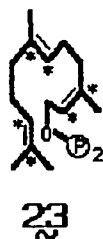
A notable application of these techniques involving infrared absorption spectroscopy was the observation that the cyanide group in kanamycin D (20) was derived from the carboxy group of 1- $^{13}\text{C}$ -acetic acid.<sup>35</sup> In labeled kanamycin D,  $^{13}\text{CN}$  absorbs at  $2139\text{ cm}^{-1}$ , while  $^{12}\text{CN}$  in the natural material absorbs at  $2155\text{ cm}^{-1}$ .



The knowledge of biosynthetic pathways in terpene biosynthesis has been dramatically expanded due to the use of  $^{13}\text{C}$  stable isotope techniques. Cane and Levin studied the biosynthesis of ovalicin (22), a sesquiterpene produced by the fungus *Pseudorotium ovalis*.<sup>36</sup>

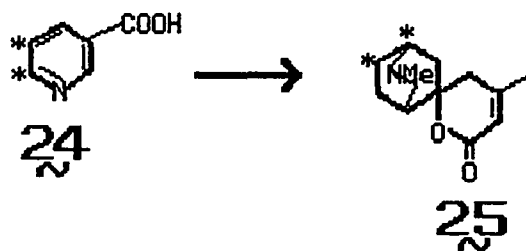


These investigators were able to show that synthetic mevalonolactone-3,4- $^{13}\text{C}_2$  (21) was incorporated into two pairs of contiguous  $^{13}\text{C}$  atoms. The detection of only two pairs of contiguous  $^{13}\text{C}$  atoms (22) via  $^{13}\text{C}$  NMR spectroscopy demonstrated that one of the three pairs of contiguous  $^{13}\text{C}$  atoms in the intermediate farnesol pyrophosphate (23) was separated during the biosynthetic sequence.<sup>37</sup>



The phenomenon of adjacent  $^{13}\text{C}$  atoms giving rise to spin-spin coupling and hence satellites in the  $^{13}\text{C}$  NMR spectrum is used in an interesting technique which has found application in a large number of biosynthetic studies carried out with sodium  $^{13}\text{C}$ -2 acetate. This particular technique has provided the investigator with a useful tool for determining whether a natural product is a polyketide. Seto and co-workers first demonstrated this technique in studies of the biosynthesis of microbial products.<sup>38</sup>

Additionally, Leete used this technique to show that 5,6- $^{13}\text{C}$ -3-nicotinic acid (24) is a direct precursor of part of the isoquinuclidine ring system found in the alkaloid dioscorine (25) produced by the tropical yam *Dioscorea hispida*.<sup>39</sup>



With the advent of Fourier transform NMR techniques, the mechanism of enzyme action has been investigated with carbon-13 labeled substrates. Mackenzie and Malthouse investigated the mechanism involved in the action of the enzyme aldolase in the conversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate to fructose-1,6-diphosphate.<sup>40</sup> Unkkefer and Blazer studied the interconversion of nicotinic-2-<sup>13</sup>C acid to the pyridine nucleotides NAD<sup>+</sup> and NADH *in vivo*, within the cells of *E. coli*.<sup>41</sup>

In addition to the biosynthetic studies described above, carbon-13 is now being routinely used for *in vivo* examination of biosynthetic pathways as a dynamic and continuing process within the cell itself. Shulman has conducted much of the early work involving the examination of live mammals, including humans, in the probe of a nuclear magnetic resonance spectrometer.<sup>42</sup> In one example, he was able to demonstrate the actual metabolism of glucose-1-<sup>13</sup>C in intact rat liver. He accomplished this by monitoring the initial signals of glucose at 96.8 and 92.3 ppm, which are the signals of the C-1 position in the alpha and beta anomers of glucose. The intensities of these signals decreased dramatically with time, with a concurrent emergence of a signal at 101 ppm. This new signal was assigned to C-1 in the polysaccharide glycogen produced in the rat liver from labeled glucose.<sup>33</sup>

## II. EXPERIMENTAL PART

### General Information Instrumental Techniques

GC-MS measurements were performed with a Finnigan OWA automated gas chromatograph (Model Sigma 3A)/mass spectrometer (Model 1030B). Finnigan Incos software was employed for real time data manipulation on the instrument's Tektronix data station. All mass spectral data were archived on nine track magnetic tape in standard EPA data format. Capillary gas chromatography was carried out using a 30 m DB-5 fused silica capillary column, operating in a 25 second splitless mode. Temperature programming was employed consisting of an initial temperature 125° C maintained for 1 min, then increased to 325° C at a rate 8° C/min, for the chromatographic separation. Chromatographic details are displayed either as total reconstructed ion current (RIC) versus time, or as mass fragmentography, which represents the ion current related to one specific mass versus time. All mass spectra are presented in normalized format and represent a resolution of one mass unit. Scan averaging (SA) and rapid single ion monitoring (SIM) or multiple ion monitoring (MIM) of 1 to 10 scans/second was utilized for isotopic averaging in order to detect incorporation of isotopically labeled precursors.

In those instances where gas chromatography without mass spectrometry was employed a Hewlett Packard 5830A gas chromatograph was employed. Chromatography was accomplished on a 2m x 6mm glass column, packed with 3% SP2100 on Supelcoport (110 to 120 mesh). Temperature programming was employed consisting of an initial temperature of 170° C maintained for 1 min, then increased to 220° C at a rate 10° C/min, for the chromatographic separation. Helium was employed as a carrier gas and maintained at a constant flow rate of 25 mL/min. Detection was accomplished by flame ionization (FID) or by Hall Electrolytic Conductivity (HECD) normally operating in the sulfur specific mode.

#### Infrared Spectroscopy

Infrared spectra were measured on a Perkin Elmer model 1430 ratio-recording Infrared Spectrometer interfaced to a Perkin Elmer model 3600 data station. Spectra were generally obtained as neat films between silver chloride plates. The absorption bands are reported in wave numbers ( $\text{cm}^{-1}$ ) and are designated as strong (s), medium (m), weak (w), shoulder (sh), sharp (sp), and broad (bd).

#### Ion Specific Electrode Analyses

Chloride ion was detected with an Orion model 94-1700 chloride selective electrode interfaced to an Orion model 901 ionalyzer. Chloride concentration was followed on a

strip chart recorder as a function of time in preliminary gelatin capsule experiments. A typical experiment involved placing capsules (100 mg content) holding solid sodium chloride (typically 50 mg) into 200 mL buffered (pH 8) distilled water at room temperature. A magnetic stirring bar was added and set to 50 rpm (medium). In the experiment described below, two separate capsule styles were evaluated, one with a single layer, the other consisting of two capsules, one encapsulated by another, with sodium chloride within the inner-most capsule. Typically a small portion of silicon grease was employed to form a water tight seal between gelatin surfaces.

#### $^1\text{H}$ NMR Spectrometry

$^1\text{H}$  NMR spectra were determined on an XL-100-FT spectrometer using tetramethylsilane (TMS) or a deuterium lock as an internal reference. All spectra were recorded in the frequency sweep mode. All signal multiplicities are denoted as singlet (s) doublet (d), triplet (t), and multiplet (m). A signal may also be occasionally designated as being sharp (sp) or broad (bd).

#### High Resolution Mass Spectrometry

High resolution mass spectra were obtained on a Varian Mat 311 high resolution mass spectrometer operating with ionizing voltages between 20 and 70 eV.

### Experimental Introduction

Porifera taxonomy is a complex and highly specialized branch of Systematics. Sponge samples were submitted to Dr. Patricia Bergquist, of the University of Auckland, New Zealand, for identification. The experimental sponge had previously been identified as *Hymeniacidon* sp.<sup>43</sup> but should be *Ciocalypta* sp.<sup>44</sup> This sponge is almost exclusively found growing in shallow, dark caves along the Pupukea shoreline of the North Shore of O'ahu. Several attempts have been made to locate this sponge in other geographical areas about O'ahu with little success. There has been some indication that the sponge also resides in the Ala Wai Channel on the South Shore of O'ahu near the Ala Wai boat harbor. However, although taxonomically identical, metabolites isolated from this sponge differ in their basic carbon skeletons.<sup>45</sup> Diving conditions at Pupukea limit *in situ* experimentation and observation of this species to the months of May to September. Water conditions are too rough for diving during the other months of the year. An early discovery of a naturally occurring isocyanosesquiterpene involved the nudibranch *Phyllidia varicosa*. This mollusk uses 9-isocyanopupukeanane (6) for protection against predators and sequesters it from its prey, a member of the sponge genus *Ciocalypta* sp. *Ciocalypta* sp. is a massive, thick, pale white to slightly yellow sponge.



### Capsular Incorporation Technique

In general, biosynthetic work involving Porifera has been plagued by the apparent lack of incorporation of labeled precursors into the metabolic pathways under investigation. One of the many reasons for this is the relative inability of the investigator to ascertain whether or not the animal is still living before or after the attempted incorporation. Obviously, minimal manipulation is desirable. In my work I attempted to address this issue by assuring that all experimentation was conducted *in situ* in the ocean. Furthermore, since *Ciocalypta* sp. is a massive, rigid sponge, simple physical insertion of a solid, gelatin encapsulated labeled precursor seemed to be the least invasive way to attempt its introduction. This was particularly true in view of the fact that these sponges grow in narrow, underwater caves constantly experiencing intense surges. Several experiments employing both single and double (encapsulated) 100 mg gelatin capsules were conducted in order to evaluate dissolution times and general capsule stability under water. Results of one such set of experiments are displayed below in Figure 4.

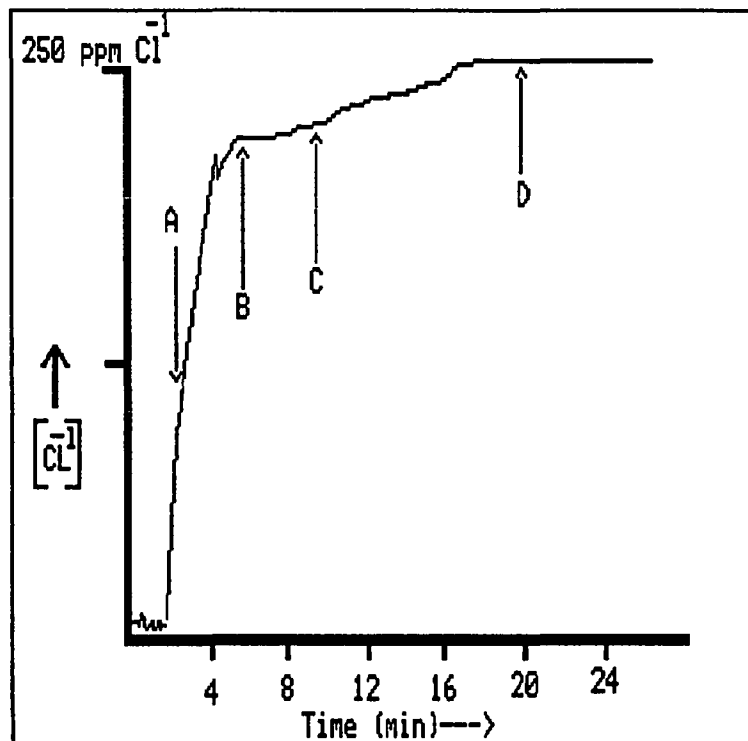


Figure 4. Chloride concentration as a function of time. A= Breakthrough of single capsule (CS). B= Complete dissolution of contents within CS. C= Breakthrough of doubly encapsulated capsule (DEC). D= Complete dissolution of contents of DEC.

In this work it was established that a single capsule would dissolve and release its contents within 5 minutes. The single capsule wall would break and begin releasing its contents within approximately 100 seconds. In the doubly encapsulated experiments it was established that capsule breakthrough would occur within 9.5 minutes, with complete release and dissolution of contents within 19.4 minutes.

### In situ Experimentation with SCUBA

A typical experiment was conducted in the following manner. Solid, labeled precursor, either synthetically produced, or directly purchased from commercial sources, was carefully packaged in double-lined 100 mg gelatin capsules. Normally two capsules were employed in each incorporation experiment. The capsules were sealed in a water-tight, screw-top bottle, which was carried by the diver. Upon locating a suitable animal, based primarily upon weight (normally a minimum of 100 g) and to some extent upon convenience of location, the precursor was embedded. This involved making a fine 5 mm incision into the sponge tissue. The gelatin capsules were then removed from the sealed container and inserted into the incision. The capsules maintained their structural rigidity for approximately 3 to 4 minutes under these conditions before disintegrating into a gelatinous mass. One had to work fairly rapidly at this stage. Then, a small portion of *Ciocalypta* sp. was removed from a distant location and used as a plug to seal the capsules within the incision. The animal was then tagged with a small wire so that it could be readily relocated.

### Sponge Extraction and Preparation

After two to three weeks, the animal was harvested and returned to the laboratory packed in Dry Ice. An inspection was made to determine whether or not the incision had indeed "healed". Generally, it had not, and was still visible to the naked eye. The sponge was then sectioned with a sharp knife, and the portion which contained the labeled precursor (normally 5 to 10 g wet weight) was diced into fine cubes for extraction. Extraction was performed with methanol (10 mL, reagent grade) to the wet, chopped sponge in a 50 mL centrifuge tube. Hexane (1 mL / g of wet sponge) was then added and the mixture was shaken vigorously for 2 to 3 min. An emulsion normally resulted, which was broken by centrifuging the extraction tube at 8000 rpm for 5 min.

The upper hexane layer was then analyzed directly via capillary gas chromatography/mass spectrometry. An example of a typical gas chromatogram resulting from the extraction outlined above is shown in Figure 5.

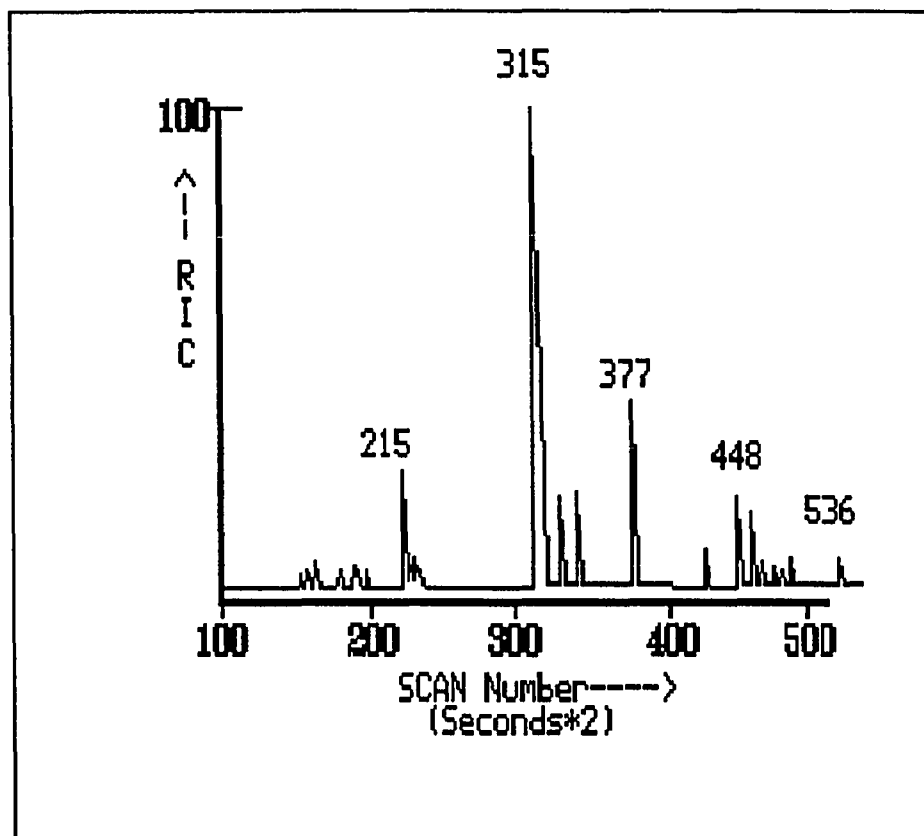


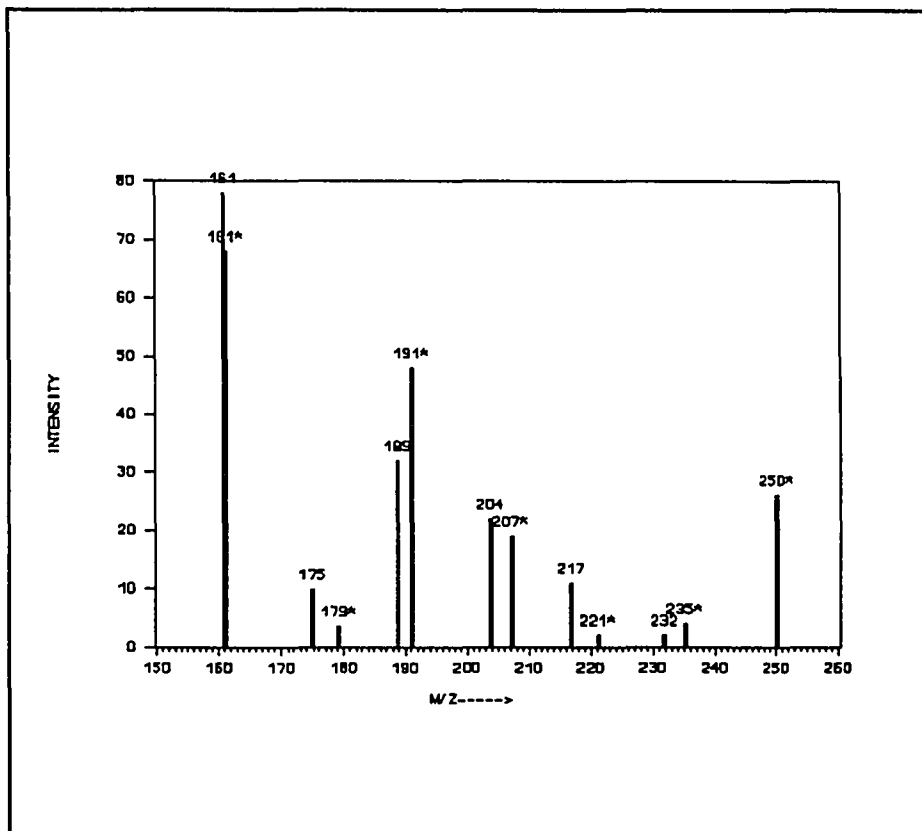
Figure 5. GC/MS of crude hexane layer.

#### Mass Spectrometry: Methodology and Techniques

Chromatographic separation of each metabolite was accomplished via time-of-flight, gas chromatography / mass spectrometry. A 30 m, 0.25 mm id, 0.25 u film thickness, DB-5 (medium polarity) capillary column was employed for all chromatographic work. Temperature programming was employed as described in the Instrumental Techniques section above. Care was taken to properly calibrate and electronically zero the mass spectrometer before each chromatographic separation.

Calibration was conducted automatically daily using Finnigan OWA software and was based on the normal ion intensities and isotope ratios of perfluorokerosene (PFK). Ion intensities and ratios were checked daily to make sure that both were within the manufacturer's specifications.

Selected ion monitoring employing rapid (less than 1 sec) scanning was employed for averaging purposes. Natural and labeled (+1) masses were viewed on a time-of-flight basis and resulting data were reduced and refined with use of Finnigan OWA data handling software. In certain instances, isonitrile metabolites were hydrolyzed to formamides in order to increase sensitivity by enhancing the intensity of the molecular ion. Figure 6 represents the relative intensities of the formamide vs the isonitrile molecular ions.



**Figure 6.** Relative intensities of molecular ions for the  $^{13}\text{C}$ -2-formamido-pupukeanane (--- \* ---, m/z 250) and  $^{13}\text{C}$ -2-isocyano-pupukeanane (m/z 232).

In an attempt to define the lower detection limit of the current methodologies to qualitatively and quantitatively detect incorporation of a labeled precursor into the normal metabolism of the sponge, the following experiment was conducted. Known weights of synthetic 2- $^{13}\text{C}$ -formamido-pupukeanane were directly added to known weights the natural product obtained from the hydrolysis of the purified isonitrile by dilute acetic acid.

Each sample was quantitatively assayed for "incorporation". Results are shown in Figure 7.

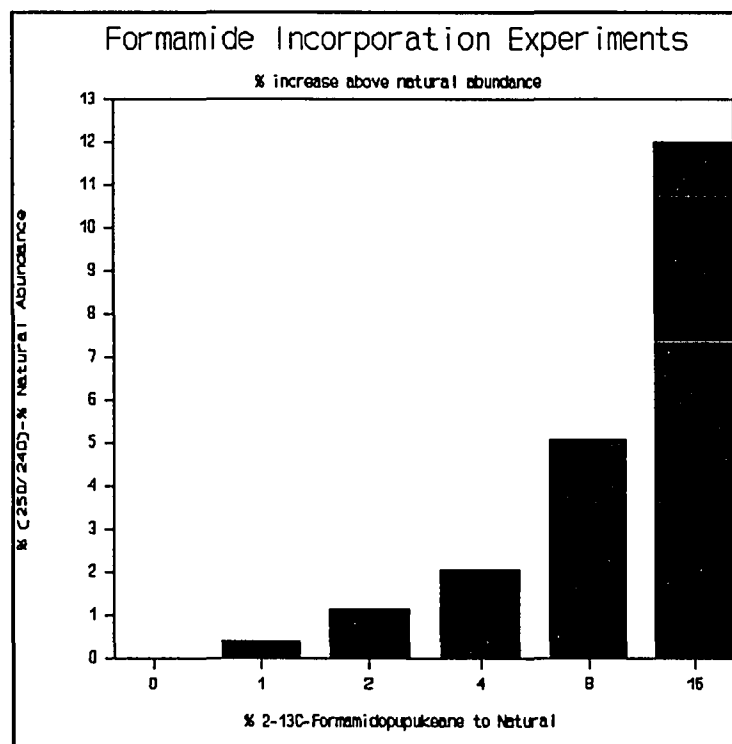


Figure 7. Formamide incorporation experiments.

The experimental data presented above suggest that one should be able to reliably detect a 2% (or greater) incorporation of a <sup>13</sup>C labeled metabolite into the normal metabolism and biosynthetic production of the isonitrile. Below 2% noise and uncertainty of measurement would likely interfere with such an assignment. This experimental work formed the qualitative and quantitative basis of the methodology employed for the formal **detection of incorporation** described in this work.

Minor Metabolites of *Ciocalypta* sp.

Infrared Spectroscopy of Crude Extracts

Crude methylene dichloride extracts of *Ciocalypta* sp. were prepared by partitioning 10 g of wet diced sponge between methanol (20 mL, reagent grade) and methylene dichloride (50:50). The crude extract was then taken to dryness, and the resinous residue, which had a pronounced odor, was scanned via infrared spectroscopy, neat on sodium chloride. The IR spectrum of the crude extract is shown in Figure 8. Several notable features are apparent in the spectrum.

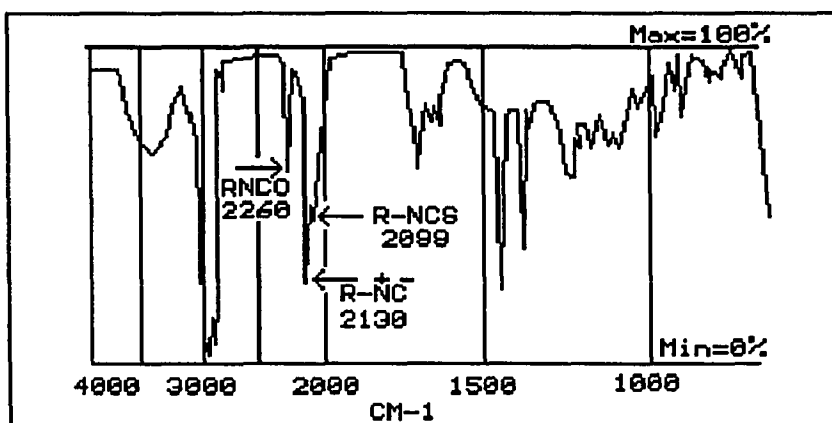


Figure 8. IR spectrum of crude methylene chloride extract, neat (NaCl).

Note the three bands present between 2500 and 2000  $\text{cm}^{-1}$ . These bands are known to be due to the presence of the isocyano ( $2130 \text{ cm}^{-1}$ ), isothiocyano ( $2120 \text{ cm}^{-1}$ ) and the isocyanato ( $2260 \text{ cm}^{-1}$ ).

These observations were to play a central role in the characterization of the minor metabolites in the crude *Ciocalypta* sp. extracts. Furthermore, the combination of the distinctive diagnostic region of the IR spectrum, from 1500 to 3500  $\text{cm}^{-1}$ , as well as absorption bands in the fingerprint region between 1500 to 650  $\text{cm}^{-1}$  became the subject of a later paper<sup>48</sup> involving the use of infrared spectroscopy in *Porifera* taxonomy.

#### GC/MS of Crude Extracts

Crude methylene dichloride extracts prepared in a similar manner (*vide supra*) were introduced into a Finnigan gas chromatograph/mass spectrometer. The resulting reconstructed ion chromatogram (RIC) is shown in Figure 9.

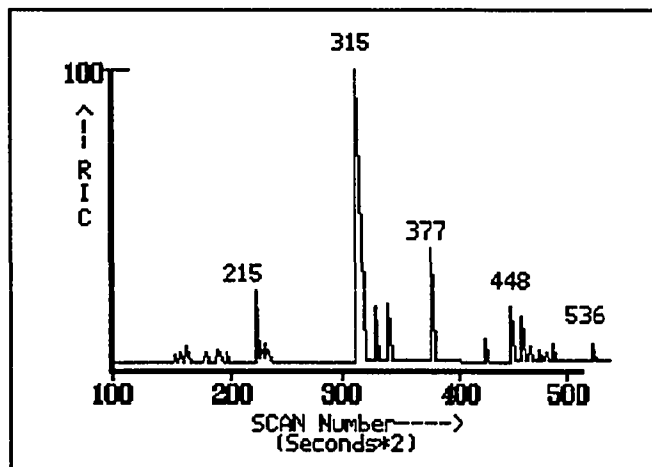


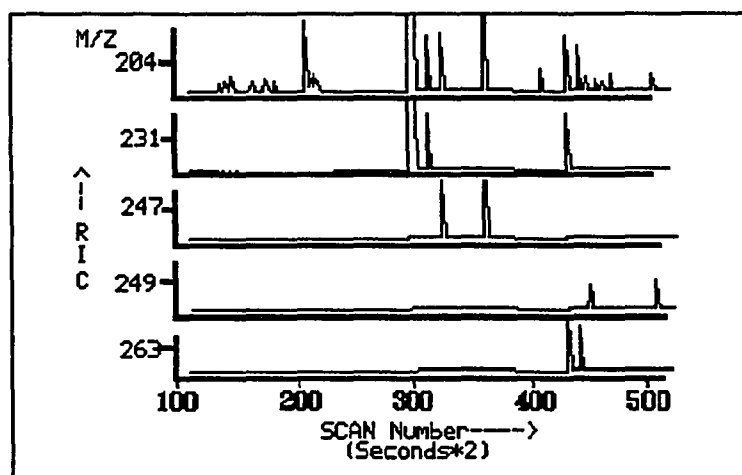
Figure 9. Reconstructed ion chromatogram of crude  $\text{CH}_2\text{Cl}_2$  extract, in  $\text{CH}_2\text{Cl}_2$ .

High resolution capillary gas chromatographic/mass spectrometric analysis of sponge extracts revealed several interesting features. Table II outlines the presumptive, structural correlation with each characteristic mass displayed in Figure 10.

**Table II. Correlation of Structure with Characteristic Molecular Ion**

<u>M/Z</u>	Presumed Structure
204	Hydrocarbon Skeleton (HS)
231	Isocyano-HS
247	Isocyanato-HS
249	Formamido-HS
263	Isothiocyanato-HS

Mass chromatograms of selected, characteristic molecular ions are shown below in Figure 10.



**Figure 10.** Mass chromatograms (m/z 204, 231, 247, 249 and 263) of crude  $\text{CH}_2\text{Cl}_2$  extract, in  $\text{CH}_2\text{Cl}_2$ .

Gas chromatography employing element specific detectors were also employed with interesting results. Figure 11 below shows a comparison of identical chromatography employing in instance "A" a general detector (FID) and in instance "B" an element specific (sulfur) detector (HECD).

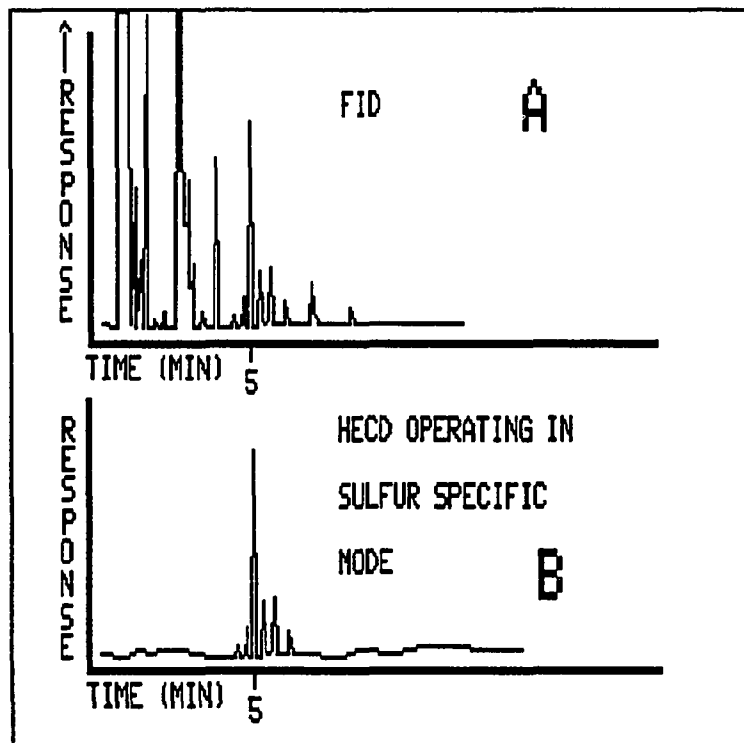
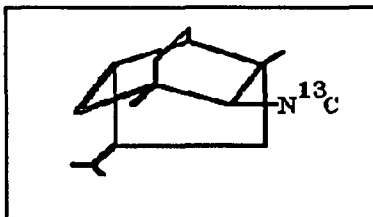


Figure 11. Comparison of identical chromatography with two different detection systems; A= FID; B= HECD (sulfur specific mode).

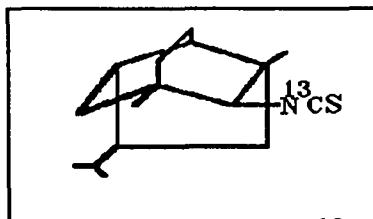
### Semi-Synthetic Metabolites

Several metabolites, which are normally present in the sponge, were labeled at the isocyano carbon with  $^{13}\text{C}$ . The synthesis of these metabolites is described elsewhere.<sup>49</sup>

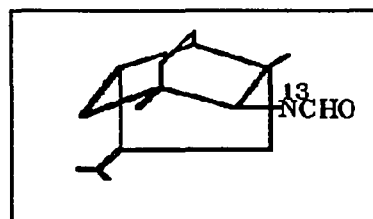
Names and structures are as follows.



**Figure 12.** 2- $^{13}\text{C}$ -isocyano-pupukeanane



**Figure 13.** 2- $^{13}\text{C}$ -isothiocyano-pupukeanane



**Figure 14.** 2- $^{13}\text{C}$ -formamido-pupukeanane

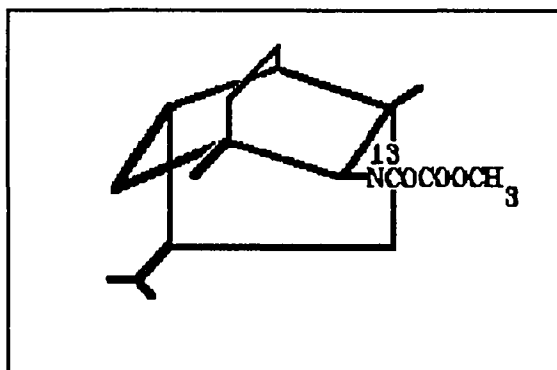


Figure 15. 2-<sup>13</sup>C-oxalato-pupukeanane

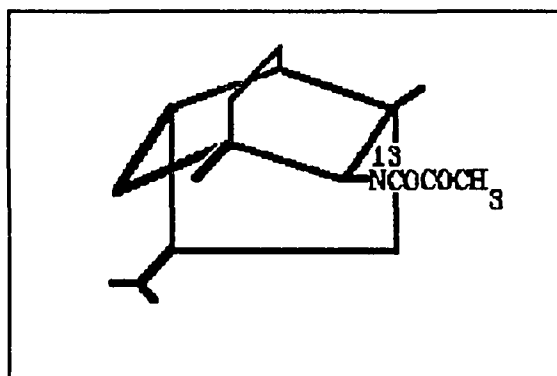


Figure 16. 2-<sup>13</sup>C-pyruvamido-pupukeanane

Mass spectra of all labeled metabolites are shown in Figures 17 through 21.

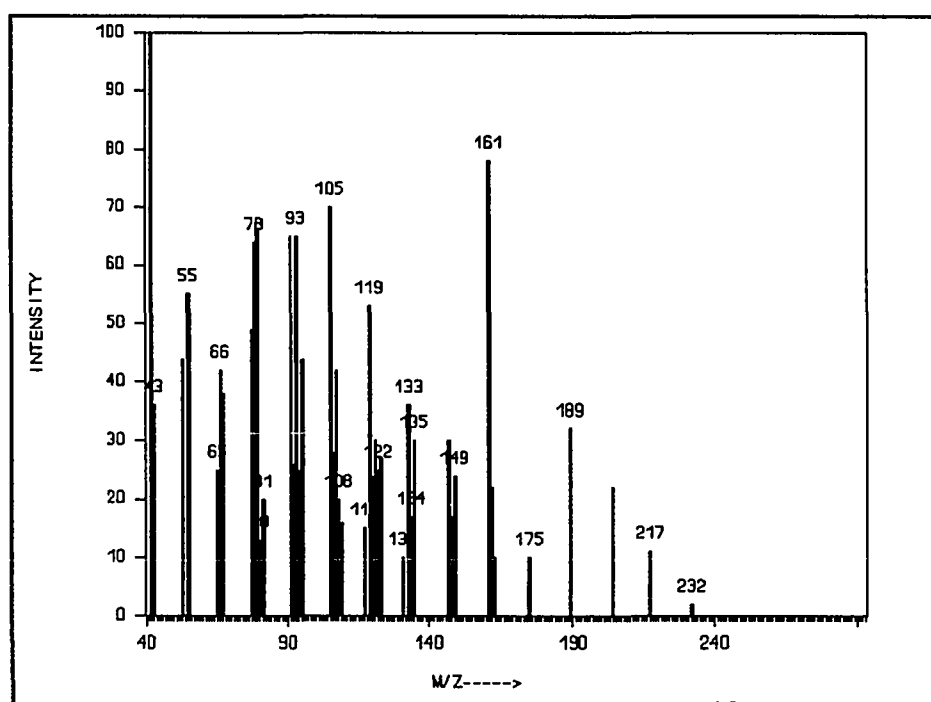


Figure 17. Mass spectrum of 2-<sup>13</sup>C-isocyanopupukeanane.

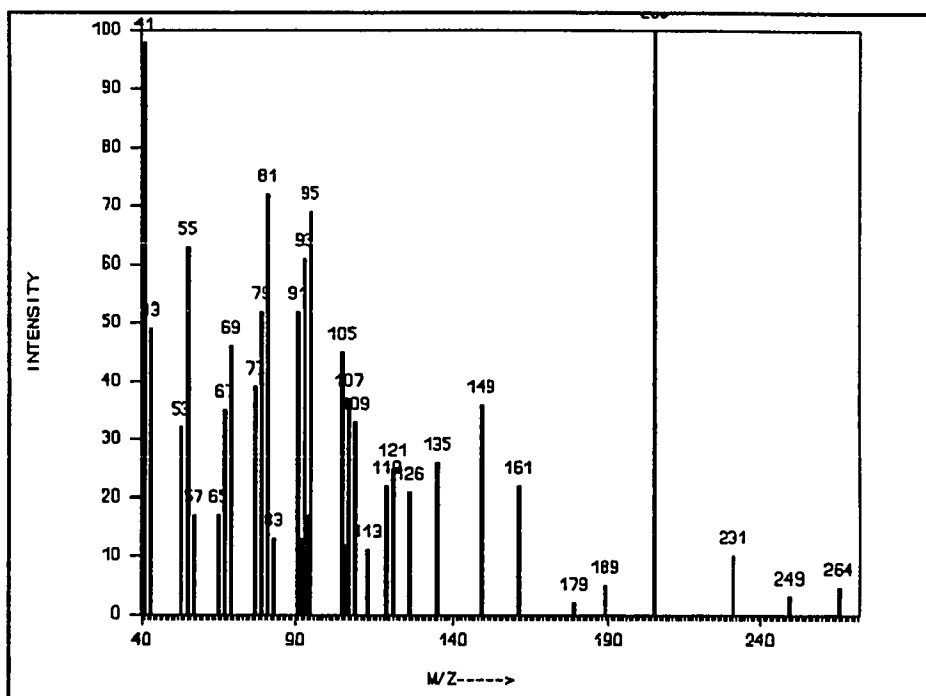


Figure 18. Mass spectrum of 2-<sup>13</sup>C-isothiocyanopupekeanane.

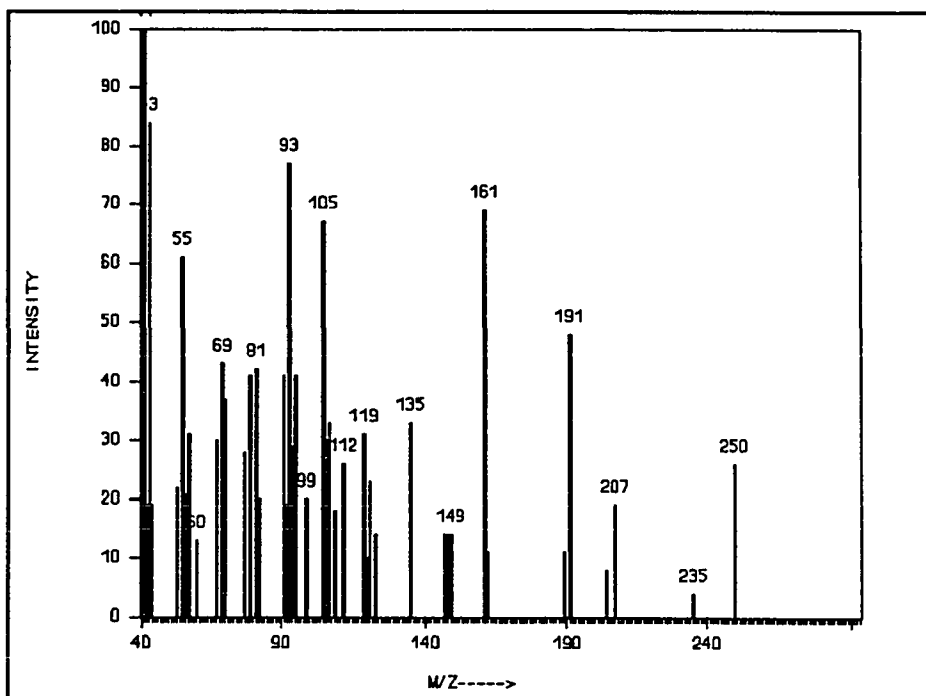


Figure 19. Mass spectrum of 2-<sup>13</sup>C-formamidopupekeanane.

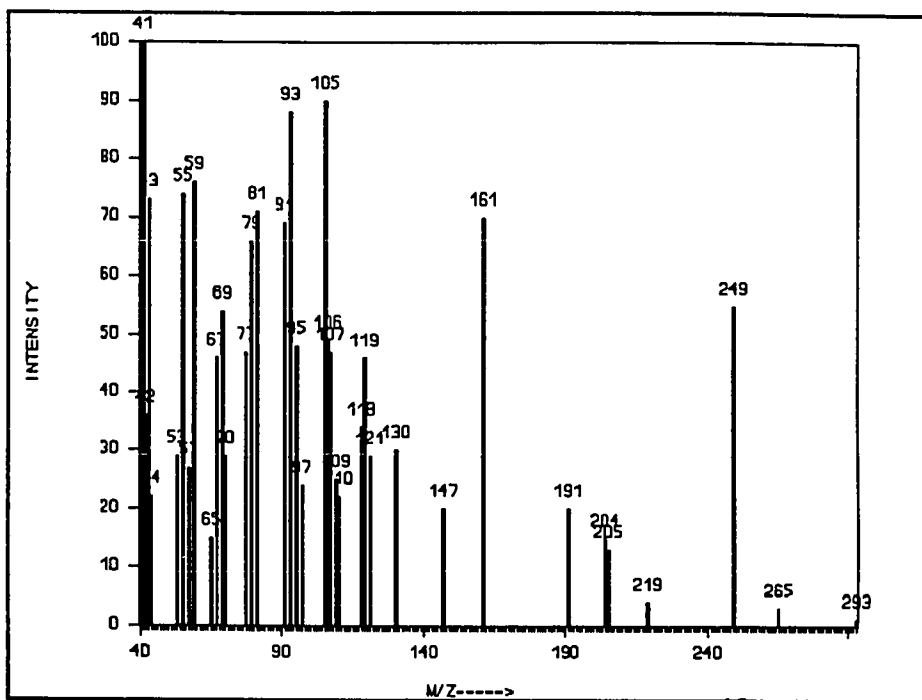


Figure 20. Mass spectrum of 2-<sup>13</sup>C-oxalato-pupukeanane.

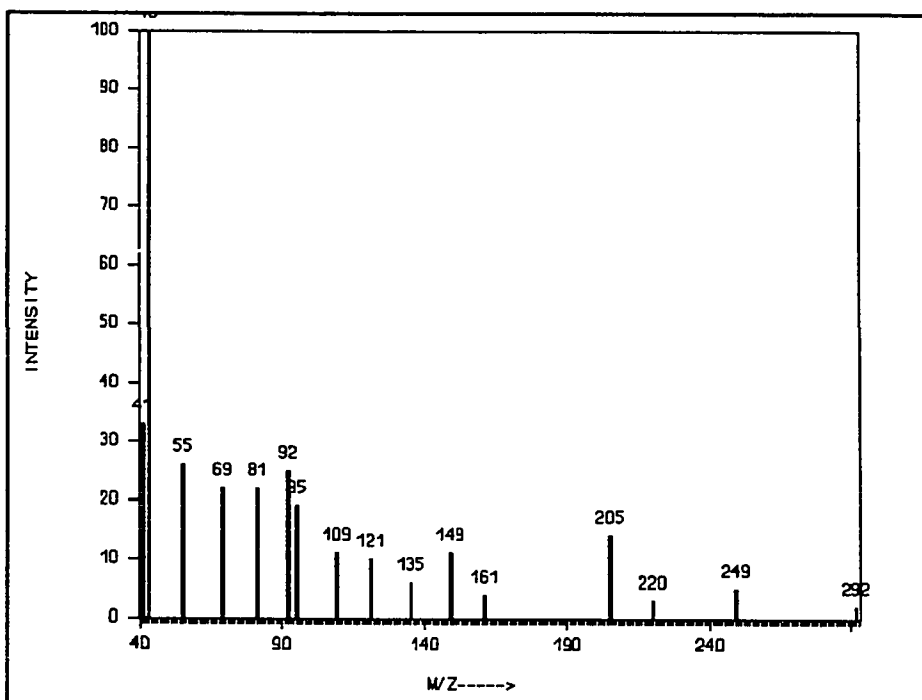


Figure 21. Mass spectrum of 2-<sup>13</sup>C-pyruvamido-pupukeanane.

Chromatographic Isolation and Purification  
of Sesquiterpene Hydrocarbons

From early spectrographic and chromatographic studies of the crude extracts obtained from *Ciocalypta* sp. it was apparent that a significant percentage of the entire weight of the extract consisted of a hydrocarbon oil. A gc/ms of the hydrocarbon fraction is presented in Figure 22 below.

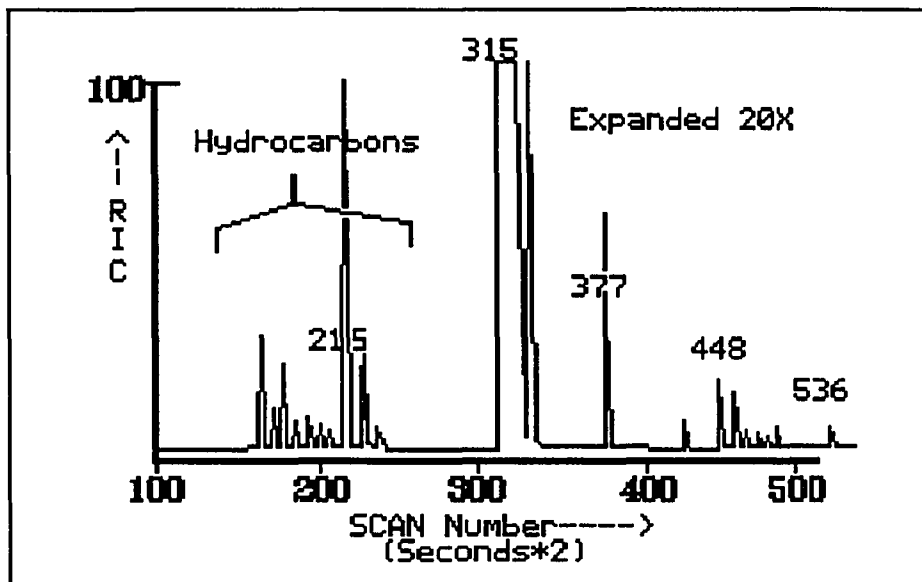


Figure 22. Gc/ms of *Ciocalypta* sp. hydrocarbon fraction.

This hydrocarbon fraction could be readily isolated as an oil by column chromatography on silica employing hexane as an eluent. The actual yields of this hydrocarbon differed from sponge to sponge, and from season to season, but were always significant. Although apparently structurally similar to the isocyanopupukeanane series of functionalized hydrocarbons,

on the basis of the fragmentation pattern alone, it was never rigorously isolated or purified for comprehensive spectrochemical studies. Because of their abundance in *Ciocalypta* sp. extracts, the hydrocarbons were later used to demonstrate the enzymatic character of cyanide incorporation.<sup>50</sup>

### 2-Isocyanopupukeanane

By far the most abundant and reliably present functionalized terpene in *Ciocalypta* sp. extracts was the previously characterized 2-isocyanopupukeanane (7).<sup>51</sup> Gram-quantities of this purified metabolite were necessary for the synthetic production of <sup>13</sup>C- labeled substrates and precursors used in this work. Because of this, considerable energy was spent in developing efficient methodologies for the isolation and purification of this metabolite. 2-Isocyanopupukeanane was typically isolated and purified in one of two ways.

#### 1. By Chromatography

The diced sponge (typically 100 g) was first thoroughly extracted (overnight) with 100 mL of a 50:50 mixture of methanol / dichloromethane. The mashed organic extract was then vacuum-filtered employing a 10 cm diameter Büchner funnel, with the wet filtrate subsequently partitioned with an equal volume of hexane. The hexane layer was separated and concentrated *in vacuo*.

The thick, pungent-odorous oil, which remained was dissolved in a minimum quantity of hexane and placed directly on the top of a 2.5 cm by 30 cm silica gel column, which had been prepared from a hexane slurry. A solvent gradient elution was performed as follows: 50 mL hexane, 50 mL dichloromethane/ hexane(1:9), 50 mL dichloromethane/ hexane(4:6), 50 mL dichloromethane/ hexane(6:4), 50 mL dichloromethane. Chromatography was conveniently followed by odor, with the isonitriles eluting in conjunction with the first 10 to 20 mL of the 4:6 solvent mixture. Elution of the isonitriles was complete with the 6:4 dichloromethane/ hexane elution step. One could also follow the chromatography with the appearance and elution of a bright yellow pigment, which co-chromatographed with the isonitrile. Metabolite elution generally was in the order hydrocarbons, isothiocyanates, isocyanates, yellow pigments, isonitriles, and formamides employing normal phase silica gel chromatography. Separation of the isothiocyanates from the isocyanates was generally the most difficult to accomplish. Separation of various yellow sponge pigments (as yet uncharacterized) from the isonitriles was also chromatographically difficult and was best accomplished via fractional recrystallization from isooctane. Once chromatographically separated, the isonitrile(s) tended to crystallize upon standing at room temperature.

2-isocyanopupukeanane could be further purified by carefully dissolving the yellow crystals in warm (100 °C) isooctane (sparingly soluble) and allowing the concentrated solution to rapidly crystallize in the freezer. There was no apparent advantage to slowly crystallize the isonitriles because of the extreme differences in solubilities of the pigments and the isonitrile. Furthermore, no serious occlusion problems were noted; the high purity of the resulting crystals was demonstrated by gas-liquid chromatography. Recrystallization was best accomplished in small 2 dram glass vials with final isooctane volumes of the order of 0.5 to 1 mL.

Once recrystallized, most of the mother liquor was removed while still cold with the use of a Pasteur pipet and rubber bulb. Any residual liquid still clinging to the crystals was removed by inverting the vial in a larger test tube and using gentle centrifugation to remove isooctane, which often tenaciously adhered to the lower half of the glass vial. This procedure typically resulted in gas chromatographic purity in excess of 97% by weight of 2-isocyanopupukeanane. The crystals would be devoid of other isonitrile impurities, as well as any yellow discoloration generated from the co-elution of the pigments described above.

## 2. By Liquid/Liquid Extraction

The second method of isolation and purification was

based upon earlier literature by Ugi,<sup>52</sup> which drew attention to the fact that isonitriles, in general, are good ligands for coordination with both transition and IB and IIB group metals. In particular, copper, silver, gold, zinc, cadmium, and mercury form bonds (with essentially *sigma* character) with isonitriles. Generally, synthesis of metal isonitrile complexes is straightforward and consists of mixing of a metal salt with the isonitrile in a solvent under mild conditions to avoid polymerization. Often an excess of isonitrile reduces the metal to a lower oxidation state which is then stable in the isonitrile complex.<sup>53</sup> This observation was employed advantageously for selective and nearly quantitative removal of all isonitriles from the crude *Ciocalypta* sp. extract. The procedure was relatively non-critical and involved the preparation of the reagents in Table III:

**Table III.** Reagents Employed for the Isolation of the *Ciocalypta* sp. Isonitriles from the Crude Metabolite Mixture

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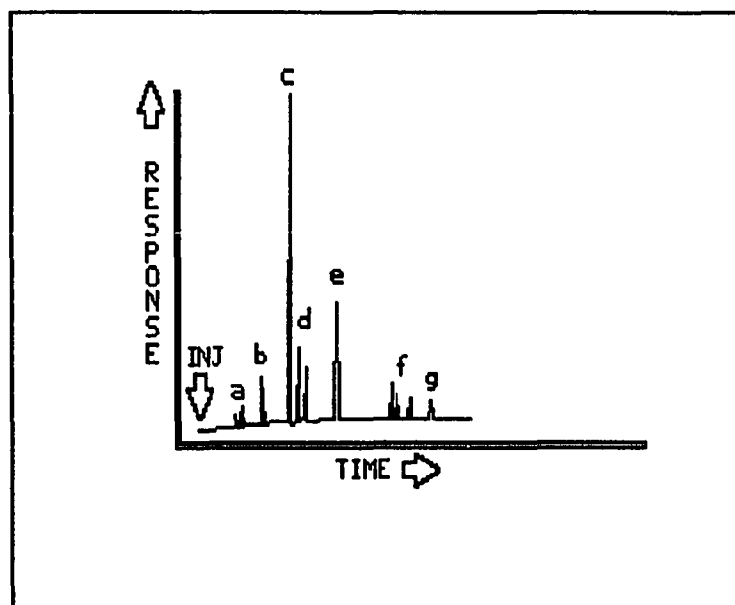
Reagent A:	A saturated solution of sodium chloride in tap water
Reagent B:	A solution of 0.1% silver nitrate in methanol/distilled water (1:1)
Reagent C:	A solution of hexane saturated with methanol

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For the preparation of these three reagents, it was

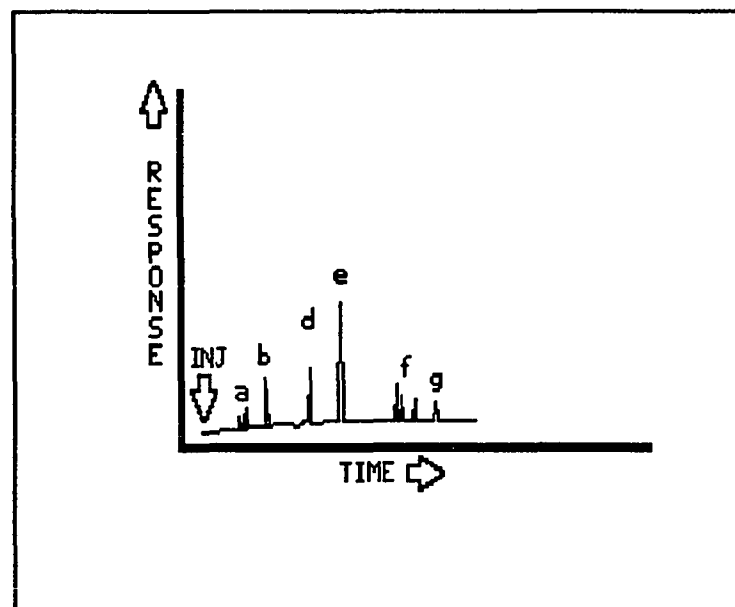
important to first dissolve silver nitrate in distilled water before adding methanol, resulting in a 0.1% concentration of silver nitrate (as silver nitrate) in methanol/distilled water (1:1). Otherwise, the dissolution of silver nitrate is exceedingly slow in the methanol/distilled water mixture. Once prepared, the silver nitrate solution should be stored refrigerated in the dark.

Isolation of the isonitriles began with dissolving the crude hexane extract (isolated from the sponge as described above) into the hexane solution (typically one gram of extract to 50 mL of hexane solution). The hexane extract was then transferred to a 500 mL separatory funnel, and washed once very gently with 50 mL distilled water to remove any residual salts carried through from the initial isolation. If vigorous shaking is employed, a stable emulsion will result because of the surfactant-like nature of the isonitriles in the extract. The aqueous (bottom) layer is discarded and 25 mL of reagent B is added to the funnel. The contents are gently shaken and allowed to separate. The aqueous bottom layer is removed and saved. The process above is repeated twice, all aqueous layers are combined for a total of 75 mL of Reagent B. The hexane layer is then discarded. Reconstructed gas-liquid-chromatograms of the hexane solution before and after the metallation of the isonitriles illustrating the efficiency of the process are shown in Figures 23 through 24.



**Figure 23.** Gc/ms of crude hexane extract before treatment with Reagent B.

a=minor hydrocarbons, b=major hydrocarbon, c=2-isocyanopupukeanane,  
 d=unknown isonitrile and unknown isocyanate,  
 e=isocyanatobisaboline, f=isothiocyantes, g=formamides.



**Figure 24.** Gc/ms of crude hexane extract after removal of isocyanate component.

Once combined, the silver extracts are protected from light and kept refrigerated to avoid the possibility of catalytic homopolymerization<sup>54</sup>.

The isonitriles exist in the methanolic layer as silver complexes with a bond order of the isonitrile C--N bond between 2 and 3. Silver in oxidation state +1 produces primarily  $\sigma$  bond character with very little back bonding (or back donation) available for contribution via classical  $\pi$  bonds. This situation is significantly reversed in the case of transition metals.<sup>55</sup> The *sigma* bonding scheme is illustrated in Figure 25.

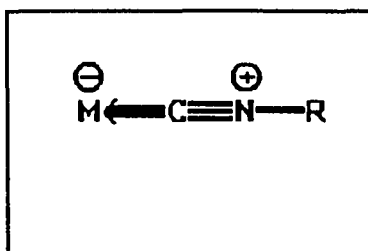


Figure 25 .  
Illustration demonstrating the major tautomeric contribution to bond order of the isonitrile CN bond upon metallation.

These relatively unstable silver complexes have been briefly characterized via infrared spectroscopy.<sup>56</sup> In this work, it was noted that these complexes are moderately soluble in methylene dichloride, but nearly insoluble in hexane.

The combined fractions were returned to a 250 mL separatory funnel and washed gently with 20 mL hexane. The upper hexane layer was discarded. Reagent A (20 mL) was then added with an immediate, concomitant precipitation of silver chloride. The aqueous/ methanolic layer was washed 3x with reagent C (25 mL ea). The lower aqueous layer was discarded. The hexane layer was then separated, concentrated *in vacuo* and a semi-solid resulted, which would eventually crystallize upon refrigeration.

#### Isocyanatobisabolene

In the course of the investigation into the structural identity of various minor metabolites in *Ciocalypta* sp. it was noted that three bands between 2500 and 2000  $\text{cm}^{-1}$  existed in the infrared spectrum of crude hexane extracts of *Ciocalypta* sp. These absorptions at 2260, 2130 and 2120 (sh)  $\text{cm}^{-1}$  were tentatively assigned to NC (2130  $\text{cm}^{-1}$ ) and NCS (2120  $\text{cm}^{-1}$ ), but the band at 2260  $\text{cm}^{-1}$  was outside the normal range for either NC or NCS. I originally presumed that the origin of the high frequency band was due to a cyano function, perhaps generated by thermal rearrangement of the isocyano metabolites. However, in further work involving gas chromatographically significant metabolites (Figures 9 through 10, pages 38 through 40) present in the crude *Ciocalypta* sp. extracts,

it was noted that one major chromatographic peak consisted of a molecular ion of 247. Its mass spectrum (Figure 26) together with its fragmentation pattern, losses of 15 ( $\text{CH}_3$ ), 29 ( $\text{HCO}$ ), 43 ( $\text{C}_3\text{H}_{10}$ ), and 58 ( $\text{C}_4\text{H}_{10}$ ) mass units indicated the potential of an isocyanato functionality present on a non-pupukeanane, skeleton.

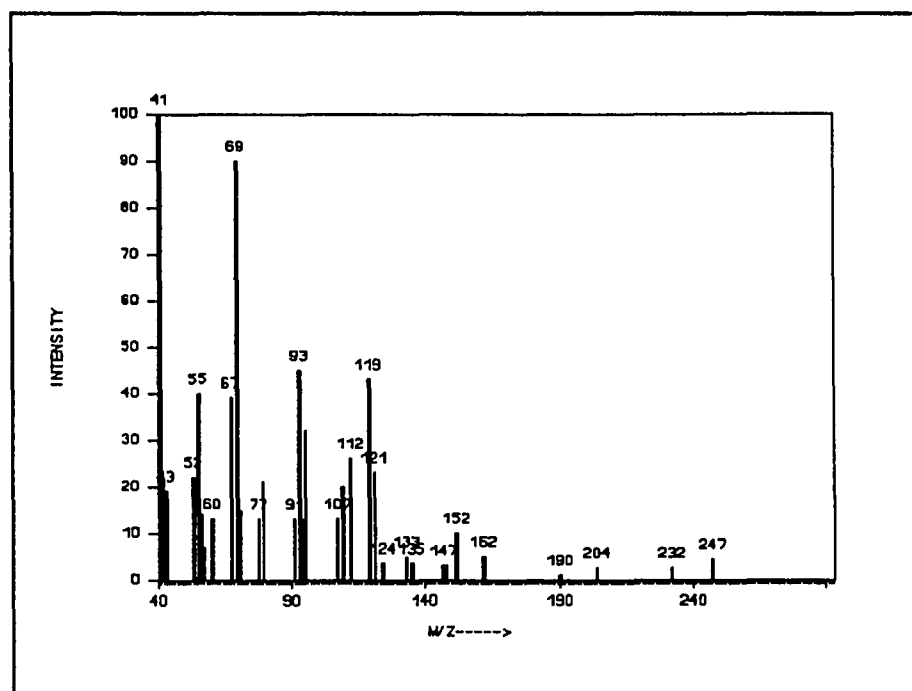


Figure 26. Mass spectrum of isocyanatobisabolene.

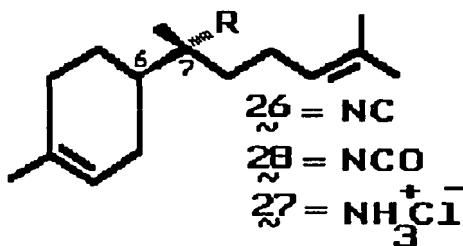
On the basis of this observation, microgram quantities of the metabolite were isolated employing thin layer chromatography (silica gel, hexane eluent,  $R_f = 0.8$ , UV visualization). Yields were very poor as considerable amounts appeared to remain at the origin, but were adequate for preliminary characterization.

Separation of the nearly co-eluting and previously characterized isothiocyanates was extremely difficult. Further characterization via infrared spectrometry,  $^1\text{H}$  NMR spectroscopy, and high resolution mass spectrometry (solid probe) were conducted on the minute quantities isolated and purified via TLC. This work proved that the  $2260\text{ cm}^{-1}$  absorption in the infrared spectrum arose from a compound of composition  $\text{C}_{16}\text{H}_{25}\text{NO}$ . Although apparently structurally dissimilar from the isocyanopupukeanane series of functionalized hydrocarbons on the basis of the fragmentation pattern alone, it was never rigorously isolated or purified for comprehensive spectrochemical studies. Because of its major abundance in *Ciocalypta* sp. extracts it became, however, the subject of a later paper.<sup>57</sup> In this work, three nitrogenous bisabolene sesquiterpenes, an isocyano metabolite (26), an amine hydrochloride (27) and an isocyanate (28) were isolated and characterized from the marine sponge, *Ciocalypta* sp. The presence of the unprecedented isocyanate was confirmed by reacting (28) with *p*-bromobenzylamine in methylene chloride, which yielded crystals of the corresponding *p*-bromobenzylurea suitable for x-ray diffraction analysis.

An interesting observation was made regarding the nature of the functionalized C-7 position in the bisabolene skeleton.

In an accompanying paper<sup>58</sup> Faulkner, et al. reported the existence of four other bisabolene metabolites obtained from a sponge, *Halichondria* sp. The two series of bisabolene derivatives had R-stereochemistry at C-6, but were epimeric at the nitrogen-bearing carbon C-7. The antipodal nature of the C-7 carbon imply that an sp<sup>2</sup>-hybridized C-7 is the immediate precursor of the nitrogenous metabolites.

The observation of the presence of both antipodes at the nitrogen-bearing carbon is not new: It was first reported by Hagadone et al.<sup>59</sup>, and recently re-investigated and confirmed by Fusetani et al.<sup>60</sup> in the isocyanopupukeanane series of metabolites from *Ciocalypta* sp.



## Biosynthetic Studies

### The Incorporation Experiments: General Considerations

A total of 11 labeling experiments were carried out. The labeled precursors chosen and the general experimental parameters involved are summarized in Table IV. The experiments are presented chronologically in the experimental order in which they were attempted.

**Table IV.** Isotopic Labeling Experiments Conducted on *Ciocalypta* sp. *in situ*

Substrate	Amount	Incubation Time (weeks)
Sodium <sup>13</sup> C formate	200 (mg)	3
2- <sup>13</sup> C formamido-*	70	3
2- <sup>13</sup> C isocyano-*	60	2
2- <sup>13</sup> C isothiocyano-*	45	2
<sup>15</sup> N glycine	200	2
<sup>13</sup> C-1-glycine	100	4
<sup>13</sup> C-2-acetate	100	3
<sup>13</sup> C-2-pyruvamido-*	15	4
<sup>13</sup> C-2-oxalato-*	33	2
<sup>13</sup> C potassium cyanide	102	3
<sup>15</sup> N Urea	100	3

Experimental details and results of biosynthetic experimentation of the first four substrates listed in Table IV above were published in the *Journal of the American Chemical Society*.<sup>61</sup> In this work, we allowed the animal, *Ciocalypta* sp., to remain in its natural habitat until completion of the experiment.

### Capsular Incorporation Technique

We employed SCUBA to embed the  $^{13}\text{C}$ -labeled precursors encased in double gelatin capsules into the live animal. At the conclusion of the each experiment we harvested the sponge, extracted the lipophilic metabolites as previously described and analyzed by GC-MS. We were able to show, as was the case previously in xanthocillin experiments<sup>62</sup>, that formate is not used as a source of the isocyano carbon. Furthermore, we successfully confirmed Sodano's<sup>63</sup> earlier conclusion, that formamide is not a precursor of isocyanide.

In our work with  $^{13}\text{C}$  labeled isothiocyanato, and isocyano metabolites, we were able to show conclusively that isothiocyanate was not transformed into isocyanide, but that both isothiocyanate and formamide were biosynthesized from the isocyanide. With independent analyses of seven parallel sponge slices situated at or about two distinct and separate locations of labeled precursor insertion we demonstrated that little diffusion of the implanted label occurred.

Work-up and analysis of the sponge extracts derived from the second (unpublished) series of 7 substrates proceeded in nearly the same manner as previously described. Experimental modifications were made in order to increase analytical precision and thereby the chances of observing minor isotopic yield increases due to precursor incorporation.

Multiple ion detection with very fast scanning was employed to average a significant number of mass spectral scans (typically > 20) for improved precision, and some increased sensitivity. Another significant modification concerned the size of the sponge slice taken for extraction and analysis. Only the central core, typically 4-8 g wet weight, immediately surrounding the labeled precursor was taken for analysis. This significantly attenuated the isotopic dilution from surrounding natural 2-isocyanopupukeanane, and thus increased the chances of seeing a small, isotopic incorporation.

#### Homogenization Incorporation Technique

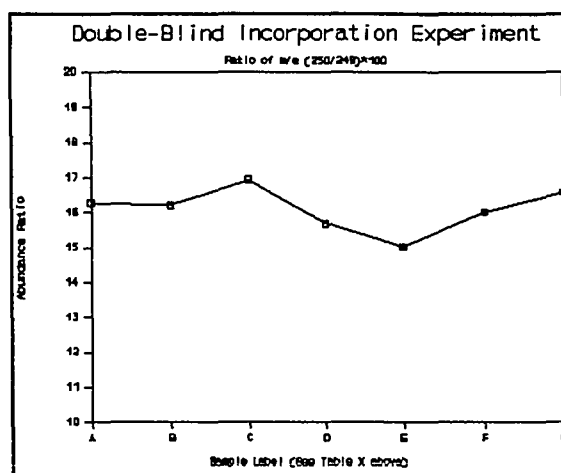
In addition to the capsular labeling experiments described above, eight other controlled, double-blind experiments involving many of the same precursors were performed. These experiments differed significantly in design from those already described. They consisted of freshly blended homogenates of *Ciocalypta* sp. prepared in the field, to which each precursor was added and incubated for periods of minutes instead of weeks. The assumption was, that the enzymes responsible for the biosynthesis were present in the homogenates, and that simple *in vitro* incubation may suffice for incorporation. Had this been shown to be successful, it would have significantly increased the number of experiments which could be examined

in a given time. After the appropriate incubation period, usually 15 minutes, the homogenates were frozen in the field on Dry Ice and returned to the laboratory for work-up and analysis as previously described. This work was accomplished in conjunction with the efforts and help of a colleague, Dr. Donald Gerhart, then a post-doctoral associate working with our research group. Due to the inherent difficulty in assaying low percentages of incorporation within *Ciocalypta* sp. the experiment was conducted in a "double blind" manner, in which the experimenter and the analyst were not aware of the identity of the samples submitted for isotopic assay. Isotopic dilution was assayed in a similar manner as described with one important exception. The isocyano functionality was efficiently hydrolyzed with dilute (1%) acetic acid (12h, rt) to the corresponding formamido functions. This was done in order to increase dramatically the intensity of the molecular ion of the resulting formamide (see page 34). This resulted in improved analytical precision and reproducibility in the mass spectrometry of these compounds. Table V shows the substrates employed in this biosynthetic experimentation. Each experiment below was originally identified (I.D.) with a letter A through G. No other information was supplied regarding the actual nature of the sample under consideration during the assay stage of the experiment.

**Table V. Isotopic Labeling Experiments Conducted on *Ciocalypta sp.* Homogenates**

Substrate	Amount (mg)	I.D.	Incubation (Min)
Control: No Label 3mL of Homogenate Not Incubated	N/A	A	N/A
Control: No label 3mL of Homogenate Incubated	N/A	B	15
1- <sup>13</sup> C-glycine	50	C	"
1- <sup>15</sup> N-glycine	54	D	"
1- <sup>15</sup> N-urea	55	E	"
<sup>13</sup> C-Na <sup>+</sup> CH <sub>3</sub> COO <sup>-</sup>	51	F	"
K <sup>+</sup> <sup>13</sup> CN <sup>-</sup>	52	G	"

Results of the Double-Blind Experiment are presented in Figure 27.



**Figure 27. Results of double-blind incorporation experiments.**

In this series of experiments the normal isotopic abundance of  $^{12}\text{C}$  to  $^{13}\text{C}$  is clearly shown in the two control homogenates labeled A and B. These values were quite close to one another and were calculated from averaged, multiple scan gc/ms data as illustrated in Figure 28 below. Clearly, the 15 minute "incubation" period did not significantly affect the isotopic ratio.

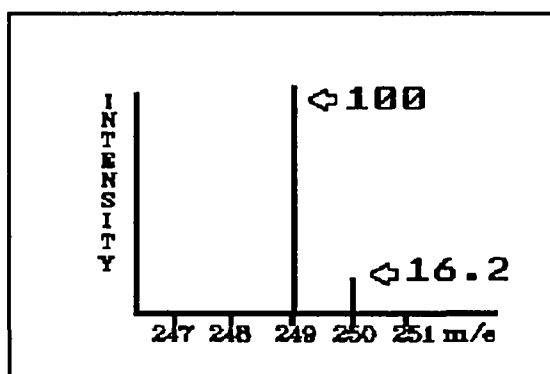


Figure 28. Scan averaged gc/ms, normalized to  $m/z$  249<sup>+</sup>, displaying  $m/z$  250/249 ratio for normal isotopic abundance.

No incorporation was detected; the average of all determinations, including the controls A and B, was 16.2% for the ratio of intensity of the M+1 ion relative to that of the M<sup>+</sup> ion. One absolute standard deviation was found to be 0.4% for all determinations. Controls A and B were 16.27 and 16.21%, respectively. The theoretical percentage ratio for an empirical formula  $\text{C}_{16}\text{H}_{27}\text{NO}$ , assuming natural isotopic abundance, is 18.14 %.<sup>64</sup>

Clearly, the values all fall too closely to one another to detect actual incorporation of labeled precursors even had it occurred at a low percentage. To be able to reliably detect incorporation by this method, one would require a determination which was at least two standard deviations above the mean of the natural background. This would translate into approximately 1% above 16.2% or 17.2% to be detected. On the basis of earlier work (page 35) it would require 2% overall incorporation on a mass basis in order to result in an instrumentally detectable enhancement (see Figure 7). Clearly, from the work above, either the homogenization technique did not operate successfully, or the analytical assay employed was not sensitive enough to detect "real" incorporation.

### III. DISCUSSION

#### The Chemical Nature of Metabolites

Organic extracts of *Ciocalypta* sp. have been shown to yield a rich variety of lipophilic metabolites. Included within this mixture one finds non-functionalized sesquiterpene hydrocarbons, isonitriles, isocyanates, formamides, amines, isothiocyanates and others of, as of yet, unassigned structure. Clearly this animal is a versatile biochemist with significant *in vivo*, biosynthetic capabilities.

The degree and intensity with which the chemistry of this animal has been studied must certainly make it one of the most thoroughly investigated members of Porifera in this respect. Its unique variety of metabolites beg biochemical questions regarding their ultimate source and origin.

The presence of the unprecedented, hydrolytically labile, isocyanates within the wet, aqueous environment of this filter feeding animal is unexpected and novel. What is the biological significance of such a compound, how is it stabilized, and what is its biogenetic relationship to the other metabolic congeners present?

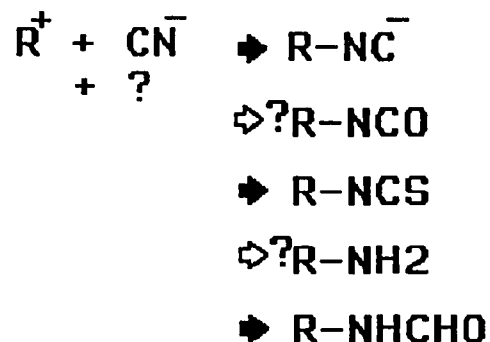
### The *In situ* Incorporation Technique

In my work I have attempted to couple simple extraction schemes and rapid gas-chromatographic assays with *in situ* biological methods. This to minimize detrimental physiological impact on *Ciocalypta* sp. as well as to rapidly screen a wide variety of different inorganic and organic precursors. These techniques have allowed a wide and diverse investigation into the source and origin of the marine isonitrile. However, a trade-off had to be made in analytical sensitivity. While sensitive by normal analytical standards, stable isotope mass spectrometry, the essence of the analytical methodology employed in this work, was only marginally effective in contrast to more recent successful experiments with radioisotope methods.

### Biogenesis of the Isonitrile

We were unable to detect incorporation of  $^{13}\text{C}$  labeled formate, glycine, acetate, cyanide, pyruvamide-, oxalato-, formamido- and isothiocyanato-pupukeanane into the marine isocyanide. Furthermore, we did not detect the incorporation of  $^{15}\text{N}$  labeled urea or glycine either. Detection would have required a minimum incorporation of 2% by weight, employing the stable isotope techniques outlined above.

From results obtained in the experimental work presented above it is clear that neither the formate, formamido, or the isothiocyanato moieties are the direct precursors of the marine isocyanide. Furthermore, we have shown that the formamido and the isothiocyanate are indeed derived from the isocyanide. The proven (bold arrows) and assumed biogenetic relationships between the various metabolites investigated thus far are outlined in Scheme 7 below.



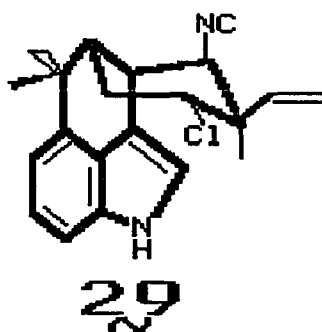
**Scheme 7**

The observation of several instances of antipodal functional groups among *Ciocalypta* sp. metabolites requires an intermediate  $sp^2$  carbocation. This is represented above with  $R^+$ .

#### IV. RECENT EXPERIMENTAL FINDINGS

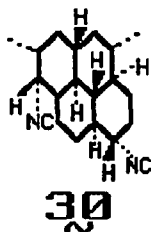
##### Biogenetic Schemes

Clearly, the source and origin of the isonitrile function in marine organisms differs from its terrestrial analog. Without exception, all terrestrial isocyanometabolites known so far have obvious or disguised amino acid precursors; In all terrestrial cases studied thus far the nitrogen atom of the isonitrile has been shown to originate from the amino nitrogen of the respective amino acid precursor.<sup>65</sup> The origin of the carbon atom, however, has been less obvious. Recently, Moore has demonstrated successful incorporation of  $^{14}\text{C}$ -cyanide, 2- $^{14}\text{C}$ -glycine, L-3- $^{14}\text{C}$ -serine, L-methyl- $^{14}\text{C}$ -methionine, and  $^{14}\text{C}$  formate into the isonitrile carbon of hapalindole A (29) from a cultured terrestrial cyanophyte *Hapalosiphon fontinalis*.<sup>66</sup> A tetrahydrofolate pathway was thus proposed with this metabolite of mixed biogenesis.<sup>67</sup>

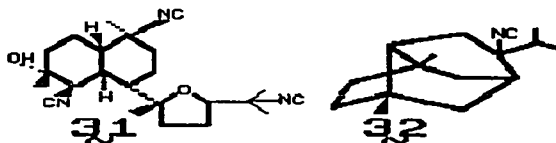


Marine Isonitriles, by contrast, possess primarily isoprenoid carbon skeletons, which poses a fundamentally

different biosynthetic question. Recently, cyanide ion has been incorporated in a marine isonitrile.<sup>68,69</sup> The sponge, *Amphimedon* sp., incorporated <sup>14</sup>C labeled sodium cyanide which was detected (1.8%) in the crystalline di-isonitrile, diisocyanoadociane (30).<sup>70</sup>



While these experiments demonstrated that a marine sponge can utilize cyanide as a carbon source, it left unanswered the question of the origin of the nitrogen atom. This question was addressed by Scheuer and coworkers,<sup>71</sup> who experimented with <sup>14</sup>C and with doubly labelled (<sup>13</sup>C<sup>15</sup>N) cyanide. Kalihinol-F (31), a tri-isocyano diterpenoid isolated from *Acanthella* sp., 2-isocyanopupukeanane (7)<sup>72</sup> and 2-isocyanoneopupukeanane (32),<sup>73</sup> both from *Ciocalypta* sp.,



showed 1.8% specific incorporation of <sup>14</sup>C-cyanide.

Furthermore, in biosynthetic experiments conducted with

*Ciocalypta* sp. collected at the south shore of O'ahu, doubly labeled  $^{13}\text{C}^{15}\text{N}$  cyanide was shown to be a precursor of both nitrogen and carbon atoms in the isonitrile of a new tricyclic sesquiterpene isocyanoneopupukeanane (32).

While strong evidence is now at hand to substantiate cyanide as a precursor of the marine isonitrile function, nothing is known of the source of the cyanide ion. Unassociated cyanide is a significantly toxic substance, to many living systems.<sup>74</sup> Because of the isocyanide fraction in *Ciocalypta* sp. and *Amphimedon* sp., either cyanide is generated and immediately consumed via biosynthesis resulting in a low steady state concentration within the animal, or an as yet undetermined amino acid or other precursor is being enzymatically degraded directly to the isocyano function, without the concomitant formation of cyanide ion.\* It may well be that free, unassociated cyanide ion is merely a competitive substrate for an already existing enzyme system, and has little, if anything to do with the actual biochemical mechanism involved in the formation of the marine isonitrile, *in vivo*.

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\* While cyanide is highly toxic to mammals and terrestrial insects,<sup>73</sup> its effect on sponges has not been systematically studied. In observations noted in this work, insertion of labeled (encapsulated) sodium cyanide into the sponge *Ciocalypta* sp. (as previously described) resulted in localized (about the embedded capsule), unhealed lesions of what appeared to be tissue necrosis. The rest of the animal appeared normal, however. From our research during nearly twenty years it has become obvious that isocyanides are tolerated by sponges and mollusks; their toxicity in mammalian systems is comparably low.<sup>75</sup>

A considerable body of biochemical precedence exists for this competitive substrate hypothesis. A review of the toxicological literature describing the primary systemic toxicology of cyanide poisoning alludes to the extremely high affinity of cyanide for methemoglobin and cytochrome  $a_3$ . As such a severe competitive inhibition of these enzyme systems occurs, leading to a rapid, catastrophic compromise of oxidative metabolism and phosphorylation.<sup>76</sup> This begs the question regarding the toxicological effects upon general cellular respiration in *Ciocalypta* sp. Perhaps, in analogy with mammals and terrestrial insects, normal cellular respiration is severely compromised, with the animal attempting to detoxify the poison (ionic cyanide) via normal enzymatic routes.

#### Future work:

##### Biogenesis of the marine isonitrile

Low level cyanide determinations could readily be accomplished via acid hydrolysis of sponge tissue and concomitant distillation of the gaseous hydrogen cyanide. A variety of sensitive analytical techniques now exist for the trace determination of cyanide ion in basic aqueous distillates.<sup>77</sup> Isonitriles and other organics would not be expected to interfere, since preliminary hydrolysis would result in the generation of amines, carbonates, and formates.

However, care must be taken to conduct thorough acidic digestion/ distillation before analytical determination via spectrophotometric or potentiometric methods. This is because both nitriles and isonitriles have been identified as potential positive and negative interferents in cyanide determinations in polluted waters, when **direct** spectrophotometric or potentiometric methods have been employed **without** prior sample cleanup by distillation.<sup>78</sup> Although unlikely, concomitant hydrogen sulfide generation (perhaps from acidic decomposition of organosulfur compounds which are occasionally found in sponge tissue), could be readily detected and subsequently removed by simple addition of lead or zinc ion prior to distillation, a standard and reliable technique in the field of analytical chemistry. Samples of surrounding reef water, entrained sponge water and wet, frozen tissue sections should be analyzed. This technique would be ideally suited to most chemical forms of cyanide, e.g., free cyanide and those complexes of cyanide which were amenable to acid hydrolysis with subsequent formation of gaseous hydrogen cyanide.

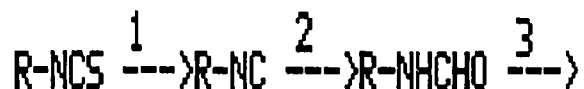
Absence of free cyanide in sponge tissue would argue against the supposition of high cyanide production by symbionts<sup>79</sup> with concomitant rapid, utilization by the sponges. This is particularly true because a high rate of cyanide production would be required in order to support the existence of such high concentrations of isonitriles in

*Ciocalypta* sp. and *Amphimedon* sp. At these levels, it would be difficult to miss the free cyanide in a "snapshot" analysis as would occur upon freezing the sponge and immediately analyzing its tissue.

Because of the extensive chelating ability of isonitriles (and cyanides) resulting in coordination with both transition and IB and IIB metals, one would expect profound biochemical consequences upon modifying normal concentration ranges of these ions found within animal's ocean environment. Once coordinated with copper, for instance, the aqueous solubility (and toxicity) of the isocyanopupukeananes would be expected to increase. It is highly likely that organometallic chemistry plays a significant role in the biosynthesis of marine isonitriles.<sup>80</sup> It is inconceivable to me that *Ciocalypta* sp. and other members of the order Halichondrida do not sequester a considerable amount of heavy metal during their normal filter feeding. Essentially, one can envision a dynamic cellular interface between the movement of sea water containing traces of inorganic metal ions over a cellular surface encapsulating a highly chelating non-mobile organic phase. As such it would be useful to analyze sponge tissue for the presence of heavy metals, in an attempt to correlate organometallic chemistry with biosynthesis. It would also be of interest to vary common heavy metal concentrations (zinc and copper) in the animals' aqueous environment.

Extremely small sections of the sponge could be removed by coring and rapidly analyzed for metabolite profile as a function of time and heavy metal content/dosage.

Of further interest in the investigation of potential precursors for the isocyano moiety would be the establishment of the actual biogenetic origin of the nitrogen in isonitrile in relationship to that of carbon. Although cyanide has been shown to be a precursor of the nitrogen in *Ciocalypta* sp.,<sup>81</sup> it has not been conclusively shown that it also is the source of nitrogen in aminopupukeanane which is also present in the sponge. Aminopupukeanane could result from simple aqueous hydrolysis of the formamide in *Ciocalypta* sp. or, less likely in view of formate and formamido experiments already conducted, it could be an intermediate on the way to isocyanide. This would also play an important role in the biogenetic investigation of those instances, where co-occurrence of isocyano-, isothiocyano-, and formamidoterpenoids has not been observed. Two interesting cases are represented by the sponge *Pseudaxinyssa* sp. from Fiji, and *Epipolasis* sp. from the rocky north shore of O'ahu, in which isothiocyanates are the sole nitrogenous constituents.<sup>82,83</sup> Semi-synthetic labeled precursor could be obtained by isolation of isothiocyanates, degradation to labeled <sup>15</sup>N amine via <sup>15</sup>N amination of the corresponding (in the case of 2-aminopupukeanane) ketone (Scheme 8)<sup>84</sup>.

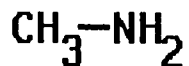


**Scheme 8.** Proposed synthetic route to  $^{15}\text{N}$  labeled amine from isothiocyanate. 1) Pd on C/20 C, sealed tube. 2) AcOH/rt. 3) HCl. 4) N-chlorosuccinimide. 5,6) NaEtO/H<sub>2</sub>O. 7)  $^{15}\text{NH}_3/\text{H}_2\text{O}$ .

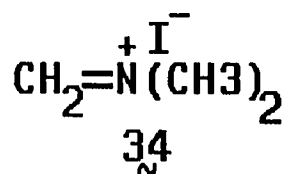
Clearly, this would be a simple way to study the "direction" of biogenesis. In analogy to the sodium cyanide experiments, it would be of interest to investigate the incorporation of singly, or if commercially available, doubly or triply labeled sodium thiocyanate ( $\text{Na}^{15}\text{N}^{13}\text{C}^{34}\text{S}$ ) or sodium cyanate ( $\text{Na}^{15}\text{N}^{13}\text{C}^{18}\text{O}$ ). The multiply labeled substrates would be particularly suitable to the gas chromatographic / mass spectrometric method, because of virtually nonexistent interference from background isotopic abundance.

Additional insight may be gained from experimentation utilizing different degrees of unsaturation and molecular size of cyanide analogs.

For instance, saturated, bulky, labeled methylamine (33) or unsaturated, *Eschenmoser's salt* (34) might make interesting candidates.



33



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#### Potential Commercial Applications

Several interesting possibilities exist for commercial applications involving marine isonitriles or their hosts.

Coordination with copper or tin could result in a second generation of marine paints, whereby the active "organic and natural" ingredient (in this case, the isonitrile) slowly bleeds or leaches from a solidified paint matrix permanently encapsulating an insoluble form of copper. Reactivation of the anti-feedant/toxic properties of the paint could be readily accomplished by re-spraying the original painted surface with an organic solution of the isonitrile. The copper in this case merely serves to coordinate with the isonitrile holding on to it for a predetermined period of time before loss of effective paint layer concentration occurs.

Indeed, marine paint, formulated with the isonitriles could also serve as an effective natural anti-fouling surface.

There are significant indications that the isocyanopupukeanane series may serve as an important biochemical tool for the study of the immune system. In various thymocyte assays 2-isocyanopupukeanane has been shown to stimulate significantly the growth and production of macrophages *in vitro*.<sup>85</sup>

Recently a great deal of effort has been expended to find, albeit without much success, alternatives to classical cyanide leaching and stabilizing baths commonly employed in metal plating operations. This has traditionally involved the precious metal industry. Isocyanides, with similar chelating properties and for the most part absence of toxic side-effects could well provide the answer for the safety and hazardous waste disposal problems which now plague the industry.

If indeed *Ciocalypta* sp. is found to sequester and effectively remove heavy metals from contaminated sea water, this animal holds great potential in waste recovery systems, detoxification of hazardous waste and water treatment. Furthermore, because of the huge volume of water processed by these active filter feeders, sponges of the order *Halichondrida*, may play an as yet, unappreciated role in the stabilization, control and purification processes inherent in the quality of reef ecosystem.

In organometallic chemistry the isonitrile ligand represents an interesting alternative to carbon monoxide or nitrogen. As neutral linear two-electron donors (isoelectronic with carbon monoxide and nitrogen) they are better *sigma*-donors and analogously poorer *pi* acceptors than carbon monoxide. The consequence of this mixture of electronic properties results in the ability to stabilize higher oxidation state metal complexes than the corresponding carbonyl species. Because of this property, isonitriles have recently been employed in model metal-containing biological systems of cytochrome c<sup>86</sup> and indolamine 2,3-dioxygenase,<sup>87</sup> in studies of the binding constants of different active sites on each enzyme system.

Isonitriles have also generated interest as novel synthetic reagents in chemical reactions involving coordinated ligands. These include coupling, migratory insertions and dealkylation applications.<sup>88</sup>

Because of its potential application as a tool for the critical study of both biological and chemical systems, Sigma Chemical Company and CalBiochem currently offer for sale 2-isocyanopupukeanane to the biochemical research community.<sup>89</sup>

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