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Full Papers

Novel cytotoxic peptides from the tropical marine cyanobacterium *Hormothamnion enteromorphoides*. 1. Discovery, isolation and initial chemical and biological characterization of the hormothamnins from wild and cultured material

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Summary. A Caribbean cyanobacterium, *Hormothamnion enteromorphoides,* was found to produce a complex mixture of ichthyotoxic peptides, perhaps explaining the apparent absence of predation upon these potentially palatable life forms. Bioassay-guided fractionation was used to isolate these toxic and antimicrobial natural products, and a variety of techniques including HR FAB mass spectrometry, 2D-NMR, traditional hydrolysis-amino acid analysis, and several chemical reactions were used to define the basic structural features of the major peptide, hormothamnin A. Hormothamnin A is a cyclic undecapeptide containing six common and five uncommon or new amino acid residues. HPLC analyses indicate that the relative proportions of these peptide natural products remain relatively constant between different collection locations and years, however, they do vary seasonally. Clonal isolates of this cyanobacterium in culture produce the full spectrum of toxic peptides.
Key words. Cyclic peptides; cytotoxin; natural products; blue green algae; cyanobacteria.

Introduction

In the last several years the chemical potential of the cyanobacteria (blue green algae) for producing structurally-novel and biologically-active natural products has been uncovered, largely due to the efforts of Moore and co-workers [1]. Cyanobacterial natural products have been reported from the following structural classes; acetogenins [2a], bromophenols [2b], fatty acids [3], terpenes and sterols [4], alkaloids (indole, purine and tropane-like) [5–7], amides [8], cyclic oligosaccharides [9], macrolides [10], and cyclic peptides [11]. The range of potentially useful biological activities reported for these isolates include antibiotic [1], antifungal [11a], and antitumor activity [12], cytotoxicity to cancer cells [8b], anti-inflammatory [13] and inflammatory activity [14], and neuromuscular toxicity [1]. Further, in a survey of crude extracts from marine cyanobacteria from the Pacific Basin, very high levels of in vitro and in vivo anticancer activity were detected [15]. More recently, the recognition of cyanobacteria as a promising resource of anticancer natural products has led to the initiation of a screening program by the National Cancer Institute [16]. In this context, our discovery of bioactive peptides from the marine cyanobacterium *Hormothamnion enteromorphoides* lends additional support to the rationale for efforts in this area.

Despite the encouraging results obtained from work with Pacific marine species and various freshwater species of cyanobacteria, there have been relatively few investigations of these prokaryotes from elsewhere. Hence, we were eager to include cyanobacteria in our survey of marine algae from Puerto Rico as potential sources of new biomedicinals and have subsequently published results of our work with several tropical cyanobacteria and other microalgae [8b, 17]. One of our most promising projects, initiated as a result of this survey effort, has occupied us over the past two years and we report here the isolation and initial chemical description of cytotoxins produced by the cyanobacterium *Hormothamnion enteromorphoides*. Furthermore, we describe the establishment of clonal isolates of *H. enteromorphoides* and the production of these toxins in cultured material, the first reported for a cultivated marine cyanobacterium.

Methods and materials

General chemical

Ultraviolet spectra were recorded on an Aminco DW-2a UV-Vis spectrophotometer and infrared spectra (IR) on Perkin-Elmer 727 and Beckman Acculab 7 spectrophotometers. Optical rotation was measured on a Perkin Elmer Model 141 polarimeter using a 10-cm microcell. Nuclear magnetic resonance spectra (NMR) were recorded on Varian EM 360, FT-80A and Bruker AM 400 NMR

spectrometers and all shifts are reported relative to an internal TMS standard. Low resolution mass spectra (LRMS) were obtained on a Varian MAT CH 7 spectrometer while linked scan FAB mass spectra were obtained on a Kratos MS 50 TC. High resolution mass spectra were obtained on a VG 70 SE double focussing mass spectrometer. High performance liquid chromatography (HPLC) employed a Waters M-6000 pump, U6K injector and R 401 differential refractometer, while thin layer chromatograms were made using Merck silica gel 60 F_{254} on aluminum sheets. All solvents were distilled from glass prior to use.

Collection

Collections of *H. enteromorphoides* for chemical and culture work were made by snorkeling at Playa de Luquillo, Puerto Rico, as well as other sites around the island (see fig. 3, A–E). Excess water was removed by firmly pressing the cyanobacteria, found growing as large tufts up to 50 cm long, just prior to their storage in solvent (IPA or MeOH). The preserved material was transported at RT and stored in the laboratory at −10 to −20 °C until workup. Material for culture was segregated at the time of collection by placing tufts of *H. enteromorphoides*, free of any apparent epiphytes, in Whirl Paks with a small volume of seawater. The Whirl Paks were maintained at ambient shade temperatures during transport to the field laboratory.

Extraction

The partially frozen cyanobacteria in alcohol/water were defrosted and steeped (30 min) in a stainless steel bucket containing an excess of $CHCl_3$ and MeOH (2:1). Upon cooling, the mixture was filtered through Whatman No. 1 filter paper and the cyanobacterial mass extracted once more in an identical fashion. The lipid extracted cyanobacteria were re-extracted for more polar constituents by steeping in 70% aqueous MeOH. The lipid and aqueous extracts were separately reduced in vacuo (400 °C), and yielded in both cases a dark green, oily tar. The extracts were placed in amber bottles and stored (−20 °C). From one collection of *H. enteromorphoides* made in March 1985, a total of 1.001 g of lipid extract was obtained from 53.4 g air-dried cyanobacteria.

General biological

Antimicrobial activity to a variety of microorganisms was assessed by employing standard antimicrobial sensitivity methods. Paper discs, 6.5 mm in diameter, were impregnated with known quantities of either crude extract, chromatography fractions, or pure compounds dissolved in diethyl ether or MeOH. Solvent vehicles were evaporated at RT and the extract impregnated discs applied to inoculated Mueller-Hinton agar plates. The inverted plates were incubated overnight at 35–37 °C and zones of inhibition (diameters in mm) measured between 16 and 24 h post-inoculation. IC_{50} values were determined graphically following spectrophotometric analysis (650 nm) of Mueller-Hinton broths containing the test microorganisms and serial 2-fold dilutions of hormothamnin A (6 h incubation)[18].

Brine shrimp toxicities were determined following the methods of McLaughlin and coworkers[19]. Toxicity to the common goldfish *Carassius carassius* was determined following published methodology[20]. In vitro cytotoxicity testing to cancer cells was performed by Bristol-Myers Company, Wallingford, Conn., USA.

Table 1. Some biological activities of hormothamnin A

Goldfish toxicity	ca. 10 µg/ml	
Brine shrimp toxicity	inactive at 20 µg/ml	
Antimicrobial[a]	Strain	(IC_{50} µg/ml)
gram positive		
Staphylococcus aureus	ATCC 12,600	inactive
Bacillus subtilis	ATCC 6081	>100
Streptococcus faecalis	ATCC 29,212	inactive
gram negative		
Pseudomonas aeruginosa	ATCC 9721	>100
Escherichia coli	ATCC 11,775	inactive
Salmonella typhimurium	ATCC 14,028	inactive
Fungi/yeasts		
Candida albicans	ATCC 14,053	inactive
Trichophyton mentagrophytes	ATCC 18,748	inactive
In vitro cytotoxicity	Cell line	
Human lung	SW 1271	0.20
Human lung	A 529	0.16
Murine melanoma	B16-F10	0.13
Human colon	HCT-116	0.72

[a] Sensitivity to hormothamnin A was determined by impregnated paper discs on seeded agar (100 µg/disc) and read 15 h post-inoculation [*P. aeruginosa* = 8 mm, *B. subtilis* = 7 mm]. IC_{50} values were explored for sensitive species by the tube dilution method (see experimental), however, due to the relative inactivity of hormothamnin A, the IC_{50}'s exceeded the solubility of the peptide in the test broth and thus, are estimated from lower levels of inhibition.

Isolation of peptides

Approximately 800 mg of crude lipid extract was applied to a short column of silica gel (40 mm diameter × 40 mm high) in a sintered glass vacuum funnel[21]. A gradient of progressively more polar solvent mixtures (isooctane/EtOAc/MeOH) was used to elute 16 fractions from the column. These were reduced in vacuo and analyzed using antimicrobial assay and TLC. The largest zones of inhibition to *Bacillus subtilis* (13 mm) were obtained for those fractions which contained a low Rf, UV-absorbing band which had been identified as antimicrobial from bioautography of the crude extract. Recombination of these fractions (10–20% MeOH/EtOAc) yielded 34.7 mg. This material was separated into 14 fractions by HPLC over a LiChrosorb RP-18 column (7 µm, 250 mm × 10 mm) employing 30% H_2O/MeOH (table 2). Hormothamnin A was the major compound (HPLC fraction 13) and was further purified from a chromatographical-

Table 2. Antimicrobial activity of *Hormothamnion enteromorphoides* peptides[a]

HPLC fraction number	Peptide(s) by TLC[b]	*B. subtilis*	*C. albicans*
14	Unknown	c	c
13	Hormothamnin A	7	–
12	Hormothamnin A′	12	11
11	Hormothamnin C,D	9	10
10	Hormothamnin E,F	–	sl
9	Hormothamnin G	10	11
8	Hormothamnin G′	7	7
7	Hormothamnin G″	10	8
6	Hormothamnin H	7	–
5	Hormothamnin I	8	sl
4	Hormothamnin J	9	7
3	Hormothamnin K	9	8
2	Hormothamnin L	9	–
1	Hormothamnin M	13	–

[a] Zones of inhibition given in mm and represent total diameters including 6.5 mm of paper disc containing 10 µg of peptide (read at 20 h, dashes indicate no growth inhibition).
[b] See experimental for TLC conditions and figure 3a for HPLC elution positions. Some hormothamnins were purified by further HPLC before antimicrobial testing.
[c] Unknown substance not tested due to insufficient amounts.

ly-similar minor compound by a second pass on HPLC employing the same conditions (ca. 10 mg, 1% of the crude extract). Additional hormothamnin A was present in related vacuum chromatography fractions.

Culture

Material for culture was collected, as indicated above, from Playa de Luquillo in January 1987 and transported in native seawater at approximately 29 °C to a laboratory at the University of Puerto Rico in Rio Piedras. The cyanobacteria were washed in filter-sterilized native seawater before transfer onto 60- and 150-mm diameter petri dishes containing 1% agar with 0.25 strength Castenholz Marine Medium (CMM)[22] in Instant Ocean (33 g/l). The plates were exposed to unidirectional light of low intensity until return to Oregon. In Oregon, the cultures were kept at constant temperature (28 ± 1 °C) and were exposed to unidirectional light of 250 ft-cd. The phototactic response of *H. enteromorphoides* enabled agar cubes containing single filaments to be cut from the plates. These were used to inoculate 125-ml Erlenmeyer flasks containing 75 ml of 0.25 strength CMM medium in Instant Ocean (33 g/l). The flasks were placed on a rotary shaker operating at 120 rpm and illuminated with 250 ft-cd of cool-white light on a 16/8 h light-dark cycle. The cultures showed healthy growth within a few days and were used for sequential inoculation into bigger flasks of 500 ml (Erlenmeyer) and 2.8 l (Fernbach) capacity. The 2.8-l flasks were grown for 6–8 weeks and then harvested by vacuum filtration through Whatman No. 4 filter paper. The harvested cyanobacteria were weighed and frozen at −20 °C until workup for analytical HPLC.

Analytical HPLC

Frozen samples of the cyanobacteria were thawed at RT and repetitively extracted (3 × 20 min) with boiling $CHCl_3$/MeOH (2:1) at which point there was no further hormothamnin A extracted as judged by TLC (silica gel F_{254}, 25% MeOH/$CHCl_3$) with UV and Cl_2/o-tolidine visualization[24]. The filtered lipid extracts were combined and reduced in vacuo or under a stream of dry N_2 in a warm water bath.

The crude extracts obtained from culture or field collected materials were dissolved in MeOH, applied to a short pipet column of C-18 RP silica gel and the peptides eluted with 1.5 ml of 20% H_2O/MeOH. From model studies with the crude extract obtained from wild collected *H. enteromorphoides*, the full complement of peptides was eluted in this volume. The solvents were removed in vacuo or under a stream of dry N_2 and the crude peptide fraction redissolved in a measured volume of MeOH for anaylsis on HPLC. Analytical HPLC of this mixture of peptides employed a 4.2 mm × 30 cm LiChrosorb RP-18 (5 µm) column and 30% H_2O/MeOH (1.0 ml/min). The eluant was monitored with a refractive index detector and the peptides came off between 7.5 and 38 min, with hormothamnin A eluting near the end at 27–30 min.

Results and discussion

A) Isolation and characterization of hormothamnin A

Emerald green masses of the tropical cyanobacterium, *Hormothamnion enteromorphoides* (fig. 1), were collected from a small bay on the northern coast of Puerto Rico in 1983 and its crude organic extract analyzed for antimicrobial activity, ichthyotoxicity and the presence of novel-appearing compounds by thin layer chromatography. The extract was growth inhibitory to *Bacillus subtilis* and the pathogenic yeast *Candida albicans*, and was qualitatively toxic to fish. Surprisingly, little of potential interest was observed by TLC employing solvent systems of mod-

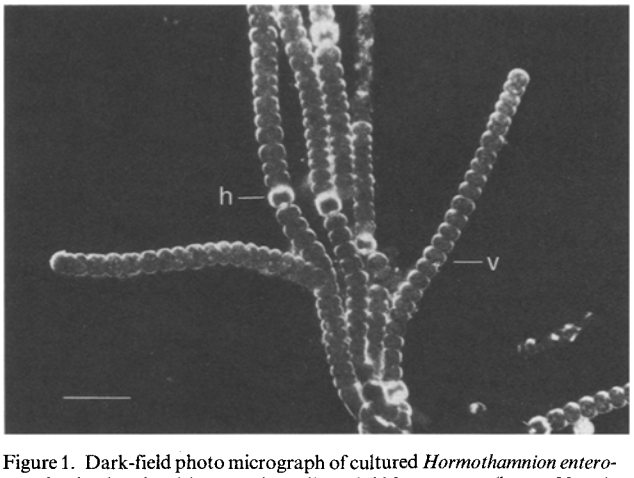

Figure 1. Dark-field photo micrograph of cultured *Hormothamnion enteromorphoides* showing (v) vegetative cells and (h) heterocysts (bar = 30 µm).

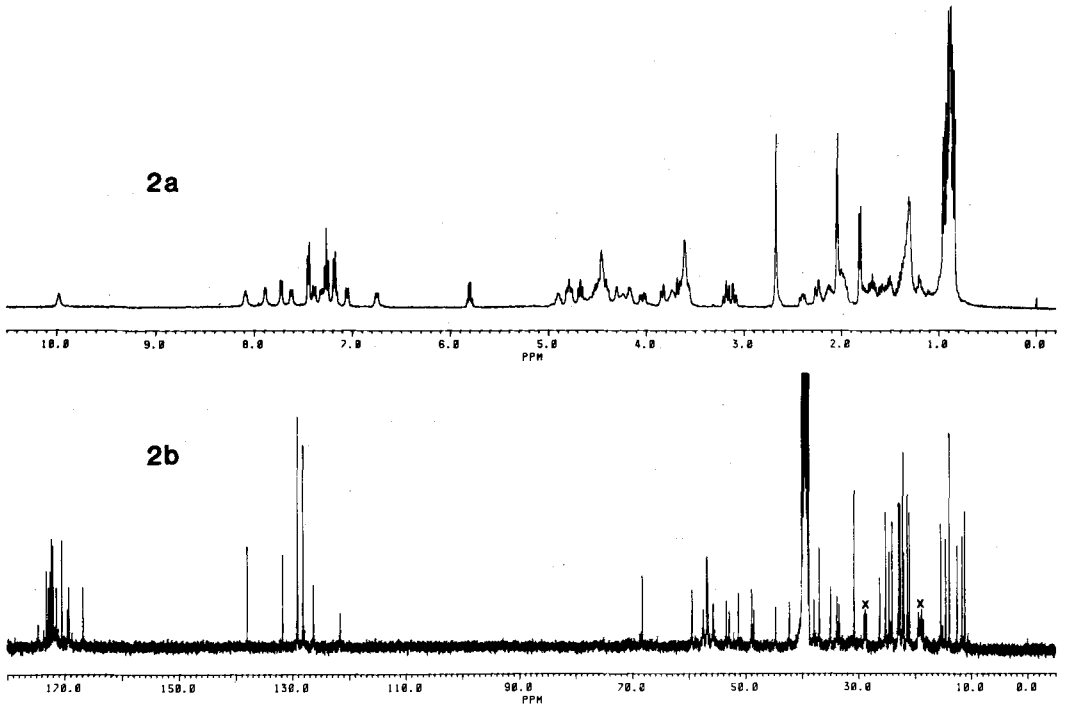

Figure 2. High field nuclear magnetic resonance spectra for hormothamnin A, approximately 25 mg in 0.4 ml d-6-DMSO with 0.1% TMS as internal standard; *a* ^{1}H NMR at 400 MHz and *b* ^{13}C NMR at 100 MHz (× indicates impurities not observed in other preparations of this hormothamnin A).

erate polarity (up to 100% Et_2O). However, by bioautography the strong antimicrobial activity of the extract was localized to the origin even in these moderately polar solvents. Employing even more polar solvent combinations (10–30% MeOH in $CHCl_3$), an UV-absorbing spot which did not char upon acidification and heating was found by bioautography to be responsible for the pronounced antimicrobial activity of the extract.

Vacuum chromatography provided an efficient means for the initial separation of the organic constituents into distinct fractions. Antimicrobial tests and TLC localized both the majority of the antimicrobial activity and the polar UV-absorbing compounds to two fractions which eluted with 10–20% MeOH in EtOAc. These were recombined and repetitively chromatographed employing reversed phase HPLC to give a complex series of related metabolites (table 2, fig. 3). The least polar of the two major compounds in this series, hormothamnin A, was also the purest as judged by HPLC, TLC visualized with Cl_2/o-tolidine[24], and high field ^{1}H NMR after recycling on HPLC.

The pure toxin, hormothamnin A ($[\alpha]_D^{25} = 47.2°$, (c = 1.22, MeOH), displayed prominent absorptions in the IR spectrum for –NH and C=O stretches at wavelengths typical for peptides (ν = 3350, 1700 cm^{-1}) and a UV absorption characteristic of a monosubstituted benzene ring (λ_{max} = 270 nm, ε = 400). By LR and HR FAB MS (+ ion) a prominent $M^+ + H$ was observed at 1196.7303 from which, in concert with high field ^{1}H and ^{13}C NMR data, a molecular formula of $C_{60}H_{97}N_{11}O_{14}$ could be deduced. The 18 degrees of unsaturation inherent in this formula were partially accounted for by 11 carbonyls (from ^{13}C NMR), one aromatic ring and one trisubstituted carbon-carbon double bond. Thus, the peptide contained two additional rings, and since hormothamnin A did not react with ninhydrin or CH_2N_2, it was probably of an overall cyclic structure, a feature consistent with other cyanobacterial peptides[11].

Amino acid analysis of the crude 6N HCl hydrolysis product from hormothamnin A revealed the presence of only seven distinct types of residues (phe, leu, ile, met, gly, hypro and an acidic unknown). However, the high field ^{1}H NMR of the crude acid hydrolysate, when compared with the intact peptide in the same solvent, did not show characteristic vinyl (δ 5.4, 1 H, q, J = 7) and methyl (δ 1.6, 3H, d, J = 7) resonances present in the intact molecule. These resonances were assignable to a dehydro amino acid which decomposed in acid. Furthermore, the acidic unknown was subsequently shown to be identical with homoserine by comparison with authentic material. Two homoserine residues were present in hormothamnin A as indicated by molar ratios (1.2) and a variety of NMR techniques (discussed below).

Other amino acid analysis data were inconsistent with results from ^{1}H and ^{13}C NMR data, including COSY, HETCOR and INEPT experiments, which did not support the presence of a methionine residue. This anomaly was explored by first oxidizing hormothamnin A with HCO_3H and then hydrolyzing the peptide with 6N HCl. Amino acid analysis of this mixture revealed that the

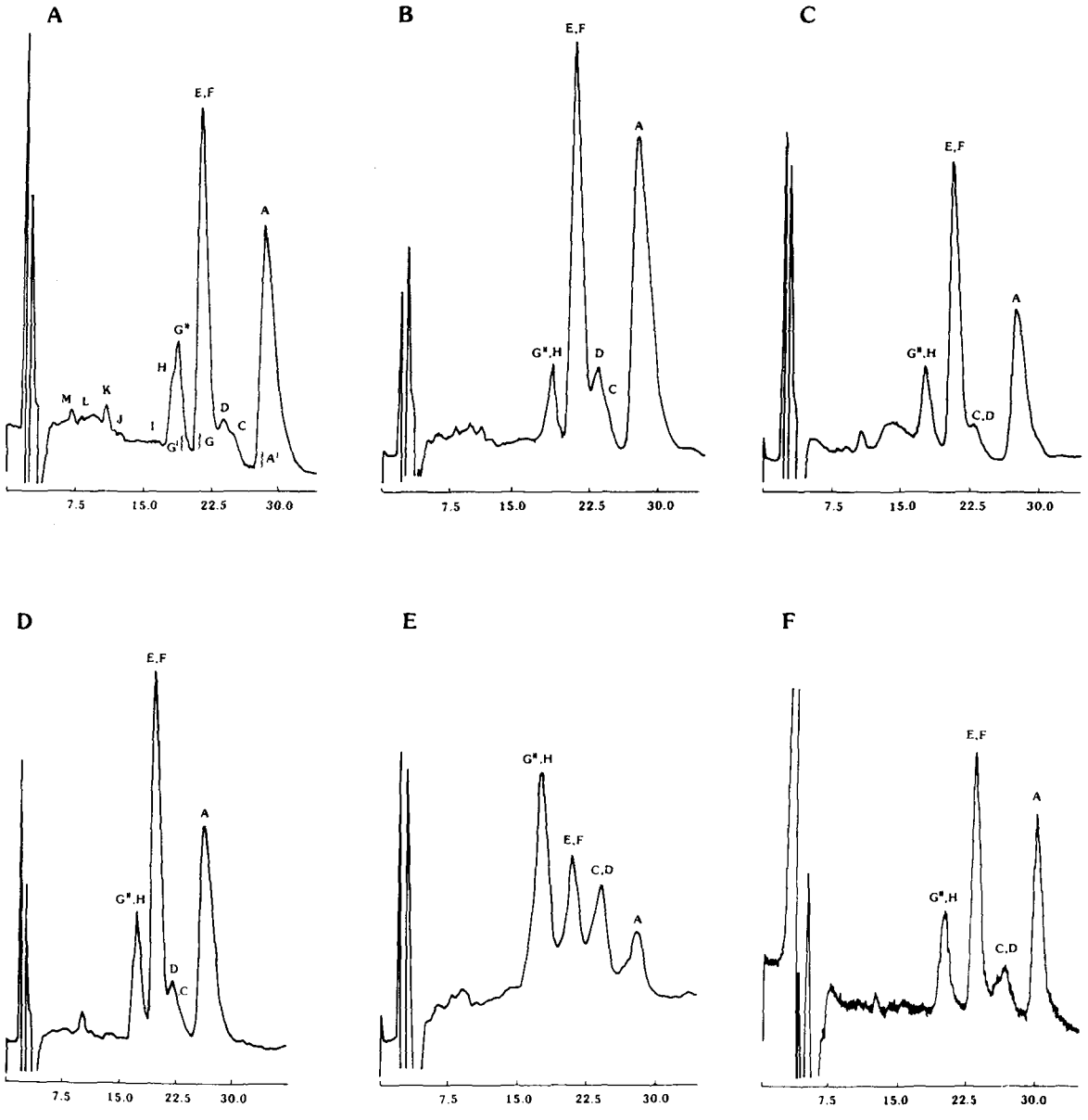

Figure 3. Hormothamnin peptide composition from different collections of *Hormothamnion enteromorphoides* by reversed phase high performance liquid chromatography (RP-HPLC) using refractive index detection (see experimental for details); samples obtained from *A* Vega Baja, Puerto Rico (Atlantic), 30 June 1986, *B* La Parguera, Puerto Rico (Caribbean), 29 June 1986, *C* Vega Baja, 17 March 1985, *D* Vega Baja, 26 March 1986, *E* Vega Baja, 3 January 1986, and *F* cultured *H. enteromorphoides* harvested November 1987.

'met' peak remained unchanged, and was therefore a second 'unknown' residue. The molar ratio of 2.3 for the leu peak was also anomalous as only 2 leu residues were suggested by four characteristic methyl group absorption in the ^{13}C NMR of the intact peptide (δ 22.92, 22.70, 21.47 and 21.09). Thus, this peak represented 2 leu residues and another 'unknown' residue with a leu-like retention time. Further, at least one of the leu residues was probably in linkage with a second aliphatic residue to explain this decreased molar ratio. In conclusion, these data were interpreted to indicate that hormothamnin A contains an acid-unstable dehydro amino acid, 1 phe, 2 leu, 1 ile, 2 homoser, one unknown with a met-like retention and a second with a leu-like retention, 1 gly, and 1 hydroxyproline. Thus, hormothamnin A is a cyclic undecapeptide consisting of 6 standard amino acid residues and 5 uncommon or unknown residues.

The high field ^1H NMR spectrum (fig. 2a) for hormothamnin A was exceedingly complex with considerable band overlap in the regions δ 0.7–2 and 3–5. Even with 2D techniques for the careful visualization of coupling interactions (COSY, relay COSY, and 2DJ experiments), there was too much signal degeneracy to permit complete interpretation. The ^1H NMR shifts of 1 phe, 1 gly and 1 leu residue were fully assignable from COSY data alone. The relay COSY experiment made it possible to

assign the ^{1}H NMR shifts for the hypro and 2 homoser residues. A vinyl proton vicinally coupled to a vinyl methyl group along with an unusual amide NH proton at δ 10.69 were assigned on the basis of comparison to model compounds to a 2,3-dehydro-homoala residue[23]. These ^{1}H-based experiments were unable, however, to clearly separate and make identifiable the ^{1}H NMR shift for 1 leu, 1 ile and the 2 unknown aliphatic residues.

While the ^{13}C NMR broad band proton decoupled (fig. 2b), DEPT and ^{1}H-^{13}C HETCOR were also extremely complex, they did provide some insight into the nature of the new residues and assignments for the known ones. The carbon assignments for the dehydro homoala residue were readily determined from the HETCOR experiment ($-CH_3$ 12.4; $=C_BH$ 121.7; $=C_\delta$ 131.8) and provided additional support for this partial structure. A total of 10 methyl groups was observed at high field, 7 of which were ascribable to the known residues enumerated above. Thus, three aliphatic methyl groups could be assigned to the two unknown residues. Collectively, these two residues contain 14 carbon atoms, 26 hydrogen atoms, 2 nitrogen atoms and 2 oxygen atoms. Further investigation of the structures of these two residues by NMR (TOCSY, DQF COSY) and synthesis are underway.

B) Biological characterization of hormothamnin A

Hormothamnin A was weakly antimicrobial to the gram positive bacterium *Bacillus subtilis* and the gram negative bacterium *Pseudomonas aeruginosa* (table 1). Further, it was found to be highly cytotoxic to a variety of cancer cell lines in tissue culture. Interestingly, pure hormothamnin A was inactive as a toxin to brine shrimp. Hormothamnin A is moderately ichthyotoxic ($LD_{50} \sim 5$ µg/ml to the goldfish *Carassius carassius*) and may thus explain the apparent absence of predation upon *H. enteromorphoides* by herbivorous reef fish.

C) Peptide analysis of cultured and native H. enteromorphoides

Thin layer chromatograms of the crude lipid extract from cultured *H. enteromorphoides* were compared with those obtained from wild material collected at the same time as the original source inoculum for culture, as well as with several other collections of wild *H. enteromorphoides*. A good spread of the hormothamnins was obtained with 25% MeOH in $CHCl_3$ and visualization was achieved through the combination of UV and Cl_2/o-tolidine detection. In all collections, the hormothamnins were major secondary metabolites of this cyanobacterium, however, the relative proportions of these peptides varied (see below).

High performance liquid chromatography of a peptide-enriched fraction from cultured and wild *H. enteromorphoides* provided a qualitative and semi-quantitative picture of peptide production in these samples (fig. 3, A–F). Notably, collections from nearly the same date but from different water masses (Caribbean and Atlantic, fig. 3, A and B) as well as from the same season and location but in subsequent years (1985 and 1986, fig. 3, C and D) showed a very consistent production of the hormothamnins. However, HPLC comparison of collections from different seasons from the same location (fig. 3, D and E) show a variation in the proportion of polar hormothamnins (G, H). Laboratory cultured *H. enteromorphoides* gave a HPLC profile of peptides (fig. 3F) remarkably similar to that obtained from wild collected material (fig. 3, A–D). Collection and HR ^{1}H NMR analysis of peak 'A' from this culture-derived material confirmed its identity as hormothamnin A. On a quantitative basis, the production of these cytotoxic peptides was reduced in culture (culture: approx. 1% of lipid extract; wild: approx. 8% of lipid extract).

Conclusion

Hormothamnion enteromorphoides is a relatively common cyanobacterium in the tropics which produces a complex mixture of peptide secondary metabolites with a variety of biological activities. Production of these peptides, although seasonally variable, appears to be quite consistent between different collections. In nature, these peptides may function to deter predation by herbivorous animals, including fish, zooplankton and molluscs. In the laboratory the major peptide, hormothamnin A, shows antimicrobial activity against two human pathogenic microorganisms and toxicity to cancer cells in vitro. Other hormothamnins display both antibacterial and antifungal activity (table 2).

Hormothamnin A is an undecapeptide and contains 1 phe, 2 leu, 1 ile, 1 gly, 1 hypro, 2 homoser, 1 dehydro-homoala and 2 undefined aliphatic residues. As only 17 of the 18 degrees of unsaturation inherent in the molecular formula of hormothamnin A can be accounted for from olefins, ketones and rings inherent in the above amino acid structures, it must be of an overall cyclic structure. A combination of field and laboratory isolation procedures were employed to obtain single filaments of *Hormothamnion enteromorphoides* from agar plates. Flasks containing liquid media were inoculated with these isolates and grown for up to 3 months on a rotary shaker. By both TLC methodology with peptide-specific stains and analytical reverse phase HPLC with refractive index detection, it was shown that a full complement of peptides was produced in culture. While smaller yields of these peptides are obtained in culture compared to wild material, the relative ratios of the peptides were similar between these two sources.

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Ultrastructural localisation of substance P and choline acetyltransferase in endothelial cells of rat coronary artery and release of substance P and acetylcholine during hypoxia

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Summary. Substance P and choline acetyltransferase have been localised in a small proportion of endothelial cells of rat coronary arteries using electron microscopic immunocytochemistry. During a hypoxic period of 1 min, coronary vasodilatation was produced in the Langendorff heart preparation and increased levels of substance P and acetylcholine were released into the perfusate. The possibility that these substances are released from endothelial cells during hypoxia and contribute to the hyperaemic response is discussed.
Key words. Heart; hypoxia; endothelial cells; substance P; acetylcholine; ultrastructure.

Introduction

It has been proposed that acetylcholine (ACh), substance P and many other neurohormonal substances act via receptors on endothelial cells causing release of endothelium-derived relaxing factor (EDRF) which acts on the underlying smooth muscle to cause vasodilatation[1–3]. Evidence has now been presented to suggest that EDRF