ALKALOIDAL CONSTITUENTS OF THE
MARINE SPONGE CLIONA CELATA

by

RICHARD J. STONARD
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Department of Chemistry

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

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Methanol extracts of the marine burrowing sponge *Cliona celata* Grant have yielded a complex mixture of 'imperfect' alkaloids. Fractionation of this mixture by a rapid acid partitioning procedure has facilitated the isolation of the major metabolite clionamide (53). The structure of this modified amino acid was determined by its conversion to tetracetylclionamide (46). The assigned structure of 46, previously isolated from acetylated extracts of *C. celata*, was confirmed by the synthesis of its ultimate hydrogenation product 49. Clarification of the absolute stereochemistry of clionamide has been accomplished by hydrolysis and subsequent hydrogenolysis of 46 to give (S)-N-acetyltryptophanamide (52) which was characterized by comparison with an authentic sample.

The remaining 18-22 alkaloids elaborated by *Cliona celata* bear close resemblance to a large family of peptide alkaloids found in higher plants. Derivatization of the crude sponge extract by acetylation and subjection of the resulting residue to repetitive silica and reversed-phase chromatography have effected the isolation of four novel linear peptide alkaloids.
Structures of the 6-bromotryptophan-containing alkaloids, hexacetylcelenamide A (58), B (57) and pentacetylcelenamide C (59), and the bisdehydrotripeptide nonacetylcelenamide D (60), were established by interpretation of spectral data and chemical degradation by ozonolysis and acid-catalyzed hydrolysis. Demonstration of the presence of the unique amino acid α,β-didehydro-3,4,5-trihydroxyphenylalanine as a constituent of each of these compounds was aided by correlation with synthetic analogues. A small scale isolation employing acetic anhydride-d6 has indicated that celenamides A (70), B (71), C (72) and D (74) are the naturally occurring sponge metabolites.

A chemical comparison of specimens of Cliona celata from Massachusetts, Southern California and British Columbia is reported.
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To Gloria, Gloria, Maurice and Nancy.
List of Abbreviations

Ac2O = Acetic anhydride
CD = Circular dichroism
DCC = Dicyclohexylcarbodiimide
DMAP = Dimethylamino pyridine
DMF = Dimethylformamide
DMSO = Dimethylsulfoxide
DCU = Dicyclohexylurea
DOPA = 3,4-Dihydroxyphenylalanine
EIMS = Electron-impact mass spectroscopy
EtOAc = Ethyl acetate
GC = Gas chromatography
HPLC = High pressure liquid chromatography
IR = Infrared
'H NMR = Proton nuclear magnetic resonance
'13C NMR = Carbon-13 nuclear magnetic resonance
PND = Proton-noise-decoupled
PTLC = Preparative thick layer chromatography
Py = Pyridine
RP = Reversed-phase
S = Solvent signal
THF = Tetrahydrofuran
TLC = Thin layer chromatography
U = Unknown impurity signal
UV = Ultraviolet
W = Water signal
INTRODUCTION

"For in and out, above, about, below, 'Tis nothing but a Magic Shadow-show, Played in a Box whose Candle is the Sun, Round which we Phantom Figures come and go." -Omar Khayyam (1)

I. Natural Products from the Sea

Studies aimed at understanding biological systems at the molecular level have resulted in the classification of carbon compounds present in living organisms into two groups - the primary and secondary metabolites. The former are molecules encountered in most, if not all, biological systems and are considered to be of fundamental importance to the existence of all organisms. The study of these substances has, in the past, been primarily the domain of the biochemist. In contrast, the structurally diverse secondary metabolites are sporadic in occurrence and are not generally considered to be quintessential to life (2). However, it is becoming increasingly apparent that the flora and fauna which host these compounds have unique metabolic and social requirements. Recent work in the areas of insect pheromones (3), plant hormones (4), phytoalexins (5), and marine allelopathic agents (6) may be cited as examples. In these instances, the secondary substances serve important physiological functions. The study of the nature, occurrence,
origin and role of these compounds constitutes the realm of the natural-products chemist. The more utilitarian goal of utilizing secondary substances for man's benefit is the responsibility of pharmacologists and other pragmatic scientists.

The search for natural products from marine organisms is a recent endeavour. The proceedings of the Food-Drugs from the Sea Conference of August, 1969 (7b) cite fewer than two dozen purified and identified marine secondary metabolites (excluding sterols, pigments and simple lipids). This series of meetings (7) stimulated interest in marine natural-products research and provided direction to a field in its infancy. Advancements in the technology of SCUBA have enabled marine chemists to collect specimens which were previously unattainable. Increased ease of collection, coupled with the development of modern separation techniques and spectroscopic methods of analysis, have resulted in the examination of thousands of marine species. From these, hundreds of novel substances have been isolated and structurally defined in the past decade. Many of the fascinating structures which have been encountered are presented in the following subsection.

Although marine organisms were originally hailed as being potential sources of 'miracle elixirs' (7,8), no true pharmaceutical of marine origin has yet been produced. The absence of immediate pragmatic results has dampened the enthusiasm of many researchers and funding agencies. However, Faulkner (9) rightfully points out that the development of a drug is a time consuming and expensive process. Time and a
substantially enhanced effort by the industrial community will be required before success is achieved. In the meantime, chemists involved in marine natural-products research will continue to provide a plethora of unique organic structures which serve to further our comprehension of nature's architecture. Of equal importance is the increased tendency towards interdisciplinary cooperation. In this context marine chemists can make substantial contributions to problems of taxonomy and to the enhancement of our knowledge of marine biological phenomena. The cooperative examination of marine phytoplankton - the ocean's 'primary producers' - should prove to be most rewarding. Research into chemical communication and related allelopathic phenomena in marine communities is just beginning.
II. Nitrogenous Secondary Metabolites from Sponges

Members of the phylum Porifera - the aquatic sponges - are among the simplest and most primitive of all multicellular animals. These sessile organisms are represented by approximately 4,500 species of which 150 reside in fresh water habitats. Generally classified in the subkingdom Metazoa, the structural and developmental uniqueness of the Porifera has led many biologists to suggest that they are evolutionary dead-ends and, as such, should be relegated to the distinct subkingdom, Parazoa (10).

Sponges exist in most marine habitats, over a wide range of depths. Their abundance, distinctive appearance, and apparent lack of defence coupled with a general absence of predation make them natural candidates for natural-products research. Of the three large classes into which the Porifera are subdivided, the Demospongiae' have received the most attention. Classification of the Demospongiae is difficult and represents a major problem for the chemist in the field.

The most prolific source of unique antimicrobial substances has been species of the order Keratosa (eg. Verongidae) (11b). This generalization may result from the fact that the Keratosa are the only demosponges which do not possess spicules. Instead, these organisms have skeletons composed entirely of protein. The

' The other two classes are the Calcarea and the Hexacrinellida.
absence of a skeletal defensive mechanism may have given rise to
the evolution of biosynthetic pathways for the production of
protective substances.

The list of marine natural products which contain nitrogen
is short in comparison with the abundance of such compounds from
terrestrial sources such as plants and fungi. A thorough
treatment of marine natural-products chemistry published in 1971
(12) included only seven nitrogen-containing sponge-derived
compounds. As a result of the rapid expansion in the number of
compounds defined in recent years, a comprehensive review of
these substances would be beyond the scope of this thesis. Reviews by Minale et al. (11b), Faulkner (11c), Faulkner and
Andersen (11a), and those in the series of books edited by
Scheuer (11d,e,f,g), have provided thorough coverage of the
marine natural-products literature to 1978. In this subsection,
an attempt has been made to bring together those compounds of
sponge origin which contain nitrogen and which have been
reported subsequent to January 1978. The intention of this brief
survey is to demonstrate the array of nitrogen-containing
compounds which have been found in the Porifera in order to
provide a framework for a discussion of the metabolites of
Cliona celata in Chapter Two. Many of these compounds have no
terrestrial counterparts, while in a few instances, close
analogy to the plant alkaloids is apparent.

The term 'alkaloid' has been used in this thesis to define
substances of natural origin which contain basic nitrogen.
Inclusion in this class has been based primarily on structural
considerations. The more restrictive taxonomic and pharmacological activity requirements of Pelletier (13) and others have been excluded. Nakanishi et al. (14) have defined modified amino acids and peptides as imperfect alkaloids.

The compounds discussed below are presented in a sequence based upon the structural groupings in which their constituent nitrogen atoms are found. Those molecules possessing nitrogen in a cycle and in an acyclic structural feature are ordered on the basis of their cyclic moiety.

A. Compounds Containing Nitrogen in an Aromatic Heterocycle.

Various collections of the Indo-Pacific sponge, *Dysidea herbacea*, have provided a number of compounds containing trichloromethyl groups. Two of these compounds were found to possess an uncommon C-terminal amino-alkyl-thiazole residue. The structure and absolute configuration of isodysidenin (1, \([\alpha]_D + 47^\circ\)) was solved by X-ray diffraction analysis of the quaternary ammonium salt 2. Compound 2 was prepared from 1 by sequential treatment with diborane-THF complex and methyl iodide (15). Dysidenin (3, \([\alpha]_D - 98^\circ\)) a diastereomer of 1 was present in small amounts in the sponge sample from which 1 was obtained. Compound 3 had previously been isolated from *D. herbacea* by Kazlauskas et al. (16) and its structure correctly deduced on the basis of spectral analysis and reduction (Zn-acetic acid) to its dechloro derivative 4. Employing chemical degradation by acid hydrolysis, Charles et al. (17) have established that dysidenin
differs from isodysidenin only in the configuration about the sole amino acid α-methine carbon in the molecule and have thus, by chemical correlation, succeeded in determining the absolute configuration of 3.

\[ \text{Image of chemical structures} \]

In contrast to the terrestrial situation, few indolic compounds have been isolated from marine organisms. The majority of the molecules which have been defined are simple indole derivatives. Prior to the investigation of Cliona celata (Chapter 2), no complex metabolites reminiscent of the plant alkaloids had been isolated from sponges.\(^1\)

\(^1\) Lynbyatoxin A (6,18), hyellazole (7,19), chlorohyellazole (8,19), flustramine A (9) and B (10,20) and surugatoxin (11,21) are among the most complex indole and indole-based alkaloids obtained from marine organisms.
The simple bromotryptamine derivatives, 12 and 12a, were isolated from *Smenospongia echina* and *S. aurea*, respectively, by Djura et al. (22). The bromine atom in 12a was positioned at C-5 on the basis of $^1$H NMR comparison with 5-bromo- and 6-bromoindole-3-carboxaldehyde.

![Structure of 12 and 12a](image)

12 $R = \text{Br}$

12a $R = \text{H}$

Three tryptophan derivatives, closely related to the sponge compound aplysinopsin (13,23), have recently been isolated. The structures of 2'-de-N-methylaplysinopsin (14) and 6-bromo-2'-de-N-methylaplysinopsin (15), obtained from the sponge *Dercitus* sp., were assigned on the basis of spectral interpretations (24). Djura and Faulkner confirmed the structures of 14 and 15 by total synthesis and in the latter case by comparison with a synthetic sample of the 5-bromo compound. The absence of meta-coupling between protons at positions 5 and 7 of the indole nucleus in the $^1$H NMR spectrum of compound 15 apparently led to an incorrect assignment for the protons at C-4 and C-5 (24). The structure of the brominated indole 16 was determined (22) by spectral comparison with 5-bromo- and 6-bromoindole carboxaldehyde and with a synthetic sample of 5-bromoaplysinopsen. Compound 16 was obtained from a collection of *Smenospongia aurea* which did not contain either of the bromo indoles 12 or 12a.
Determination of the geometry about the trisubstituted double bond in aromatic dehydroamino acid derivatives presents substantial difficulties (see Chapter four for further discussion). Kazlauskas et al. (23) suggested that aplysinopsin (13) exists as a mixture of double bond isomers (9:1), but did not assign the major isomer. Analysis of the 'H NMR data provided by Djura and Faulkner (22,24) for 14, 15, 16 and related compounds does not allow conclusive stereochemical assignments to be made.

The structure of the novel indole-containing compound 17, obtained from the Caribbean sponge Halichondria melanodocia in minute quantities, was elucidated by Gopichand and Schmitz (25) solely on the basis of spectral considerations.

Several pyrrollic compounds have been isolated from sponges including a number of bromo-pyrrolle derivatives. Stierle and Faulkner (26) have recently described a family of 5-
alkylpyrrole-2-carboxaldehydes (18-20) including the first stable, naturally occurring cyanohydrin 20. A series of 3-alkylpyrrole-2-carboxaldehydes had been isolated previously by Cimino et al. (27) from Oscarella lobularis. The cyanohydrin function present in 20 was indicated by 1H- and 13C NMR comparisons with 2-hydroxynonylcyanide.

![Structural formulae](image)

Further investigation of the aqueous extract of the sponge Phakellia flabellata by Sharma et al. (28) afforded the isolation of the novel pyrrole-containing metabolite 21. Sharma and Burkholder's original investigation of this sponge (29) gave rise to the structure elucidation of two of the earliest nitrogenous secondary metabolites obtained from marine sponges-4-bromophakellin and dibromophakellin. The stereochemistry about the exocyclic double bond in 21 was assigned on the basis of a marked downfield shift of the adjacent methylene protons resulting from the anisotropy of the closely situated amide carbonyl.
Few pyridine alkaloids have been isolated from marine organisms. Substances present in several species of the genus Haliclona are toxic to fish and mice. Working with *H. rubens*, *H. viridis*, and *H. erina* Schmitz et al. (30) demonstrated that this activity was caused by a polymeric compound composed of 3-alkylpyridine units as shown in 22.

The substance responsible for the antibacterial activity of the blue sponge *Reniera sp.* has been isolated by McIntyre et al. (31). Renierone (23) was defined by X-ray crystallography.
B. Compounds Containing Nitrogen in a Nonaromatic Heterocycle.

In addition to the thiazole derivatives 1 and 3 *Dysidea herbacea* is known to produce two other nitrogenous metabolites possessing trichloromethyl groups. The structure of the diketopiperazine 24 (32), derived from trichloroleucine, was deduced by spectral analysis and sequential reduction to its saturated dechlorinated derivative 25. The E configuration was assigned to the dehydroamino acid double bond on the basis of the chemical shift of the allylic isopropyl methine proton in 24 and 25. The position of these resonances (δ 5.18 24 and 3.96 25) was attributed to deshielding by the adjacent carbonyl group. An earlier investigation of this sponge afforded dysidin (26, 33). The diverse nature of the natural products isolated from separate collections of *D. herbacea* has been attributed to the presence of different blue-green algal symbionts (32, 15).
Three novel toxic macrolides, latrunculin A (27), B (28) and C possessing an unique 2-thiazolidinone terminus have been obtained by Kashman et al. (34) from the Red Sea sponge *Latrunculia magnifica*. Structural determination of these metabolites was facilitated by X-ray diffraction analysis of the methyl ether 29. The authors speculate that these compounds are derived from a mixed polyacetae-amino acid source.

![Chemical Structure](image)

In the report describing the indolic lactam 17, Gopichand and Schmitz (25) detail the structure elucidation of a second lactam 30, also obtained from *H. melanodocia*. Confirmation of the structure proposed for 30 from spectral analysis was provided by acetylation and selective reduction of the ketone function.

![Chemical Structure](image)
Sponges of the order Verongidae have been a rich source of brominated tyrosine-derived metabolites (11b), many of which contain an isoxazole moiety fused in a spiro-sense to a carbocyclic ring. The structure of aerothionin (31) originally isolated from *V. aerophoba* in 1970 (35) has been confirmed by X-ray analysis (36). The results of the X-ray diffraction experiments in conjunction with circular dichroism measurements enabled McMillan et al. to establish its absolute configuration. Recently, several novel metabolites belonging to this class have been acquired from two Verongidae sponges. *Aplysina fistularis* provided fistularin-1 (32), -2 (33) and -3 (34, 37). These compounds were elucidated principally by $^1$H NMR. The second Verongidae sponge recently investigated produced metabolites bearing only acyclic nitrogen. These will be presented below.
C. Compounds Containing Acyclic Nitrogen.

A bioassay guided fractionation procedure enabled Carter and Rinehart (38) to isolate the potent antibiotics, \textit{35-37}, from the red-orange sponge \textit{Acarnus erithacus}. The structure elucidation of the acarnidines (35-37) was aided by protection of the guanidine function with 2,4-pentanedione. The hydrogenation product of one of the protected derivatives was synthesized in order to confirm the homospermidine skeleton.

\begin{align*}
35 & \quad R = \text{CO} (\text{CH}_2)\text{CH}_3 \\
36 & \quad R = \text{CO} (\text{CH}_2)\text{CH=CH}(\text{CH}_2)\text{CH}_3 \\
37 & \quad R = \text{CO} \text{C}_{13}\text{H}_{21}
\end{align*}

From an unidentified black sponge Yunker and Scheuer (39) isolated a series of fatty acyl derivatives (38-41) of 2-methylene-\(\beta\)-alanine. The amino acid itself and related fatty acyl derivatives had previously been reported by Kashman et al. (40) from the sponge \textit{Fasciospongia cavernosa}. Holm and Scheuer (41) subsequently described the synthesis of this toxic amino acid and its amide 39 \((n=13)\).

\begin{align*}
38 & \quad R = \text{Me}, \text{R}_1 = \text{COCH}_2(\text{CH}_2)_n\text{CH}_3 \\
39 & \quad R = \text{Me}, \text{R}_1 = \text{COCO}(\text{CH}_2)_n\text{CH}_3, \text{n} = 11-13 \\
40 & \quad R = \text{Me}, \text{R}_1 = \text{COCHOH}(\text{CH}_2)_n\text{CH}_3, \text{n} = 11-13 \\
41 & \quad R = \text{H}, \text{R}_1 = \text{COCH}_2(\text{CH}_2)_n\text{CH}_3
\end{align*}
Methanol extracts of the Verongidae sponge, *Ianthella basta*, gave the highly modified peptides, bastidin-1 (42) and bastidin-2 (43, 42). Several complex related compounds were also observed.

III. Natural-Products History of the Clionidae

The sponge family Clionidae have been largely overlooked by natural-products chemists as a result of the substantial collection problems they generally present. The vast majority of clionids are endolithic and if visible (many are not) must be chiselled or pried from their substrates as blocks consisting mostly of coral or rock. It is not surprising, therefore, that it has been primarily those sponges which attain the later, beta or gamma, stages of growth (see Chapter three for explanation) which have received attention. However, interest in the
burrowing sponges is intensifying (43) and even the resistant endolithic organisms will soon become the subjects of closer scrutiny.

**Cliona celata**, identified by Grant in 1826, was first studied for its chemical constituents by Dorée in 1909 (44) as part of his general survey of cholesterol in animals. His collections afforded an unique sterol which he named clionasterol. Bergmann repeated this isolation in 1941 (45) and suggested a partial structure for this sterol in 1942 (46). Bergmann also demonstrated the presence of clionasterol and poriferasterol in *C. carriboea* (47). Recently, Erdman and Thomson (48), employing modern GC separation techniques, have shown that Dorée's 'clionasterol' was a mixture of clionasterol (44) and poriferasterol (45).

A New Zealand specimen of *C. celata* has been investigated for its free amino acid content (49). Taurine and hypotaurine, amino sulfonic acids commonly encountered in marine invertebrates, were shown to be relatively abundant constituents. Taurocyamine was also observed.

![Chemical structures](image1)

**44**

**45**

The demonstration that marine sponges contain substances which influence the growth of bacteria was first made by Nigrelli in 1959 (50). He subsequently showed that antimicrobial
activity was associated with the organic solvent extracts of a variety of sponges including *C. celata* (gamma stage) (51). *C. carteri* and *C. copiosa* were reported by Burkholder and Ruetzler (52) to contain chemicals which inhibited the growth of several test bacteria, although *C. viridis* extracts did not. The chemicals responsible for these activities have not been determined.
Figure 1. *Cliona celata* (Grant) (yellow).
I. Tetracetylclionamide (46)

Andersen initiated our study of Cliona celata in 1977 when he observed that ethanol extracts of sponge collected in Barkley Sound showed marked in vitro antibiotic activity against the gram-positive bacterium, Staphylococcus aureus. His initial attempts at isolating the ethyl acetate soluble substance responsible for this activity were unsuccessful owing to its failure to undergo chromatography by conventional methods. The presence of phenolic compounds in the sponge extract was indicated by an intensely positive ferric chloride reaction. This discovery led to the proposal that derivatization might provide a viable solution to the isolation problem. Acetylation employing acetic anhydride-sodium acetate proved to be the most successful method in terms of ease of performance and yield. The derivatized residue was subjected to routine fractionation on a column of silica gel to give the major acetylated metabolite, tetracetylclionamide (46,53).
Tetracetylclionamide (46) crystallized from THF-isopropyl ether as very fine thread-like clusters which were not amenable to X-ray crystallographic examination. The molecular formula C_{27}H_{26}BrN_{3}O_{8} was determined by elemental analysis (C,H,N) and was confirmed by HR analysis of the parent ion doublet observed at m/e 599/601 (1:1) in the LRMS (figure 2). The base peak observed at m/e 210/208 (1:1) was attributed to the bromoindole ion shown below (54):

![Bromoindole structure](attachment:image.jpg)

Analysis by 'H NMR (figure 3, table 1) suggested that the molecule contained a bromotryptophan residue. This was confirmed by hydrolysis in MeOH-H_{2}SO_{4} to give the methyl ester 47. The structure of 47 was identified by comparison with N-acetyl-5-bromotryptophan methyl ester (48) prepared from commercially available (±)-5-bromotryptophan (Aldrich). The esters 47 and 48 differed in their IR and 'H NMR spectra (figure 4) and were distinguishable by silica-HPLC.

The remaining features of this molecule were determined by spectral analysis and two classical degradative reactions. Ozonolysis of 46 afforded 3,4,5-triacetoxybenzaldehyde which was shown to be identical to an authentic sample prepared from gallic acid (see appendix 1). This result implied the presence of the following partial structure:
Figure 2. EIMS of tetracetylclionamide (46).
Figure 3 a. Partially exchanged 270 MHz $^1$H NMR spectrum of tetracetylclionamide (46) in acetone-$d_6$. b. Expansion (1000 Hz) of the non-exchanged aromatic region of 46.
Figure 4. Comparison of the aromatic regions of the 270 MHz $^1$H NMR spectra of 47 and 48.
The $^1$H NMR spectrum of 46 in CDCl$_3$ (53) also contained signals attributable to two coupled olefinic protons; a doublet at $\delta$ 6.09 ($J=14$ Hz), and a doublet of doublets at $\delta$ 7.32 ($J=10, 14$ Hz). Exchange of a signal (bd) at $\delta$ 8.64 ($J=10$ Hz) with D$_2$O collapsed the signal at $\delta$ 7.32 to a doublet ($J=14$ Hz). These observations together with the results of double resonance experiments suggested that the remainder of the molecule not accounted for by the N-acetyltryptophan moiety, was an unusual trans-disubstituted enamide (N-vinylamide, N-acylstyrylamine).

Hydrogenation of 46 in the presence of a palladium catalyst afforded the expected dihydro-debromo derivative 49 (figure 5), thereby concluding Andersen's structure elucidation of tetracetylclionamide (46).
Figure 5. a. 270 MHz $^1$H NMR spectrum of 49 (natural) in acetone-$d_6$. b. Vertical expansion c. Offset. Impurity signals arise from the presence of the incompletely reduced degradation product 50.
II. The Objectives

The continuation of the natural-products investigation of C. celata initiated by Dr. Andersen, constitutes the subject matter of this thesis. This study was originally envisaged to be of three parts: the confirmation of the tetracetylclionamide (46) skeleton and determination of its absolute configuration; the isolation of underivatized 46, assessment of its biological activity, and identification of the source of the antimicrobial activity attributed to C. celata extracts; and finally, the isolation and structure elucidation of the remaining substances present in the acetylated sponge extract.

During the course of this investigation these objectives were expanded to encompass several other features of interest. These included: a partial survey of the chemistry of North American clionids; experiments aimed at exploring the nature of the burrowing process of C. celata; and studies towards the synthesis of dehydropeptides to be used in assessing the role of dehydroamino acid residues in metal binding, to aid in the structure assignment and for biological testing.

The results of this work are described and discussed in the following sections of this chapter and are continued in two subsequent chapters.
III. Confirmation of the Structure of Tetracetylclionamide (46)

In order to verify the carbon skeleton of tetracetylclionamide (46) it was deemed necessary to synthesize either 46 itself, or its hydrogenation product 49 which contained all of the skeletal elements of 46. In view of the difficulties associated with the synthesis of N-vinylamides in sensitive molecules (to be discussed) the hydrogenation product 49 containing the stable phenethylamine moiety was chosen as the target molecule.

N-Acetyltryptophan was prepared from tryptophan by acetylation with acetic anhydride (55). Coupling of the above with 5-hydroxydopamine hydrochloride in the presence of DCC and triethylamine afforded, upon workup, an oil which failed to react with ninhydrin. Acetylation of this material with Ac$_2$O-py gave, in 30 percent overall yield, a product (49a) (figure 6) identical (TLC, UV, MS), except for optical rotation, with 49.
Figure 6 a. 270 MHz $^1$H NMR spectrum of 49a (synthetic) in acetone-d$_6$ b. Vertical expansion c. Offset.
The dihydro-bromo derivative 50 (see experimental) was obtained during the conversion of tetracetylclionamide (46) to its ultimate hydrogenation product 49.

IV. The Absolute Configuration of Tetracetylclionamide (46)

The determination of the absolute configuration about the sole chiral carbon atom present in 46 required the production of an optically active-debrominated degradation product which was either commercially available or readily synthesized. The methyl ester 47 produced by Andersen was ruled out owing to the very low yield of product obtained in the MeOH-H$_2$SO$_4$ hydrolysis reaction.

Synthetic N-vinyl amides have been studied in connection with their ability to polymerize. Kutner (56), among others, noted that these monomers reacted in a manner similar to vinyl ethers and esters when treated with dilute solutions of mineral acids. The mechanism of styrylacetamide hydrolysis has been studied in detail (57, 58) and one possible route is outlined in scheme 1.
Scheme 1. A possible mechanism for the acid hydrolysis of styrylacetamide (57,58).
When tetracetylclionamide (46) was reacted with HCl in acetonitrile (0.1M), 2-acetamido-3-(6'-bromoindol-3'-yl) propionamide (51, figure 7) was obtained. A maximum yield of 50-60 percent could be achieved following 48-72h at room temperature. The 3,4,5-triacetoxyphenyl acetaldehyde produced, polymerized under the reaction conditions employed.

Hydrogenolysis of 51 proved to be unusually difficult. 2-Acetamido-3-(indol-3'-yl) propionamide (52, figure 8) was eventually obtained following repeated low-pressure hydrogenation employing a palladium-on-charcoal catalyst in the presence of acetic acid. The absolute configuration of 52 ([α]_D + 15° (c 1.0, CH₃OH)) was determined to be S by comparison with authentic N-acetyl-L-tryptophanamide ([α]_D + 17° (c 1.0, MeOH); lit. (59) [α]_D + 20 ± 1° (c 2%, MeOH)) purchased from the Aldrich Chemical Company. Hence, tetracetylclionamide is (2S)-N-((1"E")-5",6",7"-triacetoxystyr-1"-yl)-2-acetamido-3-(6'-bromoindol-3'-yl) propionamide (60). The degradative and synthetic
Figure 7 a. 270 MHz $^1$H NMR spectrum of 51 in DMSO-d$_6$ b. Vertical expansion c. Offset.
Figure 8 a. 270 MHz $^1$H NMR spectrum of 52 in acetone-d$_6$. b. Vertical expansion.
reactions employed in the structure elucidation of this molecule are summarized in scheme 2.

\( \text{V. Clionamide (53,61)} \)

The solvent extracts of *C. celata* once acetylated showed no inhibitory action towards *S. aureus* or several fungi (see experimental). Similarly, the lipid containing fraction obtained by trituration of the crude EtOAc extract with ether prior to acetylation also was devoid of activity. Therefore, we sought to isolate underivatized phenolic metabolites with the hope that one of these compounds would be responsible for the *in vitro* antibiotic activity. Phenolic substances are frequently responsible for the antimicrobial properties associated with extracts of marine organisms (11g).

Purification attempts utilizing chromatography on cellulose, silica, Sephadex LH-20, Sephadex G-10 or reversed-
Scheme 2. Summary of the degradative and synthetic reactions employed in the determination of tetracetylclionamide (46).
phase materials in a variety of solvents failed to provide discrete compounds. RP-HPLC on a small scale was capable of partially separating the underivatized metabolites (figure 9). However, attempts to scale up this procedure led to substantial loss of column efficiency because of the high percentage of the extract which was extremely slow to elute. The reluctance of these compounds to be purified by chromatography was attributed to their high polarity and instability and to the complex nature of the mixture.

The EtOAc soluble-ether triturated extracts of *C. celata* exhibited positive reactions not only towards ferric chloride but also to ninhydrin. When a small sample of sponge (40 g, wet weight) was worked up using acetic anhydride-\textsubscript{d6} as the acetylation agent, tetracetylc1ionamide-\textsubscript{d12} (54) and a deuteroacetylated mixture of the remainder of the sponge metabolites were isolated.

![Chemical structure of 54](image_url)
Figure 9. RP-HPLC of the underivatized Cliona metabolites. Analysis conditions; μ Bondapak-C18, 4 x 250 mm; solvent CH3CN-H2O, gradient 35-100% at 1%/min; flow rate, 1.0 mL/min; room temperature; A 290 nm.
Analysis of 54 (figure 10) and the mixture by \(^1\text{H} \) NMR indicated the absence of signals arising from acetamides (-\(\text{NHCOCH}_3\)) and phenol acetates (\(\text{PhOCOCH}_3\)), thereby proving that these were not natural features of the \textit{Cliona} compounds (see also figure 11). These results led to the suggestion that a classical alkaloid extraction with acid might be useful in isolating clionamide (53). Execution of this procedure in the presence of nitrogen afforded a pale yellow solid residue, which was shown to be practically pure clionamide (-80\%). Attempts to crystallize this material were unsuccessful due to its instability.

Clionamide (53, figure 12) was isolated in pure form following rapid chromatography on silica plates although this led to substantial losses of material. Clionamide did not give an interpretable EIMS and its IR spectrum displayed little of
Figure 10. Partially exchanged 270 MHz $^1$H NMR spectrum of tetracetylclionamide-d$_{12}$ (54) in acetone-d$_6$. 
Figure 11. IR spectrum (KBr) of the underivatized Cliona metabolites.
Figure 12. 400 MHz $^1$H NMR spectrum of clionamide (53) in methanol-d$_4$. 
diagnostic value beyond a broad OH/NH absorption at 3300 cm\(^{-1}\) and a broad amide absorption at 1635 cm\(^{-1}\). Attempts to obtain \(^1\)H NMR spectra in DMSO-\(d_6\) led to decomposition of the molecule. Formation of the deuteroacetonide 55 occurred when \(^1\)H NMR spectra (figure 13, see also appendix 2) were obtained in acetone-\(d_6\). The acetonide methyl resonances were observed at \(\delta\) 1.42 and 1.47 in the non-deuterated acetonide 56 (figure 14). A \(^1\)H NMR spectrum of clionamide (53) procured in methanol-\(d_4\) showed none of the exchangeable protons. The non-exchangeable protons were trivially identified by analogy with the assigned signals for tetracetylclionamide (46). The identity of clionamide (53) was confirmed by conversion to tetracetylclionamide (46) upon acetylation.
Figure 13 a. 270 MHz $^1$H NMR spectrum of 55 in acetone-$d_6$ b. Vertical expansion c. Offset.
Figure 14 a. 270 MHz $^1$H NMR spectrum of 56 in acetone-d$_6$ b. Offset.
Clionamide (53), the major metabolite of \textit{C. celata}, showed only weak activity against \textit{S. aureus} and was inactive towards fungi. This unexpected result compelled us to re-evaluate our original sponge bioassays. When we collected, worked up, and bioassayed \textit{C. celata} in one day a result qualitatively similar to that obtained with clionamide was observed. As a result of the weak level of inhibition obtained in this experiment we concluded that the strongly positive bioassays originally found were artifacts. Decomposition of one of the \textit{Cliona} metabolites must give rise to this effect but the compound responsible was not pursued further. The underivatized \textit{Cliona} metabolites are currently being commercially tested for their pharmacological properties.

VI. The Celenamides: Isolation (62,63)

Preliminary chromatographic analysis of crude acetylated \textit{C. celata} extracts by TLC showed two ill-defined spots with \textit{Rf} values greater than tetracetylclionamide (46) upon visualization with UV light or charring with sulphuric acid. Partial purification of this extract was achieved by silica column chromatography employing a stepwise CH$_2$Cl$_2$-EtOAc gradient.

1This result is consistent with the observation that \textit{C. celata} grows in close association with numerous algae, tunicates, bryozoans and other marine organisms. The area surrounding sponges which possess potent antimicrobial agents is usually free of other forms of marine life. However, we have never observed signs of predation of \textit{C. celata}.
Examination of the column eluate by RP- (eg. figure 15) and silica-HPLC indicated that the fraction eluting with 10-50 percent EtOAc in CH₂Cl₂ contained approximately ten compounds. 

¹H NMR investigation of this material subsequently caused us to increase our estimation of the number of acetylated celenamides present to 18-22. However, the majority of these are exceedingly minor components.

Careful repetition of the column purification step facilitated the partial fractionation of this mixture (eg. figure 16). The most polar constituent, hexacetylceleanamide B (57), was obtained in pure form following repeated silica PTLC including multiple developments of each plate. This method failed to separate hexacetylceleanamide A (58) and pentacetylceleanamide C (59). These metabolites were isolated by repeated chromatography on reversed-phase plates. Nonacetylceleanamide D (60) was obtained in an analogous fashion. The remaining Cliona alkaloids proved to be extremely difficult to purify and consequently were studied as mixtures.

Figure 16. Silica HPLC of partially purified acetylated Cliona metabolites a. Fraction eluting with 25% EtOAc b. Fraction eluting with 50% EtOAc. Analysis conditions: LiChrosorb Si60; 10 x 4 x 250 mm; solvent. CH₂Cl₂-CH₃CN (4:1); flow rate, 1.2 mL/min; room temperature; A 290 nm.
Figure 15. RP-HPLC of crude acetylated methanol extracts of Cliona celata a. β-stage b. α-stage. Analysis conditions: μBondapak-C18, 4 x 250 mm; solvent, CH₃CN-H₂O (3:2); flow rate, 1.2 mL/min; room temperature; λ 290 nm. Chromatogram 'a' from 5.59-11.36 min is identical to the chromatogram displayed by a silica column fraction eluting with 10-50% EtOAc.
VII. The Celenamides: Structure Determination

All of the purified acetylated celenamides were white powders and none have crystallized to date. The homogeneity of these compounds was established by spectral analysis (\(^{1}H\) NMR, MS) and particularly by HPLC on silica and reversed-phase supports using several solvent combinations. These powders decomposed on heating and on prolonged standing in solvents and did not give acceptable elemental analyses.

The peptidyl nature of the acetylated celenamides was evident from intense amide absorptions in their infrared spectra (\(\nu_{\text{max}}\) 3300 (NH stretch), 1660 cm\(^{-1}\) (amide I band)) (figures 17 and 18) and peptide alpha-methine and NH signals present in the \(^{1}H\) NMR spectrum of each compound (figures 19, 20, 29, 33, table 1) (see also appendix 3, 4 and 6). Many of the spectral features observed were reminiscent of those exhibited by tetracetylclionamide (46). However, the abundance of signals in the \(^{1}H\) NMR spectra suggested much greater complexity. The preliminary spectral data confirmed our suspicions that chemical degradation would be required to simplify, and so deduce, these structures. Hexacetylcelenamide A (58, 0.03% wet weight) and hexacetylcelenamide B (57, 0.02% wet weight) were the most abundant of this new class of Cliona metabolite and therefore, were the obvious choices for detailed chemical examination. The structures of pentacetylcelenamide C (59) and nonacetylcelenamide D (60) were solved largely by comparison with 57 and 58. The proposed structures of the minor
Figure 17. IR spectra a. Hexacetylcelenamide A (58) b. Hexacetylcelenamide B (57).
Figure 18. IR (KBr) spectra a. Pentacetylcelenamide C (59) b. Nonacetylcelenamide D (60).
Figure 19  a. 400 MHz $^1$H NMR spectrum of hexacetylcelenamide A (58) in acetone-$d_6$, b. Vertical expansion, c,d,e. Expansions (600 Hz) of the aromatic region of 58.
Figure 20  a. 270 MHz $^1$H NMR spectrum of hexacetylcelenamide B (57) in acetone-d$_6$ b. Vertical expansion  c,d. Expansion (1000 Hz) of the aromatic region of 57. See appendix 5 for partial 400 MHz spectrum and computer simulation.
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<tr>
<td>-DAc</td>
<td>2.26 6H</td>
<td>2.25 3H</td>
<td>2.28 9H</td>
<td>2.24 3H</td>
<td>2.26 9H</td>
<td>2.28 9H</td>
<td>2.23 6H</td>
<td>2.24 6H</td>
<td>s</td>
</tr>
<tr>
<td>Phenyl H</td>
<td>7.13 2H</td>
<td>7.17 2H</td>
<td>7.41 2H</td>
<td>7.23 2H</td>
<td>7.43 2H</td>
<td>7.21 2H</td>
<td>7.42 2H</td>
<td>7.38 2H</td>
<td>s</td>
</tr>
<tr>
<td>Phenyl H</td>
<td>7.15d.</td>
<td>7.12d.</td>
<td>7.04d.</td>
<td>8.1H</td>
<td>8.1H</td>
<td>9.1H</td>
<td>7.21d.</td>
<td>7.21d.</td>
<td>7.38d.</td>
</tr>
<tr>
<td>Phenyl H</td>
<td>2.1H</td>
<td>2.1H</td>
<td>9.1H</td>
<td>2.1H</td>
<td>7.23dd.</td>
<td>7.17dd.</td>
<td>7.28dd.</td>
<td>2.8.1H</td>
<td>2.8 1H</td>
</tr>
<tr>
<td>Phenyl H</td>
<td>7.47 2H</td>
<td>7.44 2H</td>
<td>s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-CO(NHR)C=CH</td>
<td>6.80</td>
<td>7.16</td>
<td>6.84</td>
<td>7.06 7.59</td>
<td>6.88</td>
<td>7.22</td>
<td>7.09 7.41</td>
<td>s</td>
<td></td>
</tr>
</tbody>
</table>

1 All 1H NMR spectra were recorded in (CD3)2CO. In ppm from internal tetramethylsilane. Assignments were made by extensive decoupling, D2O exchange experiments, comparison with model compounds and in several instances examination of expansions obtained at 400 MHz and computer simulated spectra (see appendix 5). Derived from 57 or 59.
constituents, not amenable to purification, were suggested solely on the basis of \(^1\)H NMR considerations.

Molecular ions were not observed in the EIMS (10-70 eV) of hexacetylcelenamides A (58, figure 21) and B (57, figure 22). The elemental compositions of \(\text{C}_{46}\text{H}_{48}\text{BrN}_{5}\text{O}_{14}\) and \(\text{C}_{45}\text{H}_{46}\text{BrN}_{5}\text{O}_{14}\) were inferred from \(^1\)H NMR (58, 43 CH and 5 NH; 57, 41 CH and 5 NH), \(^{13}\)C NMR (58, 46C, appendix.4), and mass spectra [58 and 57 displayed the fragment ion \(\text{C}_{12}\text{H}_{13}\text{NO}_{4}\) \(^+\) for C-terminal diacetoxystryrylamine (53,64,65, see scheme 3)] in conjunction with the molecular formulas, \(\text{C}_{31}\text{H}_{38}\text{BrN}_{5}\text{O}_{7}\) and \(\text{C}_{30}\text{H}_{36}\text{BrN}_{5}\text{O}_{7}\), of their methylated hydrolysis products 61 and 62 determined from exact mass measurements.

![Chemical structures](image.png)

The \(^1\)H NMR spectrum of peptide 58 (table 1) displayed resonances at \(\delta 6.52 \text{ (d, 1H, J=15Hz)}\), 7.50 (dd, 1H, J=10, 15Hz), and 9.50 (d, 1H, J=10Hz, exchangeable) indicative of a trans -
Figure 21. EIMS of hexacetylcelemamide A (58).
Figure 22. EIMS of hexacetylcelenamide B (57).
Scheme 3. Interpretation of the EIMS of hexacetylcelenamide A (58) and B (57) a. R=CH$_2$(CH$_3$)$_2$, b. R=CH(CH$_3$)$_2$, c. Consecutive losses of C$_2$H$_2$O are observed from these ions, d. Exact mass measurement obtained on daughter ions only (-C$_2$H$_2$O), e. Exact mass measurement not obtained, f. Fragment ion intensities vary with instrument conditions, g. Base peak m/e 43, h. Calcd formulas are within 0.0015 amu of obsd values.
disubstituted enamide (53) as in 46. The observation of intense fragment ions at m/e 235, 193, and 151 in the mass spectrum (figure 21, scheme 3) suggested that the enamide substituent was a diacetoxyphenyl group. Evidence for the same residue was apparent in the spectral data of peptide 57 (table 1, figure 22, scheme 3). The most intense fragments observed in the EIMS of peptides and peptide-related metabolites (66) arise from the C- and N-terminal residues. Ozonolysis of enamides 57 and 58 in methanol at -78°C followed by reduction of the ozonides with dimethyl sulfide yielded equimolar amounts of 3,4,5-triacetoxybenzaldehyde (63) and 3,4-diacetoxybenzaldehyde (64) plus polar products resulting from the remainder of the molecule. The substitution patterns about the benzene rings of these aldehydes were determined by comparison with authentic samples (see appendix 1). 3,4,5-Triacetoxybenzaldehyde was prepared from gallic acid by acetylation, formation of the acid chloride, and Rosenmund reduction (67), while 3,4-diacetoxybenzaldehyde was obtained simply by acetylating commercially available 3,4-dihydroxybenzaldehyde (Aldrich). The production of 64 in this ozonolysis reaction is consistent with the presence of (E)-1-amino-2-(3',4'-diacetoxyphenyl)ethene as the C-terminal residue of both hexacetylcelenamide A (58) and B (57).

![63](image1)

![64](image2)
Treatment of enamides 58 and 57 with 0.1 M HCl, as described for 46, gave the peptide amides 65 and 66. The richly detailed $^1$H NMR spectra of these products (figures 23, 24; table 1) revealed a number of structural features. Two peptide alpha-methine protons were observed at $\delta$ 4.28 and 4.66 for 65, and at $\delta$ 4.39 and 4.71 for 66. Both compounds showed uncoupled olefinic signals (65, $\delta$ 6.88; 66, $\delta$ 7.22) and highly deshielded NH singlets (65, $\delta$ 9.33; 66, $\delta$ 9.46) characteristic of phenyl-substituted dehydroamino acid residues (68). A table of comparative values from synthetic (see experimental) dehydroamino acid derivatives is provided on the following page.

![Chemical Structures](image)

Also discernible in the $^1$H NMR spectra were an acetamide resonance (65, $\delta$ 1.98; 66, $\delta$ 1.86) and two primary amide hydrogens 65, $\delta$ 6.51, 7.21; 66, $\delta$ 6.54, 7.22), indicating that 65 and 66 were tripeptides with the N-terminus acetylated and the C-terminus protected as a primary amide.

The nature of the two alpha-amino acid residues was
Figure 23  a. 270 MHz $^1$H NMR spectrum of 65 in acetone-$d_6$.  
   b. Expansion (1000 Hz) of the aromatic region of 65.
Figure 24 a. 270 MHz $^1$H NMR spectrum of 66 in acetone-$d_6$ b. Expansion (1000 Hz) of the aromatic region of 66.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_2C=CHAr$ (s)</th>
<th>ArH (2H, s)</th>
<th>NH (s)</th>
<th>NHAc (3H, s)</th>
<th>Deuterated solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcATPheNH$_2^2$</td>
<td>7.06$^3$</td>
<td>6.90</td>
<td>9.37</td>
<td>2.00</td>
<td>DMSO</td>
</tr>
<tr>
<td>AcATPheGlyOMe</td>
<td>7.19</td>
<td>6.88</td>
<td>8.69</td>
<td>2.12</td>
<td>Acetone</td>
</tr>
<tr>
<td>AcATPheValOH</td>
<td>6.99</td>
<td>6.89</td>
<td>9.46</td>
<td>2.01</td>
<td>DMSO</td>
</tr>
<tr>
<td>AcATPheLeuOH</td>
<td>6.99</td>
<td>6.87</td>
<td>9.38</td>
<td>2.00</td>
<td>DMSO</td>
</tr>
<tr>
<td>AcATPheATPhe$\text{NET}_2$</td>
<td>7.29</td>
<td>6.83</td>
<td>9.77</td>
<td>2.13</td>
<td>Acetone</td>
</tr>
<tr>
<td></td>
<td>5.94 (4H, s)</td>
<td></td>
<td>8.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcATPheATPheNH$_2^{tBu}$</td>
<td>7.32</td>
<td>6.92</td>
<td>9.38</td>
<td>2.21</td>
<td>Acetone</td>
</tr>
<tr>
<td></td>
<td>7.03</td>
<td>6.84</td>
<td>8.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcATPheATPheOMe</td>
<td>7.23</td>
<td>7.02</td>
<td>9.58</td>
<td>2.02</td>
<td>DMSO</td>
</tr>
<tr>
<td></td>
<td>7.15</td>
<td>6.91</td>
<td>9.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcATPheATPhe-DMD$^5$ (400 MHz)</td>
<td>7.35</td>
<td>6.94</td>
<td>10.06</td>
<td>2.11</td>
<td>DMSO</td>
</tr>
<tr>
<td></td>
<td>6.97</td>
<td>6.92</td>
<td>9.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcATPheATPhe-DMD</td>
<td>7.37</td>
<td>6.9 (m)</td>
<td>9.68</td>
<td>2.23</td>
<td>Acetone</td>
</tr>
<tr>
<td></td>
<td>7.00</td>
<td></td>
<td>9.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$Spectra were recorded at 270 MHz unless otherwise indicated.

$^2$See experimental for preparation procedures.

$^3$All chemical shifts are in ppm from internal tetramethylsilane.

$^4$ATPhe refers to $\alpha,\beta$-didehydro-$(3,4,5$-trimethoxy)phenylalanine.

$^5$DMD refers to O,O-dimethyldopamine.
Figure 25. EIMS of 65.
Figure 26. EIMS of 66.
apparent from the spectral data of hexacetylcelenamides A (58) and B (57). An abundant fragment ion at m/e 210/208, characteristic of bromoindole derivatives (54, figures 21 and 22) was observed in the mass spectra, and comparison of UV (λmax 289 nm) and 'H NMR spectra (table 1) with the corresponding spectra of tetracetylclionamide (46; UV λ max 290 nm) clearly indicated that this moiety was present as a 6-bromotryptophan residue. Strong ions at m/e 86 (58, 34%) and 72 (57, 78%) in the mass spectra and signals at high field in the 'H NMR spectra corresponding to isopropyl groups (58, δ 0.97, 0.99; 57, δ 0.99, 1.01 (each d, 3H, J=7Hz)) suggested the presence of a leucine and valine residue in 58 and 57, respectively. Hydrolysis of peptides 58 and 57 in 6N HCL at 100°C for 18h afforded leucine and valine as expected. When milder hydrolytic conditions were employed an amino acid having chromatographic (cellulose, and silica 2D-TLC) and ninhydrin colouring properties similar to (±)-5-bromotryptophan was also observed. All the required signals for the 6-bromotryptophan, leucine, and valine residues could be found in the 'H NMR spectra of the hydrolysis products 65 and 66 (table 1, see also appendix 7 and 8).

The exact nature of the third amino acid residue, known to be a phenyl-substituted dehydroamino acid from the 'H NMR data was determined by chemical degradation. Ozonolysis of the primary amides 65 and 66 gave 3,4,5-triacetoxybenzaldehyde, indicating that an α,β-didehydro-3,4,5-trihydroxyphenylalanine residue must be present. This result excluded the possibility of
having an $\alpha,\beta$-didehydro-6-bromotryptophan residue. Additional evidence for the dehydroamino acid was provided by the isolation of oxalic acid from an acid hydrolysate of the polar products (eq. 67) obtained from the ozonolysis of 57 and 58. Reduction of a small sample of a 1:1 mixture of the trimethylated hydrolysis products 61 and 62 with sodium borohydride gave in high yield a mixture of the expected dihydro derivatives (see experimental). Sodium borohydride reductions are characteristic reactions of dehydroamino acids (69c).

\[
\begin{align*}
\text{NHAc} & \\
\text{R} & \\
\text{NHCO} & \\
\text{O} & \\
\end{align*}
\]

67

We could conclude from the above data that tripeptide 65 contained 6-bromotryptophan, $\alpha,\beta$-didehydro-3,4,5-trihydroxyphenylalanine and leucine residues, while tripeptide 66 contained 6-bromotryptophan, $\alpha,\beta$-didehydro-3,4,5-trihydroxyphenylalanine, and valine residues. Hence, the structures of hexacetylcelenamides A (58) and B (57) must consist of tripeptides 65 and 66 bearing C-terminal (E)-1-amino-2-(3',4'-

$\alpha,\beta$-Didehydrotryptophan residues are components of several marine (23,24) and terrestrial natural products (69d). Typical UV spectral characteristics are $\lambda_{\text{max}}$ 360 nm ($\epsilon \times 10^5$).
diacetoxyphenyl)ethene residues in place of the primary amides. The mild acid hydrolysis of the trans-substituted enamide in 58 and 57 generates the primary amide functionalities in 65 and 66 (see also appendix 9).

The amino acid sequences of hexacetylcelenamide A (58) and B (57) were deduced from their mass spectra (figures 21, 22 and scheme 3). As mentioned earlier, in the mass spectra of most peptide alkaloids the strongest ions originate from the N-terminal amino acid (66) and intense ions originate from the C-terminal grouping. Abundant fragment ions for 58 at m/e 86 (34%) and for 57 72 (78%) suggested, therefore, that leucine and valine represented the N-terminal residues of these compounds, respectively. This was confirmed by the presence of diagnostic ions observed at m/e 128 (15%) and 156 (5%) in the spectrum of 58 and at m/e 114 (38%) and 142 (9%) in the spectrum of 57, originating from N-acetylleucine and N-acetylvaline. These interpretations were substantiated by the mass spectra of several model compounds (table 3).
### TABLE 3. EIMS OF SYNTHETIC MODEL COMPOUNDS

<table>
<thead>
<tr>
<th>Compound</th>
<th>m/e</th>
<th>rel%</th>
<th>m/e</th>
<th>rel%</th>
<th>m/e</th>
<th>rel%</th>
<th>other m/e</th>
<th>rel%</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcValNH₂</td>
<td>72</td>
<td>100</td>
<td>114</td>
<td>67</td>
<td>142</td>
<td>&lt;1</td>
<td>158 (M⁺)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>43</td>
<td>14</td>
</tr>
<tr>
<td>AcLeuNH₂</td>
<td>86</td>
<td>90</td>
<td>128</td>
<td>76</td>
<td>156</td>
<td>2</td>
<td>172 (M⁺)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>Ac(3,4,5-tri-MeO)Δ PheValOH¹</td>
<td>72</td>
<td>18</td>
<td>114</td>
<td>&lt;1</td>
<td>142</td>
<td>6</td>
<td>207</td>
<td>100</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>43</td>
<td>76</td>
</tr>
<tr>
<td>Ac(3,4,5-tri-MeO)Δ PheLeuOH²</td>
<td>86</td>
<td>3</td>
<td>128</td>
<td>1</td>
<td>156</td>
<td>5</td>
<td>340</td>
<td>100</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>43</td>
<td>25</td>
</tr>
</tbody>
</table>

¹ N-Acetyl-α,β-didehydro-(3,4,5-trimethoxy)phenylalanylvaline.

² N-Acetyl-α,β-didehydro-(3,4,5-trimethoxy)phenylalanyleucine.
The 6-bromotryptophan and \( \alpha,\beta \)-didehydro-3,4,5-trihydroxyphenylalanine residues were sequenced on the basis of two diagnostic fragment ions. Each of these fragment ions lead to a series of daughter ions resulting from losses of \( \text{C}_2\text{H}_2\text{O} \) typical of phenol acetates. The mass spectra of both 58 and 57 displayed an \( m/e \) 484/482 ion (scheme 3) resulting from the elimination of \( \text{NHCOR} \) and \( \text{H} \) which is commonly observed in tryptophan-containing metabolites (53,70). The presence of this characteristic ion suggested that 6-bromotryptophan was linked to the C-terminal styrylamino group. Support for this hypothesis comes from the observation of \( m/e \) 432 and 418 ions in the spectra of 58 and 57, respectively, indicating that the \( \alpha,\beta \)-didehydro-3,4,5-trihydroxyphenylalanine residue was linked to the N-terminal leucine and valine residues. One other structure in which the relative positions of the 6-bromotryptophan and dehydroamino acid residue are reversed, could be considered for both 58 and 57. The ions expected from the fragmentation of these hypothetical structures are not observed in the MS of 58 or 57. Only the structures proposed for these two alkaloids are consistent with the data obtained.

Electron-impact MS of the nonvolatile Cliona alkaloids are in general difficult to obtain and difficult to reproduce. The hydrolysis product 65 failed to give an interpretable field-
desorption mass spectrum.¹

The geometry about the dehydroamino acid double bond has not been determined. Nuclear Overhauser effect experiments proved to be ambiguous and comparison with synthetic model compounds (to be discussed) did not allow us to draw definitive conclusions.

The isolation of hexacetylceleanamide-δ₁₈ A (68) (figure 27) and hexacetylceleanamide-δ₁₈ B (69, figure 28) from deuteroacetylated C.celata extracts, as discussed in connection with tetracetylclionamide-δ₁₂ (54), led to the conclusion that celenamides A (70) and B (71) were the naturally occurring sponge metabolites.

¹ Peptide amide 65 crystallized from CHCl₃-acetone as very fine needles (mp 153-156°C). Elemental analysis suggested the molecular formula C₃₄H₃₈BrN₅O₁₀·2/3H₂O. The adhesion of solvents of crystallization to this material is characteristic of peptide alkaloids (64,65) and dehydropeptides (69).
Figure 27. 270 MHz $^1$H NMR spectrum of 68 in acetone-$d_6$. Discrete signals attributable to pentacetylcelenamide C-$d_{15}$ are indicated by the letter c.
Figure 28. 270 MHz $^1$H NMR spectrum of 69 in acetone-$d_6$. Signals arising from 68 are designated by the letter a.
The $^1$H NMR spectrum of pentacetylcelenamide C (59, figure 29, table 1) showed remarkable similarity to the spectrum displayed by hexacetylcelenamide A (58). Comparison of the two spectra indicated the presence of resonances corresponding to a 1,4-disubstituted and a 1,3,4,5-tetrasubstituted phenyl ring in the spectrum of 59, in contrast to the presence of signals arising from 1,3,4-trisubstituted and 1,3,4,5-tetrasubstituted phenyl rings in the spectrum of 58. Hence, the structure elucidation of the gross skeleton of 59 required only the positioning of the two phenolic substituents in order to differentiate between the two possible structures shown below:

---

1 Ozonolysis of a mixture of acetylated celenamides similar in composition to that shown in figure 15 afforded 3,4,5-triacetoxy, 3,4-diacetoxy and 4-acetoxybenzaldehyde as the only isolable products. The latter was identified by comparison with an authentic sample prepared by acetylation of 4-hydroxybenzaldehyde (Sigma).
Figure 29. 270 MHz $^1$H NMR spectrum of pentacetylcelenamide C (59) in acetone-$d_6$. b. Vertical expansion (1000 Hz) of the aromatic region of 59.
This problem was trivially solved by hydrolysis of 59 with HCl in acetonitrile to give the peptide amide 65 (figures 30 and 31) which proved to be identical (table 1) to the peptide amide obtained when 58 was treated in a similar fashion. The amino acid sequence of 59, which corresponded to that of 58, was assigned on the basis of its EIMS (figure 32 and scheme 4).

Pentacetylcelenamide-d_{15} C was not separated from hexacetylcelenamide-d_{18} A (68) but was clearly visible in the 'H NMR spectrum of a mixture of the two compounds (figure 27). Thus we could conclude that celenamide C (72) was present in the underivatized sponge extract.

![Chemical structure of 72](image)

It was immediately apparent from 'H NMR studies (table 1) that unlike hexacetylcelenamide A (58), B (57) and pentacetylcelenamide C (59), nonacetylcelenamide D (60, figure
Figure 30 a. 270 MHz $^1$H NMR spectrum of 65 in acetone-$d_6$ b. Vertical expansion c. Expansion (1000 Hz) of the aromatic region of 65.
Figure 31. EIMS of 65.
Figure 32. EIMS of pentacetylcelenamide C (59).
Scheme 4. Interpretation of the EIMS of pentacetylcelenamide C (59) a. Fragment ion intensities vary with instrument conditions, b. Base peak m/e 43, c. Consecutive losses of C$_2$H$_2$O are observed from these ions, d. Calcd formulas are with 0.00015 amu of obsd values.
33) did not possess 6-bromotryptophan and that leucine (δ CH, 4.48, m) represented the sole alpha-amino acid present in the molecule. Intense ions at m/e 86 and 128 (figure 34, scheme 5) defined N-acetylleucine as the N-terminal residue.

Characteristic resonances at δ 6.60 (d, J=15 Hz), 7.59 (dd, J=10, 15 Hz), and 9.65 (d, J=10 Hz) in the 1H NMR spectrum of 60 suggested the presence of a C-terminal styrylamine residue. Abundant fragment ions at m/e 235, 193 and 151 in the EIMS further suggested that the C-terminus was an (E)-l-amino-2-(3',4'-diacetoxyphenyl)ethene group as in 57 and 58. This was confirmed by hydrolysis to the peptide amide 73. The 1H NMR (δ 6.53 and 7.30 (each 1H, s); figure 35, table 1), IR (3640, 3540 cm⁻¹), and MS (figure 36) of 73 indicated that the C-terminal styrylamine residue of 60 was absent in 73 and that the
Figure 33  a. 270 MHz $^1$H NMR spectrum of nonacetylcelenamide D (60) in acetone-$d_6$ b. Expansion (1000 Hz) of the aromatic region of 60 c. Partial expansion (1000 Hz) of the aromatic region of a further purified sample of 60.
Figure 34. EIMS of nonacetylcelenamide D (60).
Scheme 5. Interpretation of the EIMS of nonacetylcelenamide D (60) a. Fragment ion intensities vary with instrument conditions, b. Base peak m/e 43, c. Consecutive losses of C₂H₂O are observed from these ions, d. Calcd formulas are within 0.00015 amu of obsd values.
Figure 35  a. 270 MHz $^1$H NMR spectrum of 73 in acetone-$d_6$  b. Expansion (1000 Hz) of the aromatic region of 73.
Figure 36. EIMS of 73.
The remaining structural features of nonacetylcelemamide D (60) were elucidated by $^1$H NMR (table 1). Two uncoupled olefinic signals at δ 7.06 and 7.59, two highly deshielded NH singlets at δ 9.18 and 9.89, and two 2H aromatic singlets at δ 7.43 and 7.47 clearly indicated the presence of two α,β-didehydro-3,4,5-triacetoxyphenylalanine residues. The observation of fragment ions at $m/e$ 638, 432 and 319 (figure 34) in the EIMS of 60 furnished conclusive evidence for this proposal.

Nonacetylcelemamide-d$_{27}$ D was not isolated. However, as previously mentioned, $^1$H NMR analysis of the PTLC purified deutoacetylated celenamide mixture did not display any resonances corresponding to phenol acetates or acetamides. This
result, in conjunction with absence of these signals in all of the partially purified deuteroacetylated celenamides (68,69) and tetracetylclionamide-d_{12} (54) led to the assumption that celenamide D (74) was a naturally occurring sponge metabolite.

Substantial effort has been expended in attempting to purify the least polar acetylated celenamides. The RP-HPLC of this complex mixture (below: see figure 15 for general conditions) clearly indicates the nature of the purification problem.
Several successive PTLC steps, each involving multiple solvent development, and RP-TLC have been employed. This combination of procedures occasionally generated material which was substantially pure by HPLC criteria. However, $^1\text{H}$ NMR indicated that in each case a mixture of 2-4 compounds remained. Enhancing this problem was the gradual deterioration of these substances when handled excessively, the small quantities present in the sponge extracts, and the slight variation in the composition of the minor constituents of \textit{C. celata} from collection to collection.

Owing to our inability to procure these compounds in a homogeneous state only a brief overview of the general features discernible from the $^1\text{H}$ NMR spectra of mixtures will be presented.

Several of the minor constituents bear close resemblance to hexacetylcelenamides \textit{A} (58) and \textit{B} (57), pentacetylcelenamide \textit{C} (59), and nonacetylcelenamide \textit{D} (60). The principal site of variation appears to be the aliphatic amino acid residue. Amino acid analysis of the hydrolyzed (6NHC\textsubscript{1}, reflux) acetylated celenamide mixture demonstrated the presence of a variety of common alpha-amino acids, including: threonine, serine, glycine, valine, isoleucine and leucine.

Two classes of novel metabolites have been detected. From one collection what is possibly a modified tetrapeptide, similar to 58 but containing one unique residue, was obtained. Unfortunately, this compound decomposed during routine spectral examination and has not been encountered in subsequent
collections. The second group of novel compounds studied are distinct in that, unlike 57-60, they appear to lack a typical N-terminal acetamide grouping (ca. 2.10-1.80 ppm) (eg. figures 37 and 38).

![Figure 37. Aliphatic region of the 270 MHz 'H NMR spectrum of a partially purified sample of unknown acetylated celenamides in acetone-d$_6$.](image)

This observation can be explained by three structural possibilities: an N-terminal dehydroamino acid (cf. table 2); a cyclic molecule or a cyclic N-terminal residue; or an N-alkylated N-terminal residue. Structural conclusions could not be drawn from the 'H NMR spectra alone and the materials
responsible for the $^1$H NMR spectrum shown in figure 40 did not give an interpretable EIMS. Analysis of this material by $^{13}$C NMR (appendix 10) did not aid the structure assignment due to the small amount of material available and the high molecular weight. It is clear from the information obtained thus far that these molecules all possess a 6-bromotryptophan residue and a C-terminal trans-disubstituted enamide moiety. The nature of the rest of the structural features of these distinct molecules remains to be determined.
Figure 38. 270 MHz $^1$H NMR spectrum of a partially purified unknown peptide alkaloid in acetone-$d_6$. 
VIII. Discussion

Carle and Christophersen (20) recently stated that "the literature on marine alkaloids is very scarce; moreover, the few bromosubstituted members of this class seem to bear no resemblance to patterns established from terrestrial sources". Celenamides A (70), B (71) and C (72) possess bromine, and their structures, as well as that of celenamide D (74), closely parallel those of the peptide alkaloids isolated from several terrestrial plant families (64,65) (eg. Rhamnaceae). The similarity is particularly apparent when celenamide B (71) and integerrin (75, 71) are compared. Lasiodine A (76, 72) is the only other example of an acyclic peptide alkaloid.
The biogenesis of the plant peptide alkaloids has not been investigated to any significant extent. Tschesche and Kaussmann (65a) suggest that the lone linear example, lasiodine A (76), might arise from secondary opening of a cyclic precursor. Alternatively, the cyclopeptide alkaloids may arise via 1,4-addition of the C-terminal phenolic residue of a linear precursor onto an appropriately situated \( \alpha,\beta \)-didehydroamino acid (65b). The styrylamine residue present in the marine and terrestrial peptide alkaloids is unique to this family of natural products. Lasiodine A (76) and all of the terrestrial metabolites possess this grouping in a cisoid arrangement in contrast to the transoid configuration found in clionamide (53) and the celenamides (70,71,72,74). No evidence of isomerization to the thermodynamically less stable cis configuration or subsequent cyclization to the phencyclopeptine\(^1\) nucleus has been encountered in the latter. The possibility exists that the naturally occurring sponge metabolites possess cisoid styrylamine residues and that the transoid configurations observed are artifacts. We feel that this is unlikely due to the fact that the harsh acid-base extraction procedure used to isolate lasiodine A did not result in isomerization and that in comparison, the procedure utilized in obtaining the Cliona alkaloids is extremely mild. The oxidative enzymes which give rise to the styrylamine moiety in the plants and in the sponge

\(^1\)Rapoport (73) has proposed the nomenclature "phencyclopeptines", to describe the 14-membered ring nucleus common to many peptide alkaloids.
may have different stereochemical specificities.

Investigation of the biosynthetic origin of the novel structural features of the *Cliona* metabolites has not been attempted for obvious logistical reasons. However, recent success by Dr. Pomponi in cultivating *C. celata* cells in suspension should facilitate the performance of biogenesis experiments at a later date.

Consideration of the biogenetic origin of a metabolite is a challenging intellectual process and can frequently be an important aid to structure determination. However, in the absence of experimental evidence over emphasis should not be given to speculative schemes. A plausible biosynthetic route to the C-terminal styrylamine residue of peptide alkaloids is outlined in scheme 6. The pathway shown is based upon the well-established biosynthesis of the catecholamines (74) and the peyote alkaloids (75). These are summarized in scheme 7. The catecholamines occur widely throughout the animal and plant kingdoms and are also known from microorganisms (74). Their mechanism of formation in insects is analogous to that in vertebrates (74). The plausibility of the biosynthetic pathway displayed in scheme 6 is substantiated by the natural occurrence of pandamine (77, 65) and pandaminine (78, 65).

![Chemical Structures]

\[ R = \text{CH(Me)Et} \]

\[ R = \text{CH(Me)}_2 \]
Scheme 6. Possible biosynthetic routes to C-terminal styrylamines.
Scheme 7. A. Biosynthesis of Mescaline (75). B. Biosynthesis of L-epinephrine (74).
The terrestrial and marine peptide alkaloids have several features in common in addition to their C-terminal styrylamine residues. Alkaloids from either source exist as complex mixtures. The principal site of variation in these molecules is the nature of the side chains of the aliphatic amino acids. The basicity of peptide alkaloids is attributable to their N-terminal α-amino group. All of the Cliona alkaloids isolated in a pure state possess a free amino terminus, as do a number of terrestrial peptide alkaloids (65). Most of the plant cyclopeptide alkaloids are strongly levorotatory (-200° to -400°, 65). The optical rotations of hexacetylcelenamide A (58, +40°), hexacetylcelenamide B (57, +22°), and pentacetylcelenamide C (59, +14°) are dextrorotatory and of similar magnitude to lasiodine A (76, +38°). Most synthetic di- and tripeptides composed of L-amino acids and one aromatic dehydroamino acid are dextrorotatory (75,76). Nonacetylcelenamide D (60, -25°) is levorotatory, a characteristic of peptides containing L-amino acids other than proline and two aromatic dehydroamino acid residues (75,76). Unfortunately, these generalizations cannot be used to predict the absolute stereochemistry of the α-amino acids of 57, 58, 59 and 60 as a result of the dextrorotation of lasiodine A (76). Lasiodine A is the only peptide alkaloid known to possess an amino acid having the D configuration.

The bromotryptophan residue present in clionamide (53) has been shown to possess the L configuration. Since this residue is destroyed during acid hydrolysis, the absolute stereochemistry of the two chiral carbon atoms present in hexacetylcelenamide A
(58), B (57) and pentacetylcelenamide C (59) has not been determined. The synthetic studies described in a subsequent chapter were designed to resolve this problem.

Several natural products containing bromoindole residues have been isolated from marine plants and animals (80-89, see also 11, 12, 15, 16, p. 9,10), as have a number of closely related compounds (9-11, p. 8; 79). The monobrominated metabolites, with one exception (12), possess a halogen atom in the 6-position. The Cliona metabolites 53, 70, 71 and 72 conform to this general pattern. A specific haloperoxidase may be active in the organisms from which these compounds were obtained.

The specificity of the enzyme systems responsible for the production of clionamide and the celenamides would not, at first glance, appear to be particularly rigid. On closer examination certain constancies are apparent. All of the celenamides possess, as part of the C-terminal trans-disubstitued enamide, a phenyl ring which is less oxidized than the corresponding group of clionamide (53). Oxidation of this residue to the trihydroxy state may inhibit further elongation of the peptide chain, or conversely, oxidation to this state may not be possible in the longer chain. Also, with the exception of celenamide D (74), all of the Cliona metabolites bear a 6-bromotryptophan residue in the penultimate C-terminal position.
Hemichordata

Porifera

Mollusca
Ribosomal protein and peptide synthesis is initiated at the N-terminal residue, however, it is intriguing to note that the maximum constancy of the Cliona alkaloids occurs at, and adjacent to, the C-terminus. This observation leads to the suggestion that the sponge alkaloids may be biosynthesized independently of the normal RNA-controlled peptide synthesis as is the case for cyclic peptide antibiotics (81).

\(\alpha,\beta\)-Didehydroamino acids have been studied extensively owing to their occurrence as constituents of many biologically active-microbial peptides, the majority of which are cyclic. \(\alpha,\beta\)-Didehydrophenylalanine has been encountered in the cyclic peptide tentoxin (82) and a modified \(\alpha,\beta\)-didehydrotyrosine residue is present in mycelianamide (83). Derivatives bearing higher degrees of substitution on the phenyl ring have not been previously reported. 3,5-Disubstituted tyrosine-derived residues are components of numerous marine (eg 33, 43) and terrestrial natural products. To date, we have been unable to locate a published article reporting the occurrence of 3,4,5-trihydroxyphenylalanine in nature (84). An amino acid bearing this substitution pattern is not involved in the biosynthesis of mescaline (75, see scheme 7). A detailed search for this amino acid and for L-dopa in the mescaline synthesizing cactus, Lophophora williamsii failed to demonstrate their presence (75).

The biosynthesis of dehydroamino acids and dehydropeptides

\[3,4,5\text{-Trimethoxyphenylalanine has been synthesized by Acheson et al.}(85)\]
has recently been comprehensively reviewed by Schmidt et al. (81). Three of the several hypothetical routes to the formation of α,β-didehydro-3,4,5-trihydroxyphenylalanine are given in scheme 8. The conversion of dopa to 5-hydroxydopa (indicated by an asterisk) is unprecedented.

Dehydropeptides have previously been considered to occur primarily in microorganisms, especially fungi.¹ Sponges frequently host a variety of microorganisms, particularly bacteria, fungi, and microscopic algae. In some instances these algae exist in a symbiotic association with the sponge and are referred to as zooxanthellae. Zooxanthellae have been reported in several clionids (86), including C. celata collected off the coast of British Columbia.² The origin of the Cliona metabolites must be considered uncertain at this time.

Solution of the remainder of the Cliona alkaloids will require very careful and tediously repetitious fractionation by RP-HPLC. The knowledge gained from the structural elucidation of these apparently unique compounds will undoubtedly justify the effort required to isolate them.

During any natural-products investigation questions invariably arise concerning the origin of the substances isolated, their function in the organisms physiology, and their distribution in the species and family being studied.

¹One exception is lasiodine A (76,72).
²S. A. Pomponi, personal communication.
Scheme 8. Three possible biosynthetic routes to α,β-didehydro-3,4,5-trihydroxyphenylalanine-containing peptides. R and R' represent amino acid residues, although, several of the steps shown may occur at the free amino acid level.
Frequently, experiments aimed at answering these questions provide only ambiguous results. However, the search for these answers should be a prime concern of all natural-products chemists as this is one of our principal means of contributing to man's increased understanding of nature.

This chapter has outlined in detail the first stage involved in natural-products research, namely the isolation and structure determination of the metabolites of interest.

The following chapter presents our preliminary efforts at unravelling some of the mysteries associated with the ubiquitous burrowing sponge *Cliona celata* Grant.
CHEMICAL ECOLOGY OF CLIONA CELATA

I. Biology of the Clionidae

The marine sponge family Clionidae, consisting of about 100 described species, is classified as follows (87):

- Phylum: Porifera
- Class: Demospongiae
- Order: Hadromerida
- Family: Clionidae (Gray).

The genus Cliona with its 65 members is the most abundant of the 13 Clionidae genera. Clionids are prevalent in most of the world's oceans but are uncommon in arctic waters. Specimens have been encountered from Alaska and Baffin Island to Chile and Brazil. Cliona species commonly occur in England, the Mediterranean, Japan, and throughout the west central Pacific (88). Owing to their distinctive yellow or yellow-orange colouration (see figure 1), species of the genus Cliona are often referred to as 'sulphur sponges'. However, a few green (e.g. C. viridus) or purple (e.g. C. schmidtii) species exist. The Clionidae are identified by the nature of their spicules and the morphological features of their excavations into submerged calcareous materials.

The Clionidae have three recognizable multicellular life stages (89): the alpha stage, in which the organism is almost completely endolithic and is exposed to the environment only by
way of incurrent and excurrent papillae (eg. figure 1); the beta stage, in which the sponge becomes exolithic as well as endolithic and the gamma or "free-living" stage. The presence of the original host is not detectable in the latter stage and the sponge no longer engages in burrowing. Extremely large specimens of gamma-stage clionids have been encountered (eg. *C. celata*).

The sponge of principal interest in our investigation, *Cliona celata* Grant¹, is perhaps the most widespread and conspicuous of the cosmopolitan Clionidae.² It is one of the most common marine invertebrates in the waters of the coast of British Columbia and is frequently found burrowing into either the shells of the giant barnacle, *Balanus nubilis*, and the red rock scallop, *Hinnites multirugosus*, or into calcium carbonate bearing rocks. The beta stage is the predominant form of *C. celata* throughout Howe Sound although gamma-stage sponge is occasionally encountered. Barkley Sound, with its abundance of shellfish, predominantly hosts alpha-stage sponge.

The burrowing behaviour of *Cliona* has been most widely documented in regard to its destructive influence on reef corals (91) and commercial shellfish (92). The honeycombed skeleton of a rock scallop which has been infested by *C. celata* is shown in figure 39.

¹ *C. celata* was identified on the basis of morphology, colour, and microscopic spicule examination (90). The spicules are slightly curved tylostyles with terminal heads.

²Two other species of *Cliona* are believed to reside in B.C. waters. B. Austin, personal communication.
The chips produced during Cliona boring are an important component of biogenic carbonate sediments (91,97). In some shallow water environments these particles constitute up to 30 percent of the total sediment (97).

Figure 39. Remains of an H. multirugosus shell inundated by C. celata. Arrow (upper left corner) indicates a sponge papillae opening on the upper shell surface (magnified 3X).

Rock and shell boring by marine organisms is a common phenomenon (93). The biological advantage obtained as a result of this behaviour is most certainly protection and is rarely associated with feeding. This is especially true of burrowing sponges which are thought to feed exclusively on unicellular algae, bacteria, and organic detritus.

Clionid burrowing has been a subject of scientific attention for over 100 years. The currently accepted mechanism
of substrate penetration is outlined in figure 40. The etching cell (a specialized amoebocyte) is believed to secrete a substance which facilitates CaCO₃ dissolution. Acids (eg. carbonic), enzymes (eg. carbonic anhydrase), and organic chelators have been variously suggested to be the responsible agents (94). Copious quantities of acids are not secreted (95) by clionid sponges. Aqueous extracts of B.C. C. celata are neutral. The siliceous spicules are not involved in the burrowing process (95), however, Yonge (93) has proposed that growth processes may assist. Rutzler and Rieger (96) have estimated that 2-3 percent of the excavated CaCO₃ is removed by solution. This estimation has been confirmed by the observation that Cliona celata grown on calcareous substrates in vitro does not influence the calcium ion concentration of aquaria water (95). The fate of the dissolved calcium has not been determined.

Figure 40. Schematic representation of the burrowing process. (A) Etching cell in contact with substrate (stippled). (B) Initial penetration of cell processes by chemical etching of substrate. (C,D) Continued dissolution of substrate. (E) Etched chip removed from pitted substrate. (Reproduced by kind permission of Dr. S. A. Pomponi).
II. Life-Cycle, Geographic and Taxonomic Distribution of the C. celata Alkaloids

The isolation of clionamide and the celenamides as the principal phenolic secondary metabolites of C. celata led to the suggestion that these alkaloids might participate in either the primary dissolution of CaCO₃ or in the subsequent transport of dissolved calcium. Prior to embarking on an investigation of the role played by these compounds in the physiology of C. celata, we sought to ascertain the nature of their occurrence within the Clionidae family. The reason for obtaining this information was to determine whether conclusions concerning the function of the C. celata alkaloids would be relevant to the Clionidae as a whole. It was also hoped that chemotaxonomic significance could be ascribed to these compounds.

Specimens of C. celata were obtained from three principal locations: Woods Hole, Massachusetts; La Jolla, California; and Howe Sound, Barkley Sound and the Straits of Georgia, British Columbia. C. lampa, C. caribbaea, and C. delitrix were collected off Plantation Key, Florida. Sponge samples were extracted as described for C. celata in the experimental section, to give EtOAc and water soluble fractions. Phenolic substances were detected by a standard FeCl₃ spray reagent test. The EtOAc

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¹We thank Dr. S. A. Pomponi (Woods Hole and Florida collections) and Dr. D. J. Faulkner (La Jolla collections) for their generous contributions of sponge samples.
fractions were acetylated in the usual fashion and the crude residues obtained were analyzed by TLC and RP-HPLC.¹

*C. celata* indigenous to British Columbian (B.C.) waters showed a constant pattern of alkaloids when sponges of the same life-stage were compared. Beta and γ-stage sponges possess an essentially identical array of metabolites. However, examination of figure 15b indicates the absence of pentacetylceleinamide C (59) in α-stage B.C. sponge. This chromatogram also shows the presence of a novel compound having a retention time between that of 58 and 57. Comparison of figure 15a and 15b further indicates that fewer less polar constituents are found in the α-stage organism. These results suggest that the biosynthesis of alkaloids occurs even in the earliest stages of the sponge's development and that subtle alterations in metabolism do take place as the organism matures. The absence of alkaloids in early α-stage sponge would have ruled out the possibility of the involvement of these molecules in the burrowing process. The reason for the anabolic changes observed is not clear. The composition of peptide alkaloid mixtures obtained from terrestrial plants has been reported to be dependent upon the maturity of the plant (73).

Seasonal variation of the minor metabolites has been observed but is in general, insignificant. Only in one instance

¹Statements regarding similarity or dissimilarity are based primarily on the occurrence of 46, 57, 58 and 59, since the less polar, minor metabolites have not, for the most part, been characterized.
has a compound failed to reappear in subsequent collections.

La Jollan γ-stage \textit{C. celata} is qualitatively identical to its B.C. counterpart. Tetracetylclionamide (46) and hexacetylcelenamide A (58) and B (57) were isolated from acetylated ethanol extracts of this sponge but the minor components were not determined. It is of interest here to note that B.C. \textit{C. celata} is encountered at an average depth of 18 m compared with 33 m for La Jollan \textit{C. celata}. The occurrence of this Pacific sponge at shallower depths in temperate as opposed to tropical environments is consistent with literature descriptions of \textit{C. celata} residing in western Atlantic waters (91, 95). The sponge's change of habitat with latitude may be due to several factors including, light or temperature requirements.

Gamma-stage \textit{C. celata} from Woods Hole does not contain peptide alkaloids or related compounds. Examination of acetylated EtOAc fractions of this sponge show only pigments and sterols. No other secondary metabolites are observed. The aqueous fraction gave an orange colouration when reacted with FeCl$_3$, in contrast to the more typical gray-black colouration obtained with phenols. Acetylation of the freeze dried material did not provide readily identifiable, discrete compounds. The nature of the water soluble component responsible for this reaction is being pursued in our laboratory.

The three Floridian endolithic sponges assayed were devoid of alkaloids. The EtOAc and aqueous fractions failed to react with FeCl$_3$ and did not inhibit the growth of \textit{S. aureus}. 
Woods Hole and B.C. *C. celata* burrow into biogenic and
geologic sources of CaCO₃ and both grow on Iceland spar¹ in vivo.
. The morphology, colouration, and spicule character of these
sponges are identical. The obvious chemical differences which
exist might arise as a result of one of the following factors:

1. Genetically distinct strains,
2. Associated symbionts,
3. Diet,
4. Social requirements,
5. Taxonomically distinct species.

The secondary metabolites of many marine and terrestrial
organisms are thought to be derived from symbiotic
microorganisms or dietary sources (98); Rapoport (73) suggests
that peptide alkaloids of the shrub family *Ceanothus* are
produced by root-associated symbiotic actinomycetes. As
mentioned, symbiotic zooxanthellae exist in *C. celata* and other
clionids.

Several fungi and one yeast have been isolated from B.C. *C.
celata* tissue and interstitial fluids.² None of these organisms
were found to produce peptide alkaloids or related compounds in
vitro (see experimental for details). Zooxanthellae from

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¹A transparent form of calcite.
²Fungal bioassays and the culturing of isolates were performed by
Gary Hewitt.
Atlantic and Pacific specimens of *C. celata* are currently being compared by Dr. Pomponi and her collaborators. A genetic comparison of the two sponges has also been proposed. Until the genetic and possibly the taxonomic relationship between these clionids is understood, the chemotaxonomic significance of the alkaloids discussed in chapter two can not be fully appreciated. In light of the biochemical contradictions observed, it may not be inappropriate to suggest that re-examination of the taxonomy of these sponges is in order.

The investigation of specimens of *C. celata* from the eastern Atlantic and western Pacific oceans would aid in the extension of the results presented above.

III. Experiments Aimed at Determining the Involvement of the B.C. *C. celata* Alkaloids in the Burrowing Process

Rapoport has recently suggested (99) that terrestrial cyclopeptide alkaloids may function as ionophores in their host plants. The basis of this suggestion was the observation that the inherently lipophilic natural and synthetic alkaloids of this class which possess a 14-membered ring system (eg. 75) were capable of binding certain divalent and monovalent metal cations. This work in conjunction with the natural propensity of *C. celata* to burrow into calcareous substrates stimulated our curiosity concerning the function of clionamide (53) and the celenamides (70, 71, 72, 74) in the sponge's metabolism. The instability of the underivatized *Cliona* alkaloids and the
difficulties associated with obtaining these in a pure state meant that, ideally, the total synthesis, or at the very least the synthesis of analogues, would be necessary in order to perform precisely controlled biological and physico-chemical experiments. These synthetic studies were initiated as part of this thesis and are described in the following chapter.

As synthetic materials were not immediately available, we sought to answer the following pertinent questions by utilizing simple and in some instances rather crude experimental methods. First, do all life-stages possess the compounds of interest? Secondly, do high concentrations of calcium exist within the sponge, and if so, where? Thirdly, are the alkaloids localized in a specific cell-type, and finally, are these compounds capable of transporting calcium across a hydrophobic barrier? The answer to the first question in this series was positive and has been dealt with in the foregoing subsection.

Before proceeding with this discussion it should be pointed out that the majority of the in vivo and in vitro burrowing studies which have been performed with C. celata as subject have utilized the western Atlantic organism. As a result of the findings presented in the preceding subsection, the possibility exists that the information provided by these studies is not pertinent to the eastern Pacific variety. Although it may be proven to have been inappropriate the current discussion will employ the assumption that all of the literature pertaining to C. celata is relevant to the present study.

If Warburton's (95) in vitro results are taken in
In conjunction with those of Rutzler and Rieger (96) and extrapolated to the in vivo situation then it can be assumed that 2-3 percent of the total calcium removed from the substrate during a sponge's lifetime is either released into the environment, or totally or partially retained within the sponge. In order to determine the possibility of either of the latter two situations, specimens were divided into four fractions as follows. Fresh sponge (wet weight 12.1 g) was extracted thoroughly with aqueous methanol. The methanolic extract was then further divided into EtOAc and water soluble fractions. The solids remaining after treatment with methanol were dried and one half was treated with hypochlorous acid to give purified sponge spicules. Subjection of each of these samples to elemental analysis produced the results indicated in Table 4.

Table 4. Calcium Analyses

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight g</th>
<th>Calcium — concentration %</th>
<th>mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic solvent soluble</td>
<td>0.04</td>
<td>0.10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Water soluble</td>
<td>0.4</td>
<td>0.04</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Dried total solids</td>
<td>3.6</td>
<td>3.9</td>
<td>~140</td>
</tr>
<tr>
<td>Spicules</td>
<td>3.0</td>
<td>1.9</td>
<td>~57</td>
</tr>
<tr>
<td>Non-spicule solid components</td>
<td>0.5</td>
<td>17</td>
<td>~83</td>
</tr>
<tr>
<td>Sand and debris</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Calcium analyses were performed by Chemex Laboratories.
2Inferred.
Few reports regarding the mineral composition of sponges are available in the literature. Schwab and Shone (100) examined the siliceous spicules of *Acarnus erithacus* and determined their calcium content to be 0.014 percent. Bowen and Sutton investigated the silicon and calcium (101) content of several whole dried sponges of the genera *Dysidea*, *Chondrilla*, and *Terpios*. Calcium concentration in these Demosponges ranged from 0.53 to 30.20 percent.

The latter study indicates that the calcium concentrations found in the structural features of *C. celata* are not anomalous. However, potential sites of calcium sequestration are implied by the results shown in Table 4 and may be in keeping with Yonge's suggestion (93) that growth processes may assist in the burrowing process. The nature of the calcium-retaining species present in high concentration in the sponge tissue has not been investigated.

The endolithic tissues of burrowing sponges are composed of several distinct types of cells including a specialized cell believed to be responsible for the secretion of a chemical etching agent (86b). In order to ascertain the involvement of the *C. celata* alkaloids in the primary dissolution process, dissociated cells from this sponge were fractionated\(^1\) into cell-types by discontinuous density gradient centrifugation (see experimental for details). Five cell sub-populations (~50 mg wet wt. each) were obtained and analyzed for the presence of

\(^1\)Cell separations were performed by Dr. S.A. Pomponi.
clionamide (53) and the celenamides. Alkaloids were not detected in any of the cell fractions. Several interpretations of this surprising result have been put forward. However, until further information is acquired, an accurate conclusion cannot be reached. At the time of writing, the necessary information was in the process of being gathered.

It is intriguing that the only cell fraction observed to contain free sterols and pigments also possessed the sponge's zooxanthellae. Sterols have been suggested as taxonomic aids to sponge identification (102). It would be important, therefore, to determine whether sponge sterols are synthesized de novo or are exclusively of symbiont origin.

Rapoport (99) has been able to show that cyclopeptide alkaloids bind certain monovalent and divalent metal cations. A similar result was obtained when CD spectra of hexacetylcelenamide B (57, appendix 11) were measured in the presence and absence of Ca^{2+}. The conformational change indicated in appendix 11 could be attributed to ion-binding associated with either the acetylated catechol or gallol residues, or the amide groupings which constitute the backbone of the peptide structure.

Simple linear peptides do not complex metal ions to a noticeable degree. Examination of molecular models suggested that celenamides A (70), B (71), and C (72) could exist in conformations similar in nature to the phencyclopeptine skeleton. The possibility exists that the sole dehydroamino acid residue in these molecules is responsible for the generation of
a conformational state which exhibits metal binding properties.

Ionophores, many of which possess amino acid residues or amide functionalities, are characterized by their ability to form highly lipophilic complexes with metal ions (103).

The Cliona alkaloids were tested for potential ionophoric properties in a simple U-tube system (104, see experimental for details). Ion translocation is displayed in this system by ionizable ligands but not by nonionizable ligands. The result of the first experiment performed is illustrated in appendix 12.

During the course of this experiment, a substantial amount of precipitate formed at the interface of the aqueous donor phase (+Ca^{2+}) and the organic phase. It was suggested, therefore, that the low percentage transport observed was due to insolubility of the alkaloid-metal complex(es). The possibility that the Cliona alkaloids are nonionizable ligands is not excluded by this experiment. Complexes of metal ions and nonionizable ionophores necessarily retain the net charge of the metal ion. Hence, the solubility of the complex is dependent upon the nature of the salt and the partitioning properties of the counterion.

In a second experiment, the solvent dielectric constant was increased by the use of nitrobenzene as a component of the organic phase and the transport distance was decreased by employment of a Pressman cell (104). The result obtained was roughly analogous to that shown in appendix 12. This confirmed the fact that clionamide and the celenamides are not ionizable ionophores. Further experimentation is necessary to determine
the nonionizable ionophoric capabilities of these molecules.

IV. Conclusion

The statement was made during the discussion of Chapter 2 that experiments aimed at elucidating the biological role of a secondary metabolite often lead to ambiguous results. The initiatory inquiry presented above, illustrates this point. Although, the metabolic function of the \textit{C. celata} alkaloids has not been clarified, many intriguing questions have arisen as a result of this brief investigation. These will without doubt stimulate further collaborative research. The chemistry of \textit{Cliona} zooxanthellae, the nature of sponge-symbiont sterol relationships, the site of production of the \textit{Cliona} metabolites, and the origin of the differences of Atlantic and Pacific \textit{C. celata} are subjects worthy of further examination.
SYNTHETIC STUDIES

I. Rationale

The advent of powerful spectroscopic methods for the elucidation of organic structures, has initiated a trend among natural-product chemists away from the use of chemical degradations and partial syntheses. This tendency has on occasion, led to incorrect structural assignments. In many instances, the application of readily available spectroscopic techniques does not give rise to the complete elucidation of a novel structure. The determination of stereochemical and subtle regiochemical features, frequently requires the implementation of more classical elucidative procedures.

Correct structures for the celenamides were not proposed until simplification of these complex molecules had been accomplished by chemical degradation. To provide further evidence for our structural hypotheses we sought to correlate the acquired spectral information with data reported for natural products and synthetic materials possessing similar structural features. Spectral information pertaining to aromatic dehydroamino acid and N-acylstyrylamine functionalities, particularly \(^1\)H NMR data, was found to be generally unavailable. Hence, the preparation of several simple model compounds was undertaken in an attempt to fill this void.

Analogues were also desired to aid in the clarification of the configurations of the celenamide dehydroamino acids.
Peptides incorporating substituted and unsubstituted dehydrophenylalanine residues were required for a proposed investigation regarding the ability of unsaturated peptides to bind metal ions.

To avoid multi-step total syntheses or the development of novel synthetic methods, we experimented with several literature procedures for preparing dehydropeptides in order to ascertain whether a method was available which encompassed only a few well-characterized synthetic steps.

II. Preparation of Model Compounds

Aromatic dehydroamino acids have most commonly been prepared via oxazolones (azlactones, oxazolinones, 105). The latter may be obtained by several routes, the most widely utilized being the classical Erlenmeyer-Plöchl synthesis (106) illustrated below. The formation and properties of oxazolones, particularly unsaturated oxazolones, have been the subjects of much scientific attention during the past 100 years. Recent reviews (105, 107, 108) attest to the continued interest in this area. In addition to dehydroamino acids, oxazolones are also important precursors to chiral α-amino acids and α-keto acids.
Condensation of acetylglycine (109) with 3,4,5-trimethoxybenzaldehyde (Sigma) in the presence of Ac₂O-sodium acetate provided bright yellow 2-acetyl-4-(3',4',5'-trimethoxybenzylidene)-5(4H)-oxazolone (90) in a maximum yield of 70 percent. This preparation was repeated several times with the average yield being 62 percent. Oxazolone 90 had previously been prepared in 30 percent yield by Acheson et al. (85). Attempts to synthesize 90 by the modified Erlenmeyer reaction (110) employing sodium bicarbonate in place of sodium acetate, resulted in lower yields (20-30%). Two other oxazolones, 91 (111) and 92, were made for comparative purposes. The phenyl oxazolone 92 was identical to that reported by Mauthner (112).
Transformation of unsaturated oxazolones into dehydroamino acids is readily achieved by reaction with water, alcohols or amines. Oxazolones derived from acetylglycine (aceturic acid) react readily with these reagents, whereas, those formed from hippuric acid (benzoylglycine) are more resistant to ring opening and frequently require the presence of acidic or basic catalysts (113). Several derivatives were prepared in 'excellent yields in this manner, including compounds 93-96. The acids 94 and 95 can be quantitatively reconverted into their respective oxazolones by treatment with Ac₂O-dimethylamino pyridine.

Peptides incorporating an N-terminal dehydroamino acid residue can be obtained by reaction of the appropriate unsaturated oxazolone with the sodium salts of amino acids or peptides (123). The (Z)-dehydrodipeptides 99-103 were prepared in this fashion.
Several bisdehydrodipeptides were prepared for structural analogy with nonacetylcelenamide D \((60)\). Application of the Erlenmeyer reaction to the glycine containing dehydrodipeptides \(99\) and \(103\) afforded the peptidyl oxazolones \(104\) and \(105\). This reaction sequence has been previously employed by Doherty et al. \((124)\) and also by Pieroni et al. \((76)\) in the synthesis of compound \(105\). Reaction of the peptidyl oxazolones \(104\) and \(105\) with nucleophiles, as described for the oxazolones \(90-92\), gave the bisdehydrodipeptides \(106-111\). Treatment of the oxazolone \(104\) with ammonia under a variety of conditions generated the amino acid \(96\) as the sole isolable product.
Descriptions of the synthesis of N-acylstyrylamines are not prevalent in the literature. Methods which have been employed include the condensation of acetamide and phenylacetaldehyde, the reaction of styrene epoxide with amides (116) and the decarboxylation of α-benzyolamido cinnamic acids in the presence of copper chromite (113) or ammonia (110a). The route envisaged for the production of these functionalities is shown in scheme 9. The avoidance of acids in the final elimination reaction is critical due to the acid-sensitive nature of the moiety being produced.
Application of Corey's epoxide synthesis (117) utilizing dimethylsulfonium methyldide gave the styrene epoxide 112 in 90 percent yield. The following three steps shown in scheme 9 were performed in immediate succession without isolation and characterization of the intermediate products 113 and 114. Treatment of the epoxide 112 with aqueous ammonia at room temperature for four days resulted in the formation of predominately the desired regioisomer. This selectivity is frequently observed in base-catalyzed nucleophilic ring-openings of epoxides (118).

For the purposes of this exploratory reaction sequence, valine (scheme 9, R=CH(CH₃)₂) was chosen as the amino acid component of the desired target molecule. This choice was made solely on the consideration of simplification of the end-product ¹H NMR spectrum. Simplification of the olefinic region of this spectrum was required for detection of possible geometrical isomerism in the styrylamine 116. Condensation of the crude amine 113 with N-acetylvaline, by the carbodiimide method introduced by Sheehan et al. (119), afforded the modified amino acid 114. Retention of optical activity was not a major concern during this first series of reactions. In future trials the coupling additive hydroxybenzotriazole should be employed in this step and the use of triethylamine eliminated (120, 121). Acetylation of the alcohol 114 afforded the acetate 115 in an overall-yield of 52 percent from the epoxide 112.

Although the syn elimination of acetates is a frequently employed reaction in organic synthesis, this procedure has not
apparently been used in the production of N-acylstrylamines. N-Vinyl-2-oxazolidone has been prepared from N-(2-acetoxyethyl)-2-oxazolidone as illustrated below (56).

\[
\begin{align*}
\text{OAc} & \quad \begin{array}{c}
580^\circ \\
85\% \\
(56)
\end{array} & \quad \begin{array}{c}
\text{N} \\
\text{O}
\end{array}
\end{align*}
\]

Attempts to eliminate acetic acid from acetate 115 have so far proven to be unsuccessful. A variety of pyrolytic conditions has been tried, including multiple passages through a hot tube (200-450°) filled with glass beads. Rather than resort to higher temperatures to facilitate elimination, we chose to pursue alternative, milder methods of double bond formation. Harsher pyrolytic conditions were considered to be undesirable for future syntheses in which \(\beta\)-hydroxymescaline or 3,4-dimethoxydopamine would be linked to sensitive amino acids or peptides.

In very recent work, we have surprisingly been unable to dehydrate the alcohol 114 to give the enamide 116. Methods tried include; refluxing in DCC-EtOAc, treatment with thionyl chloride in pyridine, and tosylate and mesylate forming reactions. No change was observed after 24 hours in the presence of DCC. Decomposition of the starting material was observed when thionyl
chloride, tosyl chloride or mesyl chloride were employed. Experiments aimed at resolving this situation are in progress.

Two other methods for forming the alcohol 114 were attempted. Refluxing the epoxide 112 in anhydrous DMF in the presence of N-acetylvalinamide (117) for several days at 180° resulted in the isolation of starting materials and small amounts (<5%) of the ketone 118 and the aldehyde 119 (apparently present as polymer). The addition of one equivalent of NaH to this reaction effected the formation of the alcohol 114 in approximately 10 percent yield (following acetylation). Conditions required to improve the yield of this reaction have not been explored. The opening of styrene epoxide with amines, amides, amino acids and aromatic heterocycles has been described (116, 122, 123).
III. Geometry Considerations

HPLC and $^1$H NMR analysis of the oxazolones 90-92 and TLC and $^1$H NMR analysis of the amino acids 93-98 indicated the presence of one geometrical isomer in each case. This observation agrees with the conclusion arrived at by Pieroni et al. (76) that, at least for benzaldehyde, the Erlenmeyer synthesis gives only one isomer having the Z-configuration. Condensations carried out in sulfuric acid or sulfuric acid-acetic anhydride give mixtures of E and Z isomers (105). The labile E-oxazolones can be opened with oxygen nucleophiles to the corresponding E-dehydroamino acids. Contact with amines or pyridine cause oxazolones possessing the E-configuration to isomerize to the more stable Z form (105).

Assignment of the stereochemistry about the double bond in compounds such as 93-98 is usually performed by comparison of the physical properties [eg. UV ($\lambda_{\max}$, $\varepsilon$), $^1$H NMR (6), IR (CO, $\nu_{\max}$)] of one isomer with the other. The chemical shift of the vinyl proton in these molecules is measurably sensitive to their geometry. Employing $^1$H NMR spectroscopy Morgenstern et al. (124) established that the stable configuration of aromatic dehydroamino acids is Z, in agreement with a much earlier X-ray assignment (125). This study corrected the erroneous conclusion of Brocklehurst et al. (126).
Morgenstern demonstrated that the olefinic hydrogen atom is deshielded in the E, relative to the Z, configuration. Subsequent confirmation of these observations has been made by Rao and Filler (105).

In the $^1$H NMR spectra of diketopiperazines which possess an aromatic dehydroamino acid residue and the synthetic cyclopenin precursors 120 (127, 128, 129), the vinyl proton of isomers with the Z configuration is observed at lower field than in the corresponding E isomers. Close spacial proximity of the olefinic hydrogen atom and the adjacent conformationally-fixed carbonyl group in the Z isomers gives rise to this deshielding effect. In marked contrast, geometrical isomers of unsaturated aromatic oxazolones show the opposite vinyl proton shifts. This has been attributed to the pronounced deshielding influence of an $\text{-N=C-R}$ moiety on a cis-olefinic hydrogen atom (105). In simple acyclic situations conformational freedom would be expected to give rise to molecules which exist predominately in the more stable s-transoid cinnamic acid conformation. An explanation for the vinyl proton shifts observed in the $^1$H NMR spectra of aromatic dehydroamino acids is evident in the work of Morgenstern et al. (124) and of Brocklehurst et al. (126). These authors clearly demonstrate that the addition of an $\omega$-acylamido group to either methyl cinnamate (126) or methyl acrylate (124) results in shielding of a trans-vinyl proton and deshielding of a cis-vinyl proton, with the latter being the most pronounced of the two effects ($\Delta \delta$ cis = +1.00, $\Delta \delta$ trans = -0.37). These observed shifts differ markedly from those employed by Pascual et al.
The empirical additive increment rules of Pascual et al. (130), and others (131), do not assist in the assignment of dehydroamino acid configurations in the acetylated celenamides 57-60. These rules originated from observations made on simple isolated chemical systems. Nuclei in complex molecules such as peptides may exist in unique chemical environments created by the tertiary structure of the molecule. In these situations, comparisons with spectral data obtained on molecules lacking this influence are difficult. Tobey (132) introduced the parent compound method of correlation in an attempt to circumvent this problem. However, chemical shifts of vinyl protons in (E)-dehydrophenylalanine-containing peptides are not available for comparison.\(^1\) Signals corresponding to vinyl protons were observed in the \(^1\)H NMR spectra of compounds 96, 97, and 99-103 at \(\delta 6.99\) to 7.19. Methyl N-acetylvalyl-\(\alpha,\beta-\)

\(^1\)H NMR data for E and Z isomers of BocMeAlaLeu \(\triangle\) PheGlyOMe have been reported but the spectra provided have not been assigned (133).
didehydrophenylalanate (to be discussed) displayed the corresponding signal at $\delta 7.25$.

In several instances, chemical shift correlations do facilitate the assignment of aromatic dehydroamino acid geometries [eg. 98, $\delta$ 7.46; 121, (Z) $\delta$ 7.44, (E), $\delta$ 8.00 (124)]. The $^1$H NMR spectra of peptides 57-59, 65 and 66 (Table 1) possess vinyl signals within a similar range ($\delta$ 6.80 to 7.22) to these corresponding resonances in compounds 96, 97 and 99-103. Although these values are suggestive of the Z-configuration, the temptation to draw this conclusion has been resisted. Isomerization of these residues has not been observed. Many authors depict their dehydroamino acid-containing synthetic and natural products as possessing the Z-configuration solely on the basis of this consideration. As a result of our analysis of many dehydroamino acids and dehydropeptides, we suggest that correlative procedures or stability assumptions alone are not satisfactory methods of determining the stereochemistry of the olefinic components of the latter. This is especially true of marine natural products which frequently display uncommon arrangements of functional groups and unexpected stereochemical and regiochemical features. With the exception of X-ray crystallography and total synthesis, comparison of isomeric analogues is the only definitive method of assignment available at this time. Unfortunately, both isomers of a natural product or closely related analogues are seldomly readily available.
The $^1$H NMR spectra of nonacetylcelenamide D (60) and its hydrolysis product 73 each display signals corresponding to two dehydroamino acid vinyl protons (Table 1). The disparity in the observed chemical shifts (60, $\delta$ 7.59, 7.06; 73, $\delta$ 7.41, 7.09) can be explained by considering a recent X-ray crystallographic analysis performed by Pieroni et al. (76). Their study indicated that the relationship between the olefins and their adjacent carbonyl groups in the two dehydroamino acid residues of a (Z,Z)-bisdehydrotripeptide (122) are different. One residue exists in an s-transoid conformation, whereas the other is s-cisoid. If, in solution, compounds 73 and 60 resided in a similar conformational state, then a marked disparity in the vinyl proton chemical shifts would be expected.
The 'H NMR spectra of the synthetic bisdehydrodipeptides, known to possess the Z, Z-configuration (76), showed vinyl proton signals having similar chemical shift separations to those observed in the spectra of peptides 60 and 73 (see experimental and Table 2). Although these observations do not facilitate assignment of the geometry of the Cliona peptide alkaloids, they do provide an alternative to the explanation that the observed chemical shift disparities result from the presence of dehydroamino acid residues having opposite configurations.

Tentoxin, a fungal metabolite recently synthesized by Rich et al. (133), is possibly the only aromatic dehydroamino acid-containing peptide in which the olefinic geometry has been conclusively determined. Celenamide D (74) is the first example of a naturally occurring dehydropeptide possessing multiple aromatic dehydroamino acid residues.

IV. Preparation of Dehydropeptides Possessing N-terminal O-Amino Acids

Syntheses of dehydropeptides are few in number and those which provide dehydropeptides with aromatic dehydroamino acids situated at other than the N-terminus are even fewer. The synthetic methods which have been employed, or which are conceivable, are outlined in abbreviated form in scheme 10 for the hypothetical tripeptide 123. It is not my intention here to discuss each of these six routes in intimate detail. A recent
A comprehensive review by Schmidt et al. (81) provides excellent coverage of this subject.

As indicated earlier, the purpose of our inquiry was to determine the availability of a rapid method to compounds such as 123. It is apparent from close examination of scheme 10 that routes A, C and E encompass the least number of synthetic steps. Routes B, D and F are not only substantially longer (9-12 steps) but they also have the added disadvantage of entailing the synthesis and manipulation of free amino acids. Methods related to route E have apparently not been reported, although, two related syntheses (128, 137) lend credance to its potential (see reaction sequence below).
Scheme 10. Summary of employed and possible routes to linear dehydropeptides possessing N-terminal $\alpha$-amino acids.
The first examples of the formation of dehydropeptides in which the dehydroamino acid component was not at the N-terminal end can be attributed to Bergmann (115, 138). In 1932 (138) \( \text{NH}_2\text{Gly} \triangle \text{PheOH} \) and in 1943 (115) \( \text{AcPhe} \triangle \text{PheOH} \) were prepared by dehydration of the corresponding \( \beta \)-hydroxyamino acid residues. Unfortunately, \( \beta \)-hydroxy aromatic amino acids cannot be routinely prepared. In this same 1943 report (115) Bergmann presented the formation of \( \text{AcPhe} \triangle \text{PheOH} \) by the condensation of \( \text{AcPheGlyOH} \) and benzaldehyde. The intermediate peptidyl oxazolone 124 was isolated in 19 percent yield.

![Image of oxazolone]

The application of this straightforward procedure has subsequently been attempted by Konno and Stammer (134a). They were able to isolate the desired oxazolone from \( \text{CbzGlyGlyOH} \) in less than 5 percent yield.

In order to determine whether the failure of this reaction was indeed a general phenomenon we prepared the protected

\[ \text{AcHN} \]

\[ \text{N} \]

\[ \text{O} \]

124

The abbreviation \( \triangle \text{Phe} \) refers to \( \alpha, \beta \)-didehydrophenylalanine.
dipeptide AcLeuGlyOH as described by Smart et al. (139) using DCC. Several attempts have been made to condense this material with 3,4,5-trimethoxybenzaldehyde by the Erlenmeyer procedure, but all have been unsuccessful. The reason for the success of the Erlenmeyer reaction when the N-acyl substituent of glycine is Ac, Bz, -CH₂Cl or a dehydroamino acid, in contrast to its failure when this group is an α-amino acid, is not obvious. Particularly, in view of the apparent ease of formation of similar saturated peptidyl oxazolones (134, 140).

The Bergmann-Grafe reaction (141, route C) entails the condensation of an α-keto acid and an unsubstituted amide. Wieland et al. (135a) exploited this reaction in the synthesis of several dehydroalanine derivatives including CbzGlyΔAlaOH. 3,4,5-Trimethoxyphenyl pyruvic acid (142, 112) failed to condense with the amide 117 under these conditions. The use of a variety of acidic catalysts in this procedure has recently been introduced by Shin et al. (135b). Employment of phosphorus oxychloride in the condensation mentioned above led to polymerization of the α-keto acid, as did the use of pyridine. Low yields (16%) of AcValΔPheOMe (125) were obtained when 117 was reacted with methyl phenylpyruvate in the presence of phosphorus oxychloride. Attempted condensation of AcValΔPheOH (126) with ethylglycinate has been unsuccessful. The acid 125 was unreactive towards hydrazine hydrate, DCC-p-nitrophenol,

'This work was performed in association with K. Leavens (Chemistry 449 student 1980-81).
DCC-Et$_3$N-hydroxybenzotriazole and it failed to form an oxazolone in the presence of Ac$_2$O-DMAP as did the dehydroamino acids 94 and 95. These results are consistent with those reported by Rich et al. (133).

Regrettably, we have been unable, to date, to discern a route to the desired dehydrotripeptides which fulfills the requirements outlined previously.
EXPERIMENTAL

General

The 'H NMR spectra were recorded on Nicolet-Oxford 270, Varian XL-100, Varian HA-100, Bruker WP-80, and Bruker WH-400 spectrometers. 'C NMR spectra were recorded on Bruker WH-400, Bruker WP-80 and Varian CFT-20 spectrometers. Tetramethysilane (δ=0) was employed as an internal reference in all instances. Low-resolution and high-resolution EIMS were measured on A.E.I. MS-902 and MS-50 spectrometers, respectively. Ultraviolet spectral data was obtained with a Cary-14 ultraviolet spectrophotometer, infrared spectra were recorded on a Perkin-Elmer model 700B spectrophotometer and optical rotations were measured on Perkin-Elmer model 241C or model 141 polarimeters. A 10 cm microcell was used for optical rotation measurements unless otherwise indicated. A Jasco J-20 spectro-polarimeter was employed for circular dichroism measurements.

Gas chromatography and high pressure liquid chromatography were performed on Hewlett-Packard 5830A and Perkin-Elmer Series 2 instruments, respectively. A Perkin-Elmer LC55 UV detector system was employed for peak detection during HPLC. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Merck silica gel 60 PF-254 was used for PTLC. The HPLC solvents were Fisher HPLC grade, Caledon HPLC grade or Burdick and Jackson UV quality; water was glass distilled; all other solvents were reagent grade.
Sponge collection

The sponge *Cliona celata* Grant was collected by hand using SCUBA in Barkley Sound, Howe Sound, and the Straights of Georgia, British Columbia, at an average depth of 18 metres. Specimens obtained from La Jolla, California were collected at 33 metres' depth and those from Woods Hole, Massachusetts were obtained at varying depths. All other species of *Cliona* studied were collected in 3 metres of water at Hen and Chickens Reef, two miles off Plantation Key, Florida.

Specimens were placed in methanol or ethanol at the dive site immediately following collection. The organisms were homogenized in a Waring Blender, and allowed to stand overnight at 3-5°C. The aqueous methanol extract obtained following filtration was concentrated in vacuo and the resultant suspension partitioned between brine and EtOAc. The EtOAc layer was dried over anhydrous sodium sulfate, filtered, and evaporated in vacuo. Trituration (hexane, ether, chloroform) of the green solid obtained afforded a light-brown solid mixture consisting of alkaloids free of sterols, pigments and lipids. British Columbian specimens yielded, on an average, 3 g (0.3%) of crude alkaloids from every 1 kg of wet sponge collected. The sole Californian collection was not quantitated.
Derivatization of the Crude Alkaloid Extract with Acetic Anhydride and Acetic Anhydride-\textsubscript{d6}

Derivatization reactions were performed under nitrogen in either Ac\textsubscript{2}O-py (5:1, v/v) or Ac\textsubscript{2}O-sodium acetate (5:1, w/w). In either case, the slurry was warmed until a clear solution was obtained and then allowed to stir overnight at room temperature. Removal of the solvents \textit{in vacuo} and partition of the resultant gum between brine and ethyl acetate provided a solid residue of acetylated alkaloids, in essentially quantitative yield, following drying (anhyd Na\textsubscript{2}SO\textsubscript{4}) and evaporation of the EtOAc layer.

A small scale isolation procedure (40 g wet weight of sponge) employing acetic anhydride-\textsubscript{d6} (10 ml, 99 + atom %, Aldrich) as the acetylating agent yielded a mixture of deuteroacetylated alkaloids. The HPLC of this mixture was analogous to that shown in figure 15a. The \textsuperscript{1}H NMR (270 MHz, acetone-\textsubscript{d6}) spectrum indicated the absence of signals arising from naturally occurring acetamides (-N\textsubscript{H}COCH\textsubscript{3}) and phenol acetates (Ph-O\textsubscript{CO}CH\textsubscript{3}).

Isolation of Clionamide (53)

The crude underivatized alkaloids were dissolved in EtOAc and rapidly partitioned with 0.5-1M H\textsubscript{2}SO\textsubscript{4}. The acid layer was covered with fresh ethyl acetate, the two phases were mixed and
deoxygenated by a rapid flow of nitrogen, and sodium bicarbonate was added until carbon dioxide evolution ceased. After drying over anhydrous \( \text{Na}_2\text{SO}_4 \) and concentration, the ethyl acetate extract was purified by PTLC (\( \text{CHCl}_3-\text{CH}_3\text{OH}, 5:2 \)) to give \( 53 \) as an unstable yellow powder; \( \text{RF} \) (silica, \( \text{CHCl}_3-\text{CH}_3\text{OH}, 3:1 \)) 0.24 (\( \text{FeCl}_3 \) and ninhydrin positive); \( [\alpha]_D + 32.1^\circ \) (c 2.12, \( \text{CH}_3\text{OH}, 1=1\text{cm} \)); \( \text{UV} \) (\( \text{CH}_3\text{OH} \) (nm) \( \lambda \max \) 227 (\( \epsilon \times 3.1 \times 10^4 \)), 291 (\( \epsilon \times 1.1 \times 10^4 \)), 296 (\( \epsilon \times 1.2 \times 10^4 \)), 311 sh (\( \epsilon \times 9.4 \times 10^3 \)); \( \text{IR} \) (KBr), \( \nu \max \) 3350-3200 br, 1635, 1605, 1500 cm\(^{-1} \); \( ^1\text{H} \) NMR (400 MHz, \( \text{CD}_3\text{OD} \), figure 12) \( \delta \) 3.01 (m, 1H), 3.17 (m, 1H), 3.66 (t, 1H, \( J=7 \)), 5.97 (d, 1H, \( J=15 \text{ Hz} \), \( \text{CH}=\text{CHNH} \)), 6.32 (s, 2H, Ph-H), 7.08 (s, 1H, indole H-2), 7.09 (dd, 1H, \( J=2, 8\text{ Hz} \), indole H-5), 7.15 (d, 1H, \( J=15 \text{ Hz} \), \( \text{CH}=\text{CHNH} \)), 7.48 (d, 1H, \( J=2 \text{ Hz} \), indole H-7), 7.50 (d, 1H, \( J=8 \text{ Hz} \), indole H-4). A 5 mg sample of \( 53 \) applied to a 0.65 cm disc of Schleicher and Schuell adsorbent paper gave a 1 mm zone of inhibition of the growth of \( \text{Staphylococcus aureus} \).

**Acetylation of Clionamide (53): Tetracetylclionamide (46)**

Excess acetic anhydride (10mL) was added to a solution of \( 53 \) (100 mg, 0.23 mmole) in pyridine (2 mL) and the reaction mixture was stirred overnight, at room temperature, under an atmosphere of nitrogen. Evaporation in vacuo and purification by PTLC (\( \text{CHCl}_3-\text{CH}_3\text{OH}, 10:1 \)) gave tetracetylclionamide (46, 82 mg, 0.14 mmole, 61%), mp 209-211° (THF-isopropyl ether); \( [\alpha]_D + 45^\circ \) (c 0.7, acetone); \( \text{UV} \) (\( \text{CH}_3\text{OH} \) \( \lambda \max \) (nm) 227 (\( \epsilon \times 5.9 \times 10^4 \)), 290 (\( \epsilon \times 3.7 \times 10^4 \)); \( \text{IR} \) (KBr) \( \nu \max \) 3418, 3280, 1760, 1627 cm\(^{-1} \); \( ^1\text{H} \)
NMR (figure 3, table 1); $^{13}$C NMR (CD$_3$CN) 20.4 (1C), 20.9 (2C), 23.1 (1C), 28.3 (1C), 55.2 (1C), 111.3 (1C), 112.1 (1C), 115.1 (1C), 115.5 (1C), 118.3 (2C), 121.0 (1C), 122.7 (2C), 125.5 (2C), 127.7 (1C), 136.3 (1C), 138.4 (1C), 145.0 (2C), 168.1 (1C), 168.9 (2C), 171.0 (1C), 171.9 ppm (1C); MS (figure 2); Exact mass calcd for C$_{27}$H$_{26}$*1BrN$_3$O$_8$, 601.0874; obsd, 601.0889. Anal calcd for C$_{27}$H$_{26}$BrN$_3$O$_8$ C54.01, H4.36, N7.00; found, C53.89, H4.40, N7.09.

**Isolation of Tetracetylclionamide d$_{12}$ (54)**

Workup of the crude deuteroacetylated alkaloid mixture as described for the isolation of the acetylated celenamides (below) gave tetracetylclionamide-d$_{6}$ (54). Signals at $\delta$ 1.85 (s,3H) for acetamide (-NHCOCH$_3$) and $\delta$ 2.23 (s,9H) for three phenol acetates (Ph-OCOCH$_3$) present in the spectrum of tetracetylclionamide (46) were absent in the $^1$H NMR spectrum of 54 (figure 10).

**Clionamide Acetonide (56) and Clionamide Acetonide-d$_6$ (55)**

Clionamide (53, 30 mg, 0.07 mmole) was dissolved in anhydrous acetone or acetone-d$_6$ for 1h at room temperature. Concentration and purification by PTLC (CHCl$_3$-MeOH, 5:2) gave the acetonide 56 (19 mg, 0.04 mmole, 57%) or the deuteroacetonide 55, respectively, as stable oils. 56; Rf
(CHCl₃-CH₃OH, 3:1) 0.54 (FeCl₃ and ninhydrin positive); UV (CH₃OH) λ max (nm) 229 (ε 2.5 x 10⁴), 297 (ε 1.7 x 10⁴), 315 sh (ε 1.4 x 10⁴); IR (KBr) ν max 3300 br, 1635 br, 1605, 1508 cm⁻¹; "H NMR (270 MHz, acetone-d₆, figure 14) δ 1.42 (s, 3H, acetonide-CH₃), 1.47 (s, 3H, acetonide-CH₃), 3.11 (dd, 1H, J=7.5, 14.5 Hz), 3.31 (dd, 1H, J=5, 14.5 Hz), 3.86 (dd, 1H, J=5, 7.5 Hz), 6.50 (s, 2H), 6.75 (d, 1H, J=14.2 Hz), 6.83 (d, 1H, J=14.2 Hz), 7.14 (dd, 1H, J=2, 8.5 Hz), 7.28 (s, 1H), 7.57 (d, 1H, J=2 Hz), 7.58 (d, 1H, J=8.5 Hz), 10.09 (bs, 1H, D₂O exchange); "C NMR ((CD₃)₂CO, 20 MHz, appendix 2, 55), 59.1 (1C), 105.7 (2C), 106.0 (1C), 120.7 (1C), 121.3 (2C), 122.6 (2C), 125.6 (1C), 125.7 (1C), 129.5 (1C), 146.8 (1C), 174.2 ppm (1C); MS (10-70 eV) m/e 471/473 (0.02, 1:1), 210/208 (100, 1:1). High resolution mass measurement was performed on the diacyl derivative of 56 obtained following acetylation with Ac₂O-py. Exact mass calcd for C₂₆H₂₆O₂BrN₂O₆, 557.0965; obsd, 557.0990.

The deuteroacetonide 55 was identical (TLC, UV, "H NMR (figure 13), MS) to 56 with the exception of the absence of "H NMR signals at δ 1.42 (s, 3H) and δ 1.47 (s, 3H) for two acetonide methyl groups and the complex nature of the signals in the mass spectrum.

**Hydrolysis of Tetracyclionamide (46) with Hydrochloric Acid**

Tetracyclionamide (46, 145 mg, 0.24 mmole) was dissolved in acetonitrile (15 mL, 0.1% H₂O) and concentrated HCl (0.25 mL in 2 mL CH₃CN) was added. After 48h at room temperature the
solvent was evaporated and the residue purified by PTLC (CHCl₃-
CH₃OH, 5:1) to give the bromo amide 5₁ (21 mg, 0.06 mmole, 27%);
[α]ᵰD + 4.6° (c 3.2, CH₃OH, 1=1cm); ¹H NMR (100 MHz, (CD₃)₂CO) δ
1.87 (s, 3H), 3.13 (dd, 1H, J=8, 14 Hz), 3.29 (dd, 1H, J=6, 14
Hz), 4.71 (dd, 1H, J=6, 8 Hz), 6.40 (bs, 1H, D₂O exchange, -
CONH₂), 6.93 (bs, 1H, D₂O exchange, - CONH₂), 7.17 (dd, 1H, J=9,
2 Hz), 7.61 (d, 1H, J=2 Hz), 7.64 (d, 1H, J=9 Hz), MS m/e, Mᵰ
325/323 (3, 1:1), 266/264 (43, Mᵰ-C₂H₅NO, 1:1), 210/208 (100,
1:1), 129 (35), 128 (12). Exact mass calcd for C₁₃H₁₄⁺BrN₃O₂,
323.0269; obsd, 323.0251.

Catalytic Hydrogenation of Tetracetylclionamide (4₆)

Palladium on charcoal catalyst (5%, 15 mg) was added to a
solution of 4₆ (60 mg, 0.10 mmole) in methanol (20 mL). The
solution was stirred under an atmosphere of hydrogen overnight,
filtered through Celite, concentrated, and purified by PTLC
(EtOAc) to give 5₀ (26 mg, 0.04 mmole, 43%) as a colourless oil;
[α]ᵰD +6.2° (c 4.37, acetone, 1=1cm); UV (CH₃OH) λ max (nm) 224
(ε 3.38 × 10⁴), 283 (ε 6.02 × 10³); ¹H NMR (100 MHz, (CD₃)₂CO) δ
1.88 (s, 3H), 2.24 (s, 9H), 2.67 (t, 2H, J=7 Hz,-CH₂CH₂NH-),
3.12 (m, 2H), 3.38 (m, 2H, D₂O-t, J=7 Hz,-CH₂CH₂NH-), 4.63 (m,
1H), 6.94 (s, 2H), 7.11 (m, 1H), 7.24 (s, 1H), 7.60 (d, 1H, J=2
Hz), 7.62 (d, 1H, J=9 Hz), 10.40 (bs, 1H, D₂O exchange); MS m/e,
Mᵰ 603/601 (0.3, 1:1), 544/542 (3, Mᵰ-C₂H₅NO, 1:1), 210/208
(100, 1:1). Exact mass calcd for C₂₇H₂₈⁺BrN₃O₈, 603.1040; obsd,
603.1020.
Hydrogenolysis of the Bromo Amide 50

Palladium on charcoal catalyst (5%, 10 mg) was added to a solution of 50 (26 mg, 0.04 mmole) in CH$_3$OH (15 mL) containing 0.2% CH$_3$CO$_2$H. The reaction mixture was stirred at room temperature, under an atmosphere of hydrogen, for 72h, filtered through Celite, concentrated, and purified by PTLC (EtOAc) to give 49 (8 mg, 0.02 mmole, 38%) as a colourless oil; IR (KBr) $\nu$ max 3400, 1760, 1635 cm$^{-1}$; $^1$H NMR (270 MHz, acetone-$d_6$, figure 5) $\delta$ 1.81 (s, 3H), 2.25 (s, 9H), 2.64 (t, 2H, J=7 Hz), 3.10 (m, 2H), 3.32 (m, 2H, D$_2$O-t, J=7 Hz), 4.48 (m, 1H), 6.85 (s, 2H), 7.00 (m, 1H, indole H-5), 7.0 (1H, D$_2$O exchange), 7.06 (m, 1H, indole H-6), 7.10 (s, 1H, indole H-2), 7.25 (d, 1H, J=8 Hz, D$_2$O exchange), 7.35 (d, 1H, J=8 Hz, indole H-7), 7.64 (d, 1H, H=9 Hz, indole H-4), 10.14 (b, 1H, D$_2$O exchange, indole H-1); Spin decoupling (100 MHz, (CD$_3$)$_2$CO-D$_2$O), $^1$Hobs-${^1}$Hirr}, $\delta$ 2.65 (s, 2H) - {3.36}, 3.12 (b, 2H) - {4.52}, 3.36 (s, 2H) - {2.65}, 4.52 (s, 1H) - {3.12}; MS m/e, M$^-$ 523 (0.3), 481 (0.5, M$^-$ - C$_2$H$_2$O), 464 (15, M$^-$ - C$_2$H$_5$NO), 422 (15), 380 (14), 130 (100). Exact mass calcd for C$_{27}$H$_{29}$N$_3$O$_8$, 523.1954; obsd, 523.1970.

Hydrogenolysis of the Bromo Amide 51

The procedure outlined for the hydrogenolysis of the bromo amide 50 was followed (5% Pd/C, 6 mg). Purification was performed by HPLC (silica A, hexane-isopropyl alcohol gradient)
to give the debromo amide 52 (figure 8) (8.4 mg, 0.03 mmole, 34% from 31.3 mg, 0.10 mmole of 51); $[\alpha]_D + 15^\circ$ (c 1.0, CH$_3$OH) (lit. $[\alpha]_D + 20 \pm 1^\circ$ (c 2%, CH$_3$OH) 50). The debromo acid 52 was identical to authentic (S)-2-acetamido-3-(indole-3'-yl)-propionamide (Sigma; $[\alpha]_D + 17^\circ$ (c 1.4, CH$_3$OH)).

**Synthesis of the Ultimate Hydrogenation Product (49) of Tetracetylc1ionamide (46)**

5-Hydroxydopamine hydrochloride (86.0 mg, 0.42 mmole) was added, as a solid to a solution of DCC (90.7 mg, 0.44 mmole), (S)-N-acetyltryptophan (105.3 mg, 0.43 mmole), and triethylamine (0.25 mL) in THF-CH$_3$CN (10:1, 50 mL). The reaction mixture was stirred at room temperature overnight, filtered through Celite, and evaporated. The crude residue was acetylated (Ac$_2$O-py, 20:1), evaporated in vacuo and purified twice by PTLC (CHCl$_3$-CH$_3$OH, 20:1) to give a colourless oil (66.5 mg, 0.13 mmole, 30%) identical (TLC UV, $^1$H NMR, (figure 6), IR, MS) to the ultimate hydrogenation product 49 of tetracetylc1ionamide (46).

**Isolation of Acetylated Celenamides**

Chromatography of the acetylated residue on a column of silica gel G provided a fraction eluting with 10-50% ethyl acetate in chloroform containing a mixture of acetylated celenamides and a second fraction eluting with ethyl acetate
containing tetracetylclionamide (46; 0.13%, wet weight). Pure hexacetylcelenamide A (58; 0.03%, wet weight), hexacetylcelenamide B (57; 0.02%), pentacetylcelenamide C (59; 0.003%) and nonacetylcelenamide D (60; 0.002%) were obtained as noncrystalline white solids by repeated chromatography on silica plates with chloroform-methanol-acetonitrile (19:1) as eluant and in several instances RP chromatography on Whatman KC_{18} plates with water-methanol (3:5:2) as eluant.

**Isolation of Deuteroacetylated Celenamides**

Deuteroacetylated celenamides were isolated by PTLC as described above in the case of the acetylated celenamides. Hexacetylcelenamide A-d_{18} (68, figure 27), B-d_{18} (69, figure 28), and pentacetylcelenamide C-d_{15} (figure 27) were isolated as partially purified mixtures. Nonacetylcelenamide D-d_{27} and the remainder of the deuteroacetylated celenamides were examined by \textsuperscript{1}H NMR as a crude mixture. \textsuperscript{1}H NMR analysis of the deuteroacetylated celenamides indicated an absence of signals arising from naturally occurring acetamides (-NHCOCH\textsubscript{3}) and phenol acetates (Ph-OCOCH\textsubscript{3}).

**Hexacetylcelenamide A (58)**

58: [\alpha]_D + 40^\circ \ (c 1.1, acetone); UV (MeOH) \lambda_{\text{max}} (nm) 227 (\pi 7.2 \times 10^4), 289 (br, \epsilon_{\text{max}} 5.1 \times 10^4); IR (CHCl\textsubscript{3}) \nu_{\text{max}} 3300
(br), 1680, 1660 (br), 1380, 1200 cm\(^{-1}\); \(^1\)H NMR (figure 19, table 1); \(^1^3\)C NMR (100.6 MHz, CDCl\(_3\), appendix 4) 20.0, 20.6 (2C), 20.7 (2C), 21.4, 22.8, 23.1, 25.2, 26.6, 39.8, 53.2, 55.3, 111.3, 112.9, 114.5, 115.6, 120.1, 120.3, 121.8 (2C), 122.8, 123.5, 123.8, 124.1, 124.4, 125.8, 126.8, 130.2, 131.4, 135.0, 136.0, 137.2, 140.6, 142.5, 143.5 (2C); 165.3, 167.0, 168.2 (4C), 169.4, 172.0, 174.1 ppm; MS (figure 21, scheme 3).

Hexacetylcelenamide B (57)

57: [\alpha]_D^+ 22° (c 1.1, acetone); UV (MeOH) \(\lambda\) max (nm) 227 (\(\varepsilon\) 8.0 \(\times\) 10\(^4\)), 289 (br, \(\varepsilon\) max 6.0 \(\times\) 10\(^4\)); \(^1\)H NMR (figure 20, table 1); IR (CHCl\(_3\)) \(\nu\) max 3340 (br), 1780, 1660 (br), 1510 (br), 1380, 1200 \(\text{cm}^{-1}\); \(^1^3\)C NMR (20.1 MHz, CDCl\(_3\)-DMSO-\(d_6\) [3:2], appendix 6) 170.2, 168.3, 166.2, 164.9, 164.5, 163.6, 161.7, 140.4 (2C, s),\(^1\) 139.0 (s), 139.2 (s), 132.7 (s), 132.0 (s), 129.0 (s), 127.4, 124.2, 123.2, 121.7, 121.3, 120.4, 120.2, 113.9 (2C), 118.5, 116.7 (2C), 111.4, 111.2, 108.1, 107.1, 56.5 (d), 51.9 (d), 39.1 (t),\(^2\) 27.2 (d), 23.5, 19.5, 17.4 (q, 3C), 17.0 (q), 16.4 (q), 15.3 (q); MS (Figure 22, Scheme 3); CD (CH\(_3\)CN), (appendix 11).

\(^1\)Multiplicities were obtained in a single frequency off-resonance decoupled spectrum (100.6 MHz).

\(^2\)This signal was not observed due to masking by the DMSO multiplet centered at 37.0 ppm. The chemical shift was obtained at 100.6 MHz in CDCl\(_3\).
Pentacetylcelenamide C (59)

59: \([\alpha]_D + 14^\circ \ (c \ 0.30, \ \text{acetone})\); IR (CHCl\(_3\)) \( \nu \max \ 3300 \) (br), 1780, 1660 (br) cm\(^{-1}\); \(^1\)H NMR (figure 29, table 1); MS (70 eV, figure 32, scheme 4).

Nonacetylcelenamide D (60)

60: \([\alpha]_D -25^\circ \ (c \ 0.54, \ \text{acetone})\); IR (CHCl\(_3\)) \( \nu \max \ 3300 \) (br), 1780, 1680 (br) cm\(^{-1}\); \(^1\)H NMR (figure 33, table 1); MS (70 eV, figure 34, scheme 5).

Hydrolysis of Acetylated Celenamides with Hydrochloric Acid

To 200 mg (0.21 mmole) of partially purified 58 was added 50 mL of acetonitrile and 0.5 mL of hydrochloric acid. The solution was stirred at room temperature for 24 h. Evaporation of the solvents gave an oil which was chromatographed on silica plates with chloroform-methanol (5:1) as eluant to give 21 mg (0.03 mmole, 14%) of the peptide amide 65; mp 153-156°C (CHCl\(_3\)-acetone); IR (CH\(_3\)CN) \( \nu \max \ 3600, \ 3530, \ 3340, \ 1780, \ 1670 \) cm\(^{-1}\); \(^1\)H NMR (figure 23, table 1); \(^13\)C NMR ((CD\(_3\))\(_2\)CO, 20 MHz, appendix 7) 175.4, 174.6, 173.3, 168.5 (2C), 167.6, 165.3, 144.6 (2C), 133.0-112.2 (complex), 55.7, 54.6, 40.4, 33.2-26.9 (masked by acetone signal) 25.5, 23.4, 22.8, 21.8, 20.6 (2C), 20.1; MS (figure 25); Anal calcd for C\(_{34}\)H\(_{38}\)N\(_5\)O\(_{10}\)Br \cdot 2/3H\(_2\)O, C 53.13, H
5.16, N 9.11; found, C 53.12, H 5.04, N 8.93 and C 53.15, H 5.01, N 8.98.

Similar treatment of 57 (150 mg, 0.16 mmole) gave the peptide amide 66 (18 mg, 0.02 mmole, 16%) as a noncrystalline white solid: IR (CH₃CN) ν max 3600, 3530, 3340, 1780, 1670 cm⁻¹; ¹H NMR (figure 24, table 1); ¹³C NMR (CD₃CN, 20 MHz, appendix 8) 175.2, 173.6 (2C), 169.4 (2C), 168.2, 165.4, 144.7 (2C), 139.0-111.3 (complex), 60.3, 56.4, 31.1, 27.1, 23.0, 20.9 (2C), 20.5, 19.7, 18.6; MS (figure 26).

Pentacetylcelenamide C (59, 3 mg, 0.003 mmole) afforded the peptide amide 65 (1 mg, 0.001 mmole, 40%). Compound 65 was identical (TLC, ¹H NMR (figure 30, table 1), MS (figure 31)) to the hydrolysis product isolated following similar treatment of hexacetylcelenamide A (58).

Nonacetylcelenamide D (60 8 mg, 0.008 mmole) gave the peptide amide 73 (3 mg, 0.004 mmole, 47%): IR (CH₃CN) ν max 3640, 3540, 3350, 1780, 1680 (br) cm⁻¹; ¹H NMR (figure 35, table 1); MS (70 eV, figure 36).

Ozonolysis of Hexacetylcelenamide A (58) and B (57)

Ozone was passed through a methanol solution (40 mL) of 58 (32 mg, 0.03 mmole) at -78°C for 4 min. Excess ozone was removed in a stream of oxygen, dimethyl sulfide (2 mL) was added, and the mixture was allowed to warm to room temperature. Evaporation of the solvents and purification on silica plates with ethyl acetate as eluant gave 3,4,5-triacetoxybenzaldehyde (4 mg, 0.01
mmole, 43%) and 3,4-diacetoxybenzaldehyde (3 mg, 0.01 mmole, 41%) which were identified by comparison (TLC and IR, $^1$H NMR, and MS) with authentic samples prepared from gallic acid (see p.61) and 3,4-dihydroxybenzaldehyde (Aldrich), respectively (see appendix 1).

3,4,5-Triacetoxybenzaldehyde (3 mg, 0.01 mmole, 47%) and 3,4-diacetoxybenzaldehyde (2 mg, 0.01 mmole, 39%) were also obtained by ozonolysis of 57 (22 mg, 0.02 mmole).

**Ozonolysis of the Peptide Amides 65 and 66**

Treatment of 65 (10 mg, 0.01 mmole) and 66 (10 mg, 0.01 mmole) as described for the ozonolysis of 57 and 58 gave 3,4,5-triacetoxybenzaldehyde (2 mg, 0.007 mmole each; 65, 54%; 66 53%; TLC, $^1$H NMR, MS) as the sole benzaldehydic product.

**Ozonolysis of a Crude Acetylated Celenamide Sample**

Ozonolysis of a mixture of acetylcelenamides similar in composition to that shown in figure 15 afforded 3,4,5-triacetoxy-, 3,4-diacetoxy-, and 4-acetoxybenzaldehyde as the only isolable products. The substitution pattern of each of the benzaldehydic products was determined by comparison with synthetic samples.
Hydrolysis of Hexacetylcelenamides \(\text{A (58)}\) and \(\text{B (57)}\) with 6N Hydrochloric Acid

A glass ampoule containing 14 mg (0.01 mmole) of 58 and 5 mL of 6 N hydrochloric acid was sealed under nitrogen and heated at 100°C for 18 h. The ampoule was cooled to room temperature, opened, and the contents were washed out with 5 mL of distilled water. The solvent was evaporated in vacuo, and the solid obtained was triturated with 1 N acetic acid. Filtration of the mixture and evaporation of the solvent in vacuo gave 1 mg of a light brown solid. Two-dimensional TLC (cellulose; first dimension, 1-butanol saturated with ammonia; second dimension, 1-butanol-acetic acid (9:1) saturated with water) indicated the presence of leucine. Similar treatment of 57 with 6N HCl gave valine.

Oxalic Acid

The complex mixture of polar products (20 mg) obtained from the ozonolysis of 58 was dissolved in 3 N hydrochloric acid (10 mL), and the solution was refluxed for 1 h. The solvent was evaporated in vacuo, and the solid obtained was triturated with methanol. The methanol-soluble material was treated with excess diazomethane in diethyl ether, and the mixture was analyzed by gas chromatography (10% DEGS, 4 mm x 2 m column, He flow rate 30 mL/min). The sole peak observed in the gas chromatogram had an
identical retention time ($R_T=4.61$ min) with authentic dimethyl oxalate.

Dimethyl oxalate was also observed when the polar material obtained from the ozonolysis of 57 (14 mg) was treated in a similar fashion.

**Methanolation of the Peptide Amides 65 and 66**

The peptide amide 65 (6 mg, 0.006 mmole) was dissolved in methanol (20 mL), and the solution was stirred at 50°C for 4 h under an atmosphere of nitrogen. The reaction mixture was cooled to room temperature, and excess diazomethane in diethyl ether was added. The solvents were evaporated to obtain 61: IR (KBr) $\nu$ max 3340 (br), 2950, 1640 (br) cm$^{-1}$; $^1$H NMR ((CD$_3$)$_2$SO) $\delta$ 0.92 (d, 1H, $J=7$ Hz), 0.98 (d, 1H, $J=7$ Hz), 1.7 (m, 3H), 1.98 (s, 3H), 3.33 (m, 2H), 3.67 (s, 3H, OMe), 3.78 (s, 6H, OMe), 4.26 (m, 1H), 4.61 (m, 1H), 6.87 (s, 2H), 7.06 (s, 1H), 7.14 (dd, 1H, $J=2$, 8Hz), 7.12 (br s, 1H, exch), 7.28 (d, 1H, $H=2$ Hz), 7.40 (br s, 1H exch), 7.50 (d, 1H, $J=2$, 8 Hz), 7.60 (d, 1H, $J=8$ Hz), 8.33 (m, 2H, exch), 9.96 (s, 1H, exch), 10.90 (br s, 1H, exch); MS (70 eV), m/e $M^+$ 673/671 (1:1, 2.4), 266/264 (1:1, 16), 210/208 (1:1, 58), 181 (30), 130 (28), 129 (53), 128 (44), 86 (100), 43 (95); Exact mass calcd for $C_{31}H_{38}$BrN$_5$O$_7$, 673.1927; obsd, 673.1934.

Treatment of 66 with methanol as described for 65 afforded the trimethoxy derivative 62; IR (KBr) $\nu$ max 3340 (br), 2950, 1640 (br) cm$^{-1}$; $^1$H NMR ((CD$_3$)$_2$SO) $\delta$ 0.86 (d, 3H, $J=6$ Hz), 0.91
(d, 3H, J=6 Hz), 1.80 (s, 3H), 1.97 (m, 1H), 2.97 (dd, 1H, J=10, 15Hz), 3.17 (dd, 1H, J=5, 15 Hz), 3.67 (s, 3H, OMe), 3.78 (s, 6H, OMe), 4.16 (dd, 1H, J=2, 8.4 Hz), 4.61 (ddd, 1H, J=5, 8.6, 10 Hz), 6.98 (s, 2H), 7.09 (dd, 1H, J=2 Hz), 7.11 (br s, 1H, exch), 7.17 (s, 1H), 7.22 (d, 1H, J=2 Hz), 7.40 (br s, 1H, exch), 7.43 (d, 1H, J=8.6 Hz, exch), 7.51 (d, 1H, J=2 Hz), 7.62 (d, 1H, J=8.4 Hz), 8.36 (d, 1H, J=7 Hz, exch), 10.01 (s, 1H, exch), 11.03 (br s, 1H, exch); MS (70 eV), m/e M+ 659/657 (1:1, 0.4), 266/264 (1:1, 32), 210/208 (1:1, 100), 181 (57), 129 (40), 114 (27), 72 (52), 43 (52); Exact mass calcd for C₃₀H₃₆BrN₅O₇, 659.1786; obsd, 659.1778.

The triphenolic compounds 127 and 128 were observed in ¹H NMR experiments in which (CD₃)₂CO solutions of 65 and 66 were warmed (50°C, 30 min) in the presence of water, D₂O or CD₃OD. For 127: ¹H NMR δ 0.91 (d, 3H, J=7 Hz), 0.98 (d, 3H, H=7 Hz), 1.7 (m, 3H), 1.89 (s, 3H), 3.31 (m, 2H), 4.42 (m, 1H), 4.72 (m, 1H), 6.44 (br s, 1H, exch), 6.72 (s, 2H), 7.08 (s, 1H), 7.16 (dd, 1H, J=2, 8.4 Hz), 7.21 (br s, 1H, exch), 7.30 (d, 1H, J=2 Hz), 7.54 (d, 1H, J=2 Hz), 7.60 (d, 1H, J=8.4 Hz), 7.79 (s, 1H, exch, ArOH), 7.83 (s, 2H, exch, ArOH), 8.12 (m, 2H, exch), 8.88
(s, 1H, exch), 10.21 (br s, 1H, exch). For 128: $^1$H NMR $\delta$ 0.94 (d, 3H, J=7 Hz), 0.97 (d, 3H, J=7 Hz), 1.9 (m, 1H), 1.97 (s, 3H), 3.3 (m, 2H), 4.35 (m, 1H), 4.78 (m, 1H), 6.76 (s, 2H), 7.14 (dd, 1H, J=2, 8 Hz), 7.20 (s, 1H), 7.35 (br s, 1H), 7.57 (d, 1H, J=2 Hz), 7.64 (d, 1H, J=8 Hz). Ferric chloride positive reactions were given by both 127 and 128.

Reduction of the Enamides 61 and 62 with Sodium Borohydride

To 2 mg of a 1:1 mixture of 61 and 62 dissolved in 15 mL of ethanol was added 100 mg of sodium borohydride. The solution was stirred at room temperature for 20h. Excess reagent was destroyed with 1 N hydrochloric acid, and the solution was concentrated and extracted with dichloromethane. The dichloromethane layer was dried over anhydrous sodium sulfate and evaporated to give a mixture of the two expected dihydro derivatives. $^1$H NMR ((CD$_3$)$_2$SO) $\delta$ 1.0 (m, (CH$_3$)$_2$CH), 1.7 (m, Leu $\beta$-CH$_2$), 1.78 (s, NHAc), 1.9 (m, Val $\beta$-CH), 2.73 (dd, J=8, 16 Hz, Trp $\beta$-CH$_2$), 2.90 (dd, J=6, 16, Trp $\beta$-CH$_2$), 3.36 (s, OMe), 2.66 (m, $\beta$-CH$_2$), 3.93 (m, Leu $\omega$-CH), 4.02 (m, Val $\omega$-CH), 4.45 (m, $\omega$-CH), 4.73 (m, $\omega$-CH), 7.09 (dd, J=2, 9 Hz, indole H$_5$), 7.11
(br s, CONH$_2$), 7.13 (d, J=3 Hz, indole H$_2$), 7.54 (d, J=2 Hz, indole H$_7$), 7.56 (d, J=9 Hz, indole H$_4$), 7.80 (d, J=8 Hz, NH), 7.92 (br s, CONH$_2$), 8.31 (s, PhH), 10.92 (br s, indole H$_1$).

**Fungistatic Assays and Fungal Isolates**

Samples composed of purified clionamide (53, 1.0 mg), clionamide and a mixture of celenamides (0.5, 2.5, 10 mg), and tetracyclonamide (46) and mixture of acetylated celenamides (20 mg) were applied to filter paper disks, dried and tested for inhibitory activity against the following fungi: *Aspergillus niger*, *Aureobasidium pullulans*, *Giberella zeae*, *Penicillium patulin*, *Polyporus versicolor*, *Trichoderma viride*, and a pink yeast isolate. No fungistatic activity was detectable following incubation at 25° for several days. Assays were performed on inoculated potato dextrose agar plates in duplicate.

In order to isolate the fungi associated with *C. celata*, fresh sponge was collected and stored in a small volume of sea water. Inocula were obtained from the sponge surface, the sea water and an internal portion obtained by surface sterilization (10 min, 70% EtOH) and dissection. The colonies which arose following incubation at 22-25° for several days were separated by picking, and incubated for a further 10 days on peptone dextrose agar (including rose bengal and aureomycin). In this fashion, twelve unidentified isolates were obtained which were
subsequently subjected to chemical analysis. Colonies were scraped from their agar plates, homogenized in MeOH and filtered. The methanolic extract was concentrated and analyzed by TLC. Samples were acetylated as described above and analyzed by TLC comparison with a sample of known Cliona alkaloids. Compounds bearing resemblance to those isolated from C. celata were not detected in any of the twelve fungal extracts.

*C. celata Cell Fractionation*

Freshly collected sponge was placed in several test tubes containing sterile calcium-free seawater and immediately submitted for cell fractionation by Dr. S.A. Pomponi.\(^1\) Cells were divided into sub-populations by the following abbreviated procedure: "The cells were dissociated in calcium-and magnesium-free seawater (CMF) and then separated in discontinuous density gradients of 6%, 12%, 18%, 24%, and 30% Ficoll/CMF. The bands were collected by pipetting, centrifuged, Ficoll supernatant decanted off, and pellets resuspended in absolute ethanol". Fractions from the 6-12%, 12-18%, 18-24% and 24-30% interfaces and the pellet were returned and analyzed as per the fungal cells described above. Alkaloids were not detected in any of the samples. Cholesterol and a green pigment were present in the cell-pellet extract but the usual array of yellow pigments found

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\(^1\)Horn Point Environmental Laboratories, Box 775, Cambridge, Maryland. 21613.
in nonfractionated *C. celata* tissue was not apparent in any of the cell fractions.

**Calcium Ion Transport Experiments**

In one experiment, the lower portion of a U-tube (id. 0.9 cm) equipped with a magnetic stirring bar was filled with 6.0 mL of CHCl$_3$-MeOH (9:1) containing 10 mg mL$^{-1}$ of crude triturated *Cliona* alkaloids. In one upper portion of the tube was placed 1.5 mL of an acceptor-phase (0.5 M sodium citrate, pH 5.5) and in the opposing upper portion was placed 1.5 mL of a donor-phase (0.5 M Tris-HCl, pH 8.5 containing 1.0 M CaCl$_2$). The apparatus was sealed under nitrogen and stirred at room temperature for 24 h. Aliquots (100 μL) were taken by syringe for analysis (flame-atomic absorption) at $T=1, 2, 7$ and 24 (500 μL) h. Two control experiments were performed, one in which the donor-phase was replaced by 0.5 M Tris-HCl (pH 8.5) and one in which the organic-phase was free of alkaloids. The results of these experiments are shown in appendix 12.

A second transport experiment was performed in a Pressman cell under conditions similar to those described above. CHCl$_3$-PhNO$_2$-MeOH (60:39:1; 5.0 mL) was employed as the organic solvent mixture and the donor and acceptor aqueous-phase volumes were 1.2 mL. The result of this experiment and the control experiment performed in the absence of alkaloids are described in the text.
(Z)-2-Acetyl-4-(3',4',5'-Trimethoxybenzylidene)-5(4H)-Oxazolone (90) and (Z)-2-Acetyl-4-Benzylidene-5(4H)-Oxazolone (91)

Acetylglycine (109, mp 207-208°, 2.39 g, 0.020 mole) and 3,4,5-trimethoxybenzaldehyde (Sigma, 4.00 g, 0.020 mole) were reacted as described by Herbst and Shemin (111), using benzaldehyde, to give the bright yellow azlactone 90 (3.80 g, 0.014 mole, 70%) following recrystallization from EtOAc. Treatment of freshly distilled benzaldehyde (5.8 g, 0.050 mole) under similar conditions afforded 91 (111, 6.68 g, 0.036 mole, 71%). 90: mp 159.5-162°; IR (KBr) v max 1790, 1765, 1660 cm⁻¹; IR (CHCl₃) v max 1800, 1770, 1660 cm⁻¹; ¹H NMR (100 MHz, acetone-d₆) δ 2.44 (s, 3H), 3.85 (s, 3H), 3.91 (s, 6H), 7.10 (s, 1H), 7.65 (s, 2H); MS m/e, M⁺ 277, 207, 192, 43; Exact mass calcd for C₁₄H₁₅NO₅, 277.0950; obsd, 277.0946. 91: mp 151.5-153 (lit. 148-150°); IR (KBr) v max 1790 sh, 1768, 1650 cm⁻¹; ¹H NMR (100 MHz, CDC₁₃) δ 2.41 (s, 3H), 7.17 (s, 1H), 7.47 (m, 3H), 8.46 (m, 2H); MS m/e, M⁺ 187, 158; Exact mass calcd for C₁₁H₉NO₂, 187.0634; obsd, 187.0634.

The azlactones 90 and 91 were shown to be single geometrical isomers by ¹H NMR and HPLC (RP and Silica-A) analyses.

Dehydroamino Acids Derived from 90 and 91

(Z)-2-Acetamino-(3',4',5'-trimethoxy)cinnamic acid methyl
ester (93) was prepared in quantitative yield by refluxing the azlactone 90 in anhydrous methanol for 48 h. 93: mp 220° (decomp.); IR (KBr) \( \nu \) max 3300 br, 1720, 1660 br cm\(^{-1}\); \(^1\)H NMR (100 MHz, DMSO-\(d_6\)) \( \delta \) 2.03 (s, 3H), 3.72 (s, 3H), 3.81 (s, 6H), 7.05 (s, 2H), 7.22 (s, 1H), 9.64 (s, 1H); MS, m/e, M\(^+\) 309, 267, 252, 192, 43; Exact mass calcd for C\(_{14}\)H\(_{19}\)NO\(_6\), 309.1213; obsd, 309.1210.

Treatment of 90 with refluxing aqueous acetone (113) afforded an 80% yield of the acid 94 after recrystallization from methanol. 94: mp 208-210°; IR (KBr) \( \nu \) max 3400 br, 1710, 1630 cm\(^{-1}\); \(^1\)H NMR (100 MHz, DMSO-\(d_6\)) \( \delta \) 2.04 (s, 3H), 3.72 (s, 3H), 3.81 (s, 6H), 7.03 (s, 2H), 7.27 (s, 1H), 9.49 (s, 1H). (Z)-2-Acetaminocinnamic acid (95) was obtained in 85% yield following similar treatment of 91. 95: mp 189-190° (lit. (111) mp 191-192°) IR (KBr) \( \nu \) max, 3280, 3050, 2950, 1724, 1600, 1540(br) cm\(^{-1}\). The acids 94 and 95 could be quantitatively converted back to the azlactones 90 and 91 by reacting with Ac\(_2\)O (1 equiv) and 4-dimethylaminopyridine (0.1 equiv) in Et\(_3\)N at 40°C for 12 h.

Ammonolysis of the azlactones 90 and 91 in anhydrous methanol at 0°C for 2 h gave the primary amides 96 and 97, respectively, in quantitative yields. 96: mp 211-213° (decomp); IR (KBr) \( \nu \) max 3200 br, 1660 br cm\(^{-1}\); \(^1\)H NMR (270 MHz, DMSO-\(d_6\)) \( \delta \) 2.00 (s, 3H), 2.68 (s, 3H), 3.78 (s, 6H), 6.90 (s, 2H), 7.06 (s, 1H), 7.11 (bs, 1H), 7.41 (bs, 1H), 9.37 (s, 1H); MS m/e, M\(^+\) 294, 276, 261, 252, 237, 207. 97: mp 202.5-204° (decomp); IR (KBr) \( \nu \) max 3250 br, 1650 br cm\(^{-1}\); \(^1\)H NMR (270 MHz, DMSO-\(d_6\)) \( \delta \)
1.96 (s, 3H), 6.98 (s, 1H), 7.11 (bs, 1H) 7.30 (m, 3H), 7.42 (bs, 1H), 7.48 (m, 2H), 9.32 (s, 1H); MS m/e, m* 204, 187, 162; Exact mass calcd for C\textsubscript{11}H\textsubscript{12}N\textsubscript{2}O\textsubscript{2}, 204.0899; obsd, 204.0897.

(Z)-2-Phenyl-4-(3',4',5'-Trimethoxybenzylidene)-5(4H)-Oxazolone (92) and (Z)-2-Benzamido-(3',4',5'-Trimethoxy) Cinnamic Acid Methyl Ester (98)

Condensation of hippuric acid (Sigma, 24.0 g, 0.13 mole) and 3,4,5-trimethoxybenzaldehyde (Sigma, 24.5 g, 0.12 mole) as described by Mauthner (112) afforded the azlactone 92 (27.1 g, 0.08 mole, 67%); mp 162.5-165° (lit. 165-166°, 112); IR (KBr) \( \nu \) max 1790, 1655, 1580 cm\(^{-1}\). The azlactone 92 underwent quantitative conversion to the methyl ester 98 upon refluxing in anhydrous methanol for 48 h. 98: mp 127-129°; IR (KBr) \( \nu \) max 3250 br, 2950, 1719, 1655, 1580 cm\(^{-1}\); \( ^{1} \)H NMR (100 MHz, acetone-\( \text{d}_{6} \)) \( \delta \) 3.88 (s, 9H), 7.06 (s, 2H), 7.46 (s, 1H), 7.60 (m, 3H), 8.14 (m, 2H), 9.13 (bs, 1H); MS m/e, m* 371, 266, 234.

N-Benzoyl-3-Benzylthio-(3',4',5'-Trimethoxy)Phenylalanine Methyl Ester (129)
The thio ether 129 was prepared as a mixture of diastereomers in quantitative yield according to the procedure of Rich et al. (133). The reaction was complete within 6 h at room temperature. 129: mp 132-140° (decomp); IR (KBr) $\nu$ max 3325, 2950, 1740, 1640, 1590, 1510 cm$^{-1}$; $^1$H NMR (100 MHz, CDCl$_3$) $\delta$ 3.8 (m, 14H), 4.27 (d, 1H, $J=6$Hz), 5.20 (dd, 0.6H, $J=5$, 6Hz), 5.33 (m, 0.4H), 6.58 (s, 2H), 6.8 (bm, 1H), 7.28 (bs, 5H), 7.50 (m, 3H), 7.78 (m, 2H), MS m/e, 371 (M$^+$-C$_7$H$_8$S), 339, 303, 246, 181, 121, 105, 91.

Several attempts were made to convert 129 into its corresponding free amino acid. Hydrolysis in refluxing 3N HCl, 1N NaOH, or mixtures of formic acid and aqueous HCl (133) failed to provide the desired product.

Dehydrodipeptides Derived from 90 and 91

(Z)-N-Acetyl-$\alpha,\beta$-didehydro-(3',4',5'-trimethoxy)-phenylalanylglycine (99, 95%) and (Z)-N-Acetyl-$\alpha,\beta$-didehydrophenylalanylglycine (103, 92%) were prepared from 90 and 91, respectively, by the method of Doherty et al. (115). 99: mp 207° (decomp); IR (KBr) $\nu$ max 3300 br, 1740, 1630 br cm$^{-1}$; $^1$H NMR (270 MHz, DMSO-$_d_6$) $\delta$ 2.01 (s, 3H), 3.70 (m, 11H), 6.88 (s, 2H), 7.11 (s, 1H), 8.20 (bs, 1H), 9.43 (s, 1H), 12.39 (bs, 1H); MS m/e, M$^+$ 352, 334, 319, 290. 103: mp 189-191° (lit. 194-195°, 115); IR (KBr) $\nu$ max 3380, 3230 br, 1700, 1630 br cm$^{-1}$; $^1$H NMR (270 MHz, DMSO-$_d_6$) $\delta$ 1.99 (s, 3H), 3.79 (d, 2H, $J=6$Hz), 7.09 (s, 1H), 7.34 (m, 3H), 7.49 (m, 2H), 8.23 (t, 1H, $J=6$Hz), 9.39 (s,
1H), 12.53 (bs, 1H). **99** was further characterized as its methyl ester **100** (CH$_2$N$_2$/CH$_3$OH); IR (KBr) $\nu$ max 3400 br, 1755, 1640 br cm$^{-1}$; $^1$H NMR (270 MHz, acetone-**d$_6$**) $\delta$ 2.12 (s, 3H), 3.67 (s, 3H), 3.74 (s, 3H), 3.83 (s, 6H), 4.00 (m, 2H), 6.88 (s, 2H), 7.19 (s, 1H), 7.73 (bs, 1H), 8.69 (s, 1H); MS m/e, M$^+$ 366, 348, 324, 309; Exact mass calcd for C$_{17}$H$_{22}$N$_2$O$_7$, 366.1427; obsd, 366.1426.

The azlactone **90** (3.0 g, 0.011 mole) was dissolved in acetone (25 mL) and 1.29 g (0.011 mole) of L-valine in 20 mL of 1N NaOH was added (115). The reaction mixture was stirred at room temperature overnight and then acidified with 2 M HCl to give the dehydroadipeptide **101** (2.76 g, 0.01 mole, 91%) as a white powder. When **90** (3.38 g, 0.012 mole) was treated in an analogous fashion with L-leucine (1.60 g, 0.012 mole) the dehydroadipeptide **102** was isolated (3.10 g, 0.01 mole, 83%). **101**: mp 221-223° (decomp); IR (KBr) $\nu$ max 3400 br, 2950, 1720 br, 1640 br cm$^{-1}$; $^1$H NMR (270 MHz, DMSO-**d$_6$**) $\delta$ 0.89 (d, 3H, J=7Hz), 0.92 (d, 3H, J=7Hz), 2.01 (s, 3H), 2.12 (m, 1H), 3.68 (s, 3H), 3.77 (s, 6H), 4.20 (dd, 1H, J=7,8Hz), 6.89 (s, 2H), 6.99 (s, 1H), 7.71 (d, 1H, J=8Hz), 9.46 (s, 1H). **102**: mp 199-201° (decomp); IR (KBr) $\nu$ max 3400 br, 3230 br, 2950, 1730, 1640 br cm$^{-1}$; $^1$H NMR (270 MHz, DMSO-**d$_6$**) $\delta$ 0.86 (d, 3H, J=7Hz), 0.90 (d, 3H, J=7Hz), 1.54 (m, 1H), 1.67 (m, 2H), 2.00 (s, 1H), 3.68 (s, 3H), 3.78 (s, 6H), 4.32 (m, 1H), 6.87 (s, 2H), 6.99 (s, 1H), 8.02 (d, 1H, J=8Hz), 9.38 (s, 1H).
Bisdehydropeptides Derived from 99 and 103

The dehydropeptide 99 (0.85 g, 2.43 mmole) was treated with 3,4,5-trimethoxybenzaldehyde (0.48 g, 2.31 mmole), excess Ac₂O and anhydrous sodium acetate (0.200 g, 2.4 mmole) at 40°C for 24 h (115) to give the peptide azlactone 104 (0.94 g, 1.83 mmole, 80%). When the dehydrodipeptide 103 (4.16 g, 0.016 mole) was reacted in an analogous fashion with redistilled benzaldehyde (1.70 g, 0.016 mole), the peptide azlactone 105 (4.46 g, 0.013 mole, 81%) was obtained. 104: mp 239-242°; IR (KBr) ν max 3400 (br), 2950, 1800, 1640, 1580 cm⁻¹; ¹H NMR (270 MHz, CDCl₃) δ 2.43 (s, 3H), 3.87 (s, 6H), 3.89 (s, 6H), 3.91 (s, 3H), 6.75 (s, 2H), 7.11 (s, 1H), 7.13 (s, 1H), 7.38 (s, 2H), 7.64 (s, 1H, NH); ¹H NMR (270 MHz, DMSO-δ₆) δ 2.07 (s, 3H), 3.71 (s, 3H), 3.75 (s, 3H), 3.79 (s, 6H), 3.83 (s, 6H), 7.13 (s, 2H), 7.23 (s, 1H), 7.44 (s, 1H), 7.66 (s, 2H), 9.77 (s, 1H, NH); MS m/e, M⁺ 512, 470; Exact mass calcd for C₂₆H₂₈N₂O₉, 512.1794; obsd 512.1770. 105: mp 184-186.5° (lit. (115) mp 184-186°); IR (KBr) ν max 3250, 1800 (sh), 1780, 1650 (br) cm⁻¹; ¹H NMR (270 MHz, acetone-δ₆) δ 2.19 (s, 3H), 7.00 (s, 1H), 7.41 (m, 7H), 7.74 (m, 2H), 8.23 (m, 2H), 8.91 (s, 1H, NH); MS m/e, M⁺ 332, 290, 244; Exact mass calcd for C₂₀H₁₆N₂O₃, 332.1161; obsd 332.1163.

The azlactones 104 and 105 were reacted with a variety of reagents to give the bisdehydrodipeptides described below. 106 (acetone/H₂O): IR (KBr) ν max 3300 (br), 2950, 1660 (br), 1584 cm⁻¹; ¹H NMR (270 MHz, DMSO-δ₆) δ 2.02 (s, 3H), 3.67 (s, 3H), 3.69 (s, 3H), 3.78 (s, 12H), 6.91 (s, 2H), 6.99 (s, 2H), 7.16
170

(s, 1H), 7.25 (s, 1H), 9.41 (s, 1H), 9.55 (s, 1H); MS m/e, 512 (M-18), 468. 107 (MeOH): IR (KBr) ν max 3300 (br), 2950, 1720, 1640 (br) cm⁻¹; ¹H NMR (270 MHz, DMSO-d₆) δ 2.02 (s, 3H), 3.66 (s, 3H), 3.68 (s, 3H), 3.70 (s, 3H), 3.78 (s, 12H), 6.91 (s, 2H), 7.02 (s, 2H), 7.15 (s, 1H), 7.23 (s, 1H), 9.54 (s, 1H), 9.58 (s, 1H); MS m/e, M⁺ 544, 526, 511; Exact mass calcd for C₂₇H₃₂N₂O₁₀, 544.2057; obsd 544.2055. 108 (NH₂C(CH₃)₃): ¹H NMR (400 MHz, acetone-d₆) δ 1.42 (s, 9H), 2.21 (s, 3H), 3.72 (s, 3H), 3.77 (s, 3H), 3.87 (s, 6H), 6.84 (s, 2H), 6.92 (s, 2H), 7.03 (s, 3H), 3.77 (s, 9H), 3.87 (s, 6H), 6.84 (s, 2H), 6.92 (s, 2H), 7.03 (s, 1H), 7.32 (s, 1H), 7.35 (bs, 1H, NH), 8.85 (bs, 1H), 9.38 (bs, 1H). 109 (NH(CH₂CH₃)₂): ¹H NMR (400 MHz, acetone-d₆) δ 1.16 (m, 3H), 1.28 (m, 3H), 2.13 (s, 3H), 3.34 (m, 2H), 3.66 (m, 2H), 3.73 (s, 3H), 3.74 (s, 3H), 3.79 (s, 6H), 3.88 (s, 6H), 5.94 (s, 1H), 6.83 (s, 4H), 7.29 (s, 1H), 8.97 (s, 1H), 9.77 (s, 1H). 111 (NH₄OH): mp 220-223° (decomp); IR (KBr) ν max 3300 (br), 1640 (br), 1490 cm⁻¹; ¹H NMR (270 MHz, DMSO-d₆) δ 2.07 (s, 3H), 6.93 (s, 1H), 7.4 (m, 9H), 7.7 (m, 4H), 9.63 (s, 1H), 9.97 (s, 1H); MS m/e, M⁺ 349, 332; Exact mass calcd for C₂₀H₁₉N₃O₃, 349.1427; obsd 349.1427. 110 (NH₂CH₂CH₂(3,4-MeO)C₅H₂): ¹H NMR (400 MHz, DMSO-d₆) δ 2.11 (s, 3H), 2.75 (t, 2H, J=7 Hz), 3.40 (m, 2H), 3.68 (s, 3H), 3.71 (s, 3H), 3.72 (s, 3H), 3.75 (s, 9H), 3.82 (s, 6H), 6.80 (d, 1H, J=8 Hz), 6.85 (m, 2H), 6.92 (s, 2H), 6.94 (s, 2H), 6.97 (s, 1H), 7.35 (s, 1H), 7.89 (t, 1H, J=7, NH), 9.65 (s, 1H), 10.06 (s, 1H).
Racemic Acetylvalinamide (117)

Acetyl-L-valine (109, 8.0 g, 0.05 mole, [α]_D + 3.4° (c 2.0 EtOAc) (lit. (143) [α]_D + 4° (c 2.0, EtOH)) was converted to its p-nitrophenyl ester by the procedure of Hassner and Alexanian (144). After 16 h at room temperature the reaction mixture was washed repeatedly with water and 5% acetic acid to give a pale yellow oil (13 g) following evaporation in vacuo. This material was dried and subjected to ammonolysis without further purification. Treatment with ammonia in anhydrous methanol overnight at 0°C afforded the amide 117 (6.2 g, 0.039 mole, 78% from acetyl-L-valine). 117; mp 239.5°; [α]_D + 0.1° (c 2.0, EtOH); IR (KBr) ν max 3250, 2960, 1650 br cm⁻¹; ^1H NMR (270 MHz, DMSO-d₆) δ 0.82 (d, 3H, J=7 Hz), 0.86 (d, 3H, J=7 Hz), 1.86 (s, 3H), 1.93 (m, 1H), 4.11 (dd, 1H, J=7, 8.5 Hz), 6.97 (bs, 1H), 7.35 (bs, 1H), 7.77 (d, 1H, J=8.5 Hz); Anal calcd for C₇H₁₄N₂O₂, C53.15, H8.92, N17.71; found, C53.25, H8.76, N17.73.

Acetyl-L-Valinamide (117a)

Acetyl-L-valine described above (14.0 g, 0.088 mole) was treated with thionyl chloride (16.8mL, 0.117 mole) in anhydrous methanol (140 mL) at 0° for 24 h and then at room temperature for 48 h (145). The solution was refluxed for 4 h, concentrated in vacuo and the oily residue obtained partitioned between EtOAc and aqueous potassium carbonate. The EtOAc layer was washed with
water, dried over anhydrous Na$_2$SO$_4$ and evaporated to give 8.2 g (0.047 mole, 53%) of the methyl ester 130 as a colourless oil; IR (KBr) $\nu$ max 3200, 2960, 1725 cm$^{-1}$. This material was subjected to ammonolysis without further purification. A cold solution of 200 mL of anhydrous MeOH saturated with ammonia was added to 130 (7.6 g, 0.044 mole) and the mixture was kept at 0°C for one week. Removal of the solvent in vacuo and trituration of the resulting oil with ether afforded the amide 117a (420 mg, 2.7 mmole, 7%). 117a was identical to racemic N-acetylvalinamide with the exception of its optical rotation; $[\alpha]_D -17^\circ$ (c 0.68, 1N HCl). The unchanged methyl ester isolated from this reaction (7.2 g, 0.041 mole) was resubjected to ammonolysis at room temperature for 1 week to give a further 0.44 g (2.8 mmole, 7%) of the desired amide; $[\alpha]_D -16^\circ$ (c 1.10, 1N HCl). The optical purity of this material has not been determined.

2-Oxo-3-Phenylpropionic Acid (131) and 2-Oxo-3-(3',4',5'-Trimethoxyphenyl) Propionic Acid (132)

Hydrolysis of the dehydroamino acid 94 (7.0 g, 0.024 mole) in refluxing 1N HCl for 3h afforded 2.7 g (0.011 mole, 45%) of the substituted phenylpyruvic acid 132. Identical treatment of $\alpha$-acetamino cinnamic acid (95, 2.0 g, 0.010 mole) gave phenylpyruvic acid (131, 1.5 g, 9.1 mmole, 91%). 132: mp 168-171° [lit. 167-168 (142, 112)]; IR (KBr) $\nu$ max 3360, 3220 (br), 1690, 1580, 1506 cm$^{-1}$; Fe$^{3+}$ positive. 131: mp 152-154° [lit. 150-154° (146)]; IR (KBr) $\nu$ max 3450, 3150b, 1700sh, 1650b, 1450
The styrene epoxide 112 (2.3 g, 11.0 mmole, 90%) was prepared from 3,4,5-trimethoxybenzaldehyde (2.4 g, 12.4 mmole) according to Corey's method of epoxide formation using trimethylsulphonium methyldide (117). The colourless oil, isolated following repeated passage through a silica plug with diethyl ether, crystallized upon cooling: mp 53-54°; IR (neat) $\nu$ max 2940, 2830, 1586, 1500, 1460, 1421, 1383, 1328, 1239, 1190, 1130, 1010, 942, 840 cm$^{-1}$; $^1$H NMR (100 MHz, CDCl$_3$) $\delta$ 2.75 (dd, 1H, $J$=3, 6 Hz), 3.13 (dd, 1H, $J$=4, 6 Hz), 3.83 (m, 1H), 3.85 (s, 3H), 3.86 (s, 6H), 6.55 (s, 2H); MS m/e, M$^+$ 210, 195, 181 (base peak).

**Conversion of the Epoxide 112 to Acetate 115**

The styrene epoxide 112 (864 mg, 4.1 mmole) was dissolved in 3 mL of DMF and the solution cooled to 0°. Ammonium hydroxide (2 mL) was added and the mixture stirred at room temperature until all of the reagent had reacted (TLC, 4 days). The solvents were removed in vacuo and the resulting crude ninhydrin positive, residue was dissolved in dry CH$_2$Cl$_2$ (5 mL) containing one equivalent of Et$_3$N. This solution was cooled to 0° and a mixture of 847 mg of DCC (4.1 mmole), 652 mg of N-acetylvaline
(4.1 mmole) and 5 mL of dry CH₂Cl₂ was added. The reaction was stirred overnight, (until ninhydrin negative) precipitated DCU was removed by filtered and the solvents evaporated in vacuo to give a pale yellow oil. Acetylation of this material overnight (Ac₂O-DMAP-Et₃N) followed by silica column chromatography (CH₂Cl₂-EtOAc) afforded the modified-amine acid 115 in 52 percent yield from 112 (874 mg, 2.1 mmole). 115: IR (neat) ν max 3270, 2950, 1735, 1640b, 1590, 1520b cm⁻¹; ¹H NMR (100 MHz, acetone-d₆) δ 0.88 (d, 6H, J=7Hz), 1.95 (s, 3H), 2.06 (s, 3H), 2.07 (m, 1H), 3.61 (m, 1H), 3.72 (s, 3H), 3.84 (s, 6H), 3.85 (m, 1H), 4.25 (m, 1H), 5.82 (dd, 1H, J=5,6Hz), 6.71 (s, 2H), 7.27 (bd, 1H, J=9Hz), 7.57 (bm, 1H); MS m/e, m⁺ 410, 350, 252 (base peak), 210, 197, 142, 114, 72. The acetate 115 would be converted into the alcohol 114 in 82 percent yield by overnight treatment with MeOH/K₂CO₃.

**Attempted Opening of the Epoxide 112 with the Amide 113**

The epoxide 112 (150 mg, 0.7 mmole) was dissolved in anhydrous DMF containing 126 mg (0.8 mmole) of N-acetylvalinamide). The reaction mixture was refluxed for 72 h, the solvent was removed in vacuo and a portion of the resulting oily residue purified by PTLC. ¹H NMR analysis of the fractions which did not contain starting materials indicated the presence of small quantities (<5%) of the ketone 118 and the aldehyde 119.

This reaction was repeated with the presence of one
equivalent (2 mg) of hexane-washed NaH. The residue obtained was acetylated as described above to give 28 mg (0.07 mmole, 10%) of a compound identical (TLC, 'H NMR) to the acetate following purification by PTLC.

Methyl N-Acetylvalyl-α,β-didehydrophenylalanate

To a 50 mL round bottom flask fitted with a Dean-Stark trap and a condenser, was added 10 mL of anhydrous benzene, 2.12 g (0.012 mole) of the α-keto acid 132, and 2.42 g (0.015 mole) of the unsubstituted amide 117. Phosphorus oxychloride (0.6 mL, 6.5 mmole) was added dropwise with stirring, and the mixture was refluxed for 19 h. Removal of the solvents in vacuo and purification by silica column chromatography (CH2Cl2-EtOAc) afforded two major products; the dehydrodipeptide 133 (0.6 g, 1.9 mmole, 16%) and the biproduct 134 (0.41 g, 1.5 mmole, 22%).

133: IR (KBr) ν max 3250b, 1720, 1640b, 1520 cm⁻¹; 'H NMR (270 MHz, acetone-d6) δ 1.00 dd, 3H, J=7Hz), 1.03 (d, 3H, J=7Hz), 2.00 (s, 3H), 2.20 (m, 1H), 3.75 (s, 3H), 4.43 (dd, 1H,
J=6.7Hz), 7.25 (s, 1H), 7.30 (bd, 1H, J=6Hz), 7.37 (m, 2H), 7.69 (m, 3H), 8.87 (bs, 1H); MS m/e, M* 318, 177 (base peak), 117, 114, 72. mp. 193.5-194.5°; IR (KBr) ν max 3300b, 2800, 1650b cm⁻¹; ¹H NMR (270 MHz, acetone-d₆) δ 0.97 (d, 3H, J=7Hz), 1.01 (d, 3H, J=7Hz), 1.13 (d, 6H, J=7Hz), 1.98 (s, 3H), 2.17 (m, 1H), 2.29 (s, 3H), 2.73 (m, 1H), 4.44 (dd, 1H, J=b,7Hz), 7.35 (bd, 1H, J=7Hz), 9.09 (bs, 1H); MS m/e, M* 281, 140 (base peak), 125, 114, 72.
Appendix 1. Comparison of the Substituted Benzaldehydes Derived from the Ozonolysis of 46, 57, 58, 59, 65, and 66 with Synthetic Samples.

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</table>

'*4-Acetoxybenzaldehyde was derived from the ozonolysis of a mixture of acetylated celenamides.
*From 58 and 59.
*From 46, 57, 58, 65, and 66.
*CHCl₃, ν max, cm⁻¹.
*70 eV, m/e.
*100 MHz, CDCl₃ or CCl₄, ppm from internal tetramethylsilane (δ=0).
*δ, J=8 Hz.
*δ, J=2 Hz.
*δδ, J=2, 8 Hz.
Appendix 2. 20 MHz $^{13}$C NMR of 55 in acetone-$d_6$, PND.
Appendix 3. 20 MHz $^{13}$C NMR of a mixture of acetylated celenamides in CD$_3$CN, PND.
Appendix 4. 100.6 MHz $^{13}$C NMR of hexacetylcelenamide A (58) in CDCl$_3$, PND.
Appendix 5  

a. Expansion of the aromatic region of the 400 MHz spectrum of 57, b. Computer simulation of the spectrum shown in 'a'. Coupling constants and chemical shifts were obtained from 57, 58, and 66 (table 1); line widths are 2 Hz. Inserts show the spin systems, chemical shifts and coupling constant patterns used for simulation.
Appendix 6: 20 MHz $^{13}$C NMR of hexacetylenediamine B (57) in CDCl$_3$-DMSO-d$_6$ (3:2), PND.
Appendix 7. 20 MHz $^{13}$C NMR of 65 in acetone-$_6$, PND.
Appendix 8. 20 MHz $^{13}$C NMR of 66 in CD$_3$CN, PND.
Appendix 9. IR (CHCl₃) and 100 MHz ¹H NMR of a mixture of oligomers derived from the acid hydrolysis of a mixture of 57, 58 and 59.
Appendix 10. 100.6 MHz $^{13}$C NMR spectrum of an unknown peptide alkaloid, PND.
Appendix 11. Circular dichroism spectra of hexacetylcezenamide B (57), \(1.54 \times 10^{-4}\) M in CH\(_3\)CN. No salt added, \(--\); 2.31 \(\times\) 10\(^{-3}\) M Ca\(_2\)(ClO\(_4\))\(_4\), \(----\)(\(\epsilon\) in L mol\(^{-1}\) cm\(^{-1}\)).
Appendix 12. Translocation of Ca$^{2+}$ through a CHCl$_3$-MeOH (9:1) layer mediated by a mixture of crude triturated Cliona alkaloids (—). C1 and c2 represent control experiments, see experimental for details.
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