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TOTAL STRUCTURE OF HORMOTHAMNIN A, A TOXIC CYCLIC UNDECAPEPTIDE FROM THE TROPICAL MARINE CYANOBACTERIUM *HORMOTHAMNION ENTEROMORPHOIDES*

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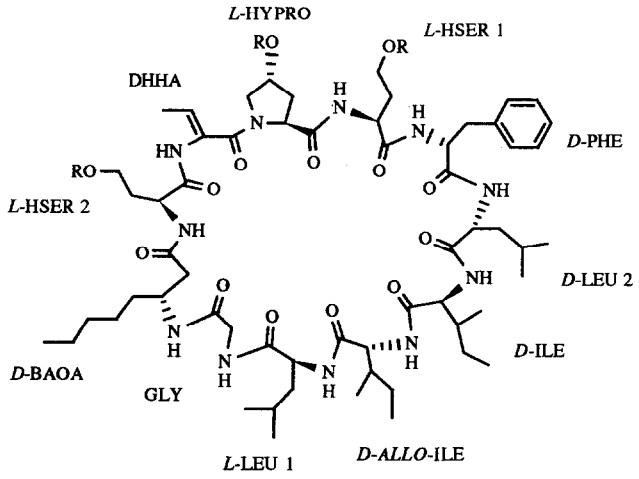
Abstract - The tropical marine cyanobacterium *Hormothamnion enteromorphoides* produces a suite of cytotoxic and antimicrobial cyclic peptides. The structure of the most lipophilic of these, hormothamnin A, was determined by interpretation of physical data, principally high field NMR and FAB MS, in combination with chemical derivitization and degradation schemes. Isolation of a key pentapeptide fragment *D*-PHE-*D*-LEU-*L*-ILE-*D-allo*-ILE-*L*-LEU, obtained under partial hydrolysis conditions, was instrumental to the final structure determination. β-*D*-aminooctanoic acid (*D*-BAOA) was characterized as a per-ester derivative following complete acid hydrolysis and Z-didehydrohomoalanine (DHHA) was spectroscopically described in the intact peptide. The remaining residues (HYPRO, 2 x HSER, GLY) were evident from amino acid and spectroscopic analysis. Sequencing of these residues made use of knowledge from fragments, high field NMR (NOESY and ROESY) and FAB MS analysis of the intact peptide. Absolute stereochemistries of the α-amino residues were determined by HPLC analysis of the acid liberated residues derivatized with Marfey's reagent. The absolute stereochemistry of the β-amino residue was shown by circular dichroism analysis, HPLC analysis of the Marfey derivative, and chiral synthesis of a homolog. By these techniques, hormothamnin A was demonstrated to possess a cyclo-[*D*-PHE-*D*-LEU-*L*-ILE-*D-allo*-ILE-*L*-LEU-GLY-*D*-BAOA-*L*-HSER-DHHA-*L*-HYPRO-*L*-HSER] structure.

Introduction

Cyclic peptides are a chemical class well recognized from bacteria and fungi,[1] and appear to represent a common chemical theme in the cyanobacteria (blue-green algae) as well.[2] Water soluble cyclic peptides have been isolated as the active hepatotoxins from the freshwater cyanobacteria *Microcystis aeruginosa*, *M. toxica*,[3] *Oscillatoria agardhii*[4] and *Nodularia spumigena*,[5] and are responsible for numerous intoxications of fish and livestock.[6,7] Several toxic and cyclic peptides of a lipophilic nature have also been isolated from two freshwater and one marine cyanobacteria (*Scytonema pseudohofmanni*,[8] an *Anabaena sp.*,[9] and *Lyngbya majuscula*[10]). An unusual structural motif common to all of these peptides is the occurrence of one β-amino acid with a large and structurally unique side chain. A range of biological activities has been reported for these cyanobacterial peptides, including insecticidal[10] and weak anticancer activity for majusculamide C,[11] weak antimicrobial but strong calcium antagonist activity for the scytonemins,[8] and positive inotropic activity for puwainaphycin.[9]



Our investigations of the cytotoxic and antimicrobial peptide chemistry of the Caribbean cyanobacterium, *Hormothamnion enteromorphoides* Grunow, have occurred over a period of several years and made use of both field collected and cultured material.[12] *H. enteromorphoides* is an exclusively marine cyanobacterium which grows abundantly in the shallow coastal waters off Northern Puerto Rico, although it has an overall pantropical distribution.[13] The distinctive emerald green color and slimy clump-like morphology assist in its field identification and collection by skin diving techniques. From this prokaryote we have detected a complex series of peptide natural products which were initially discovered as a result of bioassay guided fractionation efforts following the pronounced gram + antimicrobial activity observed in the crude lipid extract. Vacuum chromatography of this lipid extract and RP HPLC of the polar peptide containing fractions proved to be the most expeditious method for their isolation.[12] The complete structure elucidation of the most lipophilic of these, hormothamnin A (**1**), which is also the most abundant, has been a challenging problem and we report here the results of these efforts.[14]

Results and Discussion

Pure hormothamnin A (**1**), a colorless material drying to a white non-crystalline powder, was optically active and showed intense C=O and N-H absorptions at ν = 1700 and 3350 cm^{-1} typical of peptide-type natural products. It possessed an uncharacteristic UV spectrum with maxima at 240 and 270 nm (ϵ = 9500 and 400), absorptions later ascribed in concert with other data to an α,β-unsaturated amide and a mono-substituted aromatic ring, respectively. Hormothamnin A analyzed for $C_{60}H_{98}N_{11}O_{14}$ by HR FABMS [obs. (M+H)+ at 1196.7303], yielding 18° of unsaturation. From the ^{13}C NMR and molecular formula data, which showed there to be 11 amide-type carbonyls, hormothamnin A was likely an undecapeptide with a number of unsaturated and cyclic amino acid residues. Further, hormothamnin A was shown to possess three esterifiable functionalities as it

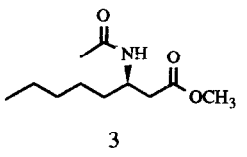
formed triacetate **2** upon acetylation.

Additional analysis of the ^{13}C NMR spectrum of **1** revealed resonances for a tri-substituted olefin (δ 131.8, 121.7) and a mono-substituted aromatic ring. The identity of this aromatic residue as PHE was conclusively given by amino acid analysis. Further, amino acid analysis showed the presence of one HYPRO residue as well, and thus, explained all but one of the degrees of unsaturation implicit in the molecular formula. Hormothamnin A also contained two HSER, one GLY, one ILE, one *allo*-ILE, and two LEU residues by amino acid analysis. Derivatization of these residues obtained from the crude hydrolysate with Marfey's reagent[15] and gradient HPLC analysis with co-injection of standards defined the absolute stereochemistry of PHE as *D*, HYPRO as *L*, both HSER's as *L*, and one LEU as *D* and the other as *L*. Because of overlap in the HPLC chromatogram, it was not possible at this point to distinguish whether hormothamnin A possessed *D*-ILE and *L-allo*-ILE or *L*-ILE and *D-allo*-ILE.

By ^{1}H and ^{13}C NMR analyses, a tenth residue was identified as didehydrohomoalanine (DHHA), and contained distinctive signals and spin systems characteristic for this unsaturated system (see table 1).[16] By estimation of the three bond coupling between the vinyl proton and carbonyl carbon in this residue,[17] and NOE experiments, the geometry of the tri-substituted olefin was shown to be *Z*. A gated decoupled ^{13}C NMR spectrum of **1** gave a complex yet narrow signal for the DHHA carbonyl at δ 166.7. The complexity of this signal is understandable in terms of the five protons to which it probably shows 3-bond couplings. The narrowness of this signal ($\Delta h_{1/2}$ = 14 Hz) implies the absence of any coupling greater than 10 Hz, which necessarily excludes the possibility of a *trans* disposed proton.[17] More conclusively, NOEDS showed bidirectional NOE's between the NH and vinyl methyl (NH to CH_3 18%, CH_3 to NH 2%) as well as significant NOE between the NH and α-proton of the adjacent HSER (30%). It is interesting to note that the DHHA residue in **1** is of the opposite geometry compared to the same residue found in another cyanobacterium cyclic peptide, scytonemin A.[8]

Hence, the eleventh and final residue was required to possess an atomic formula of $C_8H_{15}N_1O_1$ with the oxygen and nitrogen atoms being present as components of amide bonds. Importantly, this composition required the final residue to possess an aliphatic side chain, and hence, given the degrees of unsaturation in the molecule, the overall structure of hormothamnin A was required to be cyclic. This was consistent with both its lack of reactivity to CH_2N_2 in Et_2O/MeOH and that it only formed a triacetate (**2**) upon acetylation. However, due to the highly congested nature of the spectrum in the 0.9 to 1.3 ppm region in **1** or **2**, it was not possible to map out the spin system of this eleventh residue in the intact peptide.

The identity of the eleventh residue was unequivocally shown through its isolation from the crude acid hydrolysate by repetitive HPLC as a protected acetate, methyl ester derivative (**3**). LR EIMS of **3** gave a $(M)^+$ peak at *m/z* 215 indicative of a formula of $C_{11}H_{21}N_1O_3$, and which showed prominent fragmentation ions at *m/z* 172 $(M - O{=}C\text{-}CH_3)^+$, 144 $(M - CH_2CH_2CH_2CH_2CH_3)^+$, and 102 $(M - O{=}C\text{-}CH_3, CH_2CH_2CH_2CH_2CH_3)^+$. By ^{13}C NMR, all 11 atoms were easily seen and further supported the existence of carbomethoxy-ester and N-acetyl groupings. It was noteworthy that the carbon bearing the nitrogen atom was at higher field than that typical for



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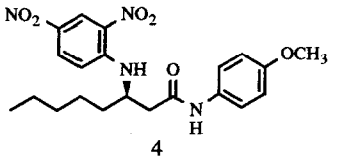

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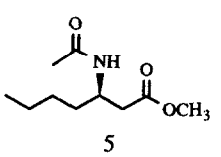

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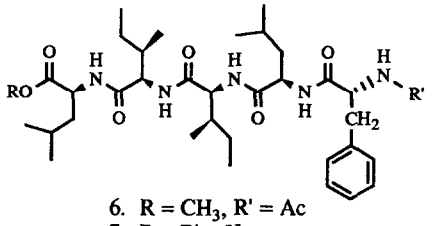
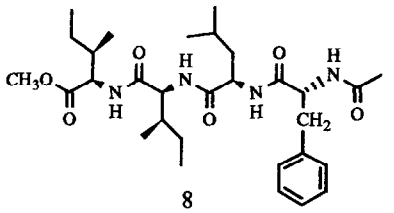


6. R = CH_3, R' = Ac
7. R = R' = H



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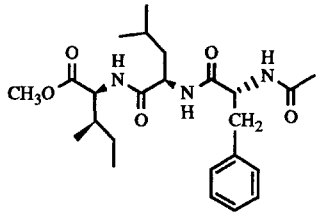

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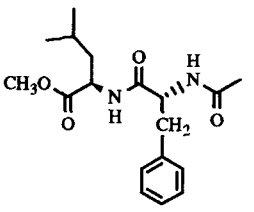

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α-amino acids (δ 44.8).[18] The ^{1}H-^{1}H COSY of this pure derivative was highly descriptive and showed that the amide proton was coupled to a complex methine proton which was in turn coupled to two different methylenes. One of these was isolated and at a shift (δ 2.53) fully consistent with its placement adjacent to the carbomethoxy-ester, thus defining it as a β-amino acid. The other methylene was at higher field (δ 1.50) and further coupled to a broad peak at δ1.25 which contained 6 protons. This 6 proton multiplet was in turn only further coupled to a high field triplet methyl group at δ 0.89. Hence, this last residue was characterized as β-amino octanoic acid.

The absolute stereochemistry of this residue was shown in three ways. Racemic synthesis of 3-aminooctanoic acid[19] followed by derivatization with Marfey's reagent and HPLC analysis gave two peaks. The later eluting of these coincided by co-injection with a late eluting peak in the chromatogram formed by analysis of similarly derivatized hormothamnin A hydrolysate. Hence, by analogy to the behavior of α-amino acids derivatized with Marfey's reagent in which the *L* series elutes earlier than the *D* series,[15] the hormothamnin A derived β-amino octanoic acid was shown to be of *D* configuration. This was further confirmed by hydrolysis of the acetate and carbomethoxy esters from derivative **3** with 6N HCl and re-derivatization to form the N-(2',4'-dinitrophenyl)-*p*-methoxyanalide (**4**).[20] In its circular dichroism spectrum derivative **4** gave a positive 267 nm Cotton effect which decreased to negative numbers at 400 nm, a spectrum highly indicative of aliphatic β-*D*-amino acids.[20] Finally, methyl-3R-aminoheptanoate acetate (**5**) was synthesized from 2*R*-aminohexanoate (*D*-norleucine)

using adaptations of literature methods[21] and its rotational characteristics compared with methyl-3-aminooctanoate acetate (3) obtained from hormothamnin A (1). Compounds 3 and 5 showed comparable positive rotations at 589 nm.

A partial sequencing of residues in hormothamnin A was obtained by analysis of the FAB MS fragmentation pattern. As expected, the peptide bond to each side of the α,β unsaturated residue (DHHA) preferentially opens with subsequent fragmentation pathways deriving by both clockwise and counterclockwise successive residue losses (figure 1). The combination of these four easily discerned fragmentation pathways yields the following sequence of residue masses beginning with DHHA: [83-113-101-147-113-113-113-113-57-141-101]. These could be interpreted to give the sequence [DHHA-113-HSER-PHE-113-113-113-113-GLY-BAOA-HSER] wherein 'mass equal to 113' residues could be either HYPRO, LEU, ILE or *allo*-ILE.

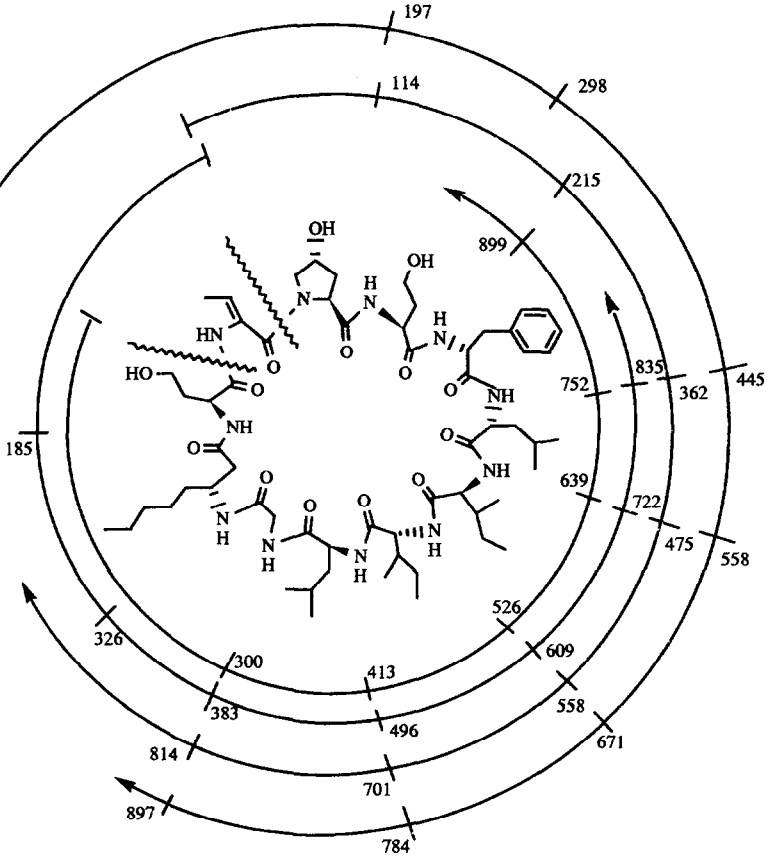

Figure 1. Positive ion fast atom bombardment mass spectral (FAB MS) fragmentation pathways obtained for hormothamnin A (1) giving partial sequence information.

However, the primary positioning of these eleven amino acids in hormothamnin A utilized data obtained from NOESY and ROESY experiments performed at high field. In turn, this required a full assignment of the ^{13}C and ^{1}H NMR spectra for hormothamnin A which was accomplished by a combination of ^{1}H-^{1}H DQFCOSY and TOCSY, ^{13}C DEPT, ^{1}H-^{13}C LR HETCOSY, HMQC, and HMBC experiments (Table 1).

Table 1. High field (500 MHz) ^1H, ^{13}C, and ^{15}N NMR data for hormothamnin A (**1**).

C#	$^1H(\delta)$	$^{13}C(\delta)$	$^{15}N(\delta)$
L-HYPRO			
C-1	-	b	
C-2	4.482	59.6	
C-3	2.252	38.0	
	1.835		
C-4	4.299	68.4	
C-5	3.318	57.6	
	3.609		
OH	5.191		
N	-		c
L-HSER 1			
C-1	-	b	
C-2	4.311	48.7	
C-3	2.015	33.9	
	1.913		
C-4	3.271	56.9	
	3.416		
OH	4.394		
NH	7.039		107.1
D-PHE			
C-1	-	b	
C-2	4.234	56.8	
C-3	2.960	37.0	
	3.037		
C-4	-	138.0	
C-5	7.208	129.1	
C-6	7.265	128.2	
C-7	7.392	126.3	
C-8	7.265	128.2	
C-9	7.208	129.1	
NH	7.650		111.5
D-LEU 2			
C-1	-	b	
C-2	4.321	51.4	
C-3	1.034	39.5	
	1.283		
C-4	1.573	24.7	
C-5	0.751	21.1	
C-6	0.806	22.8	
NH	7.225		111.7
L-ILE			
C-1	-	b	
C-2	4.761	55.8	
C-3	1.805	39.4	
C-4(Me)	0.750	15.4	
C-5	1.262	22.0	
	1.246		
C-6	0.750	11.6	
NH	6.491		c
D-allo-ILE			
C-1	-	b	
C-2	4.684	53.6	
C-3	2.068	37.1	
C-4(Me)	0.810	14.5	
C-5	1.121	26.3	
	1.195		
C-6	0.836	11.1	
NH	8.437		112.6
L-LEU 1			
C-1	-	b	
C-2	4.021	53.1	
C-3	1.365	39.2	
	1.561		
C-4	1.630	24.1	
C-5	0.919	22.6	
C-6	0.851	21.4	
NH	8.486		118.1
GLY			
C-1	-	b	
C-2	3.260	42.3	
	3.815		
NH	8.840		109.8
D-BAOA			
C-1	-	b	
C-2	1.683	39.9	
	1.901		
C-3	4.283	44.8	
C-4	1.350	35.1	
C-5	1.249	30.8	
C-6	b	b	
C-7	b	b	
C-8	0.89	13.9	
NH	6.878		121.6
L-HSER 2			
C-1	-	b	
C-2	4.702	49.0	
C-3	1.799	33.4	
C-4	3.555	56.9	
OH	4.590		
NH	7.093		118.9
DHHA			
C-1	-	166.7	
C-2	-	131.8	
C-3	5.765	121.7	
C-4	1.754	12.5	
NH	10.691		132.1

[a]Spectra obtained in DMSO-*d*-6 at 25°C.
[b]Unassigned carbonyls at δ 174.6, 173.1, 172.8, 172.5, 172.2, 172.0, 171.3, 170.4, 169.5, 169.2.
[c]Not detected.

Two NOESY (50 and 100 ms) experiments and one ROESY (50 ms) experiment were run on hormothamnin A in DMSO-d_6 at 500 MHz and 24°C. While all of these spectra gave a wealth of information on the structure of **1**, the ROESY was most descriptive of the sequence of residues in the molecule. As seen in Table 2, a series of unidirectional NH-αH interactions were observed that gave the primary connectivities. Further, a ROE interaction was observed between the NH of DHHA and the δ protons of the hydroxyproline residue, thus closing the macrocyclic ring. Through the series of adjacent leucine, isoleucine and phenylalanine residues found in **1**, NH-NH ROE interactions were also observed, indicative of additional secondary structure in this region. In addition to the expected ROE interactions between the NH of HSER2 and the β protons of the BAOA residue, interactions were also detected between the NH of LEU2 and the β protons of the *D*-PHE residue.

The remaining structural questions, the locations of the *D* and *L* leucine and normal and *allo*-isoleucine

residues, required chemical degradation coupled with amino acid analysis. Principally, this effort utilized a key pentapeptide fragment which was obtained in relatively good yield from acid hydrolysis of hormothamnin A (**1**) and subsequent acetylation and methylation. This pentapeptide derivative (**6**) results from hydrolysis of the HSER-PHE and LEU-GLY linkages with the intervening linkages between hydrophobic residues being more resistent. The HPLC purified fragment (**6**) was completely characterized by various spectroscopic methods and showed in its FAB-MS a strong series of sequence ions which confirmed the relative positions of PHE and LEU/ILE/*allo*-ILE residues (see experimental).

Table 2. Connectivities observed from a 50 ms ROESY spectrum.

NH	αH	NH	βH
DHHA	HSER 2		
HSER 2	--		BAOA
BAOA	GLY		
GLY	LEU 1		
LEU 1	*allo*-ILE		
allo-ILE	ILE		
ILE	LEU 2	*allo*-ILE, LEU 2	
LEU 2	PHE	PHE	PHE
PHE	HSER 1		

The location of ILE and *allo*-ILE residues in hormothamnin A (**1**) was shown in two ways, 1) comparison by HPLC of the amino acids remaining following two or three cycles of Edman degradation of the pentapeptide **6**, and 2) amino acid analysis of three additional peptide fragments isolated (**8-10**) from partial acid hydrolysis of hormothamnin A (**1**).

The pentapeptide derivative **6** was deprotected by acid treatment to yield *D*-PHE-LEU-(ILE-*allo*-ILE)-LEU (**7**) which was then deposited on an immobilon filter and partially sequenced on an automated gas phase protein sequencer. Two Edman degradation cycles were run with normal programming and the eluents discarded. The filter was removed and subjected to 6N HCl hydrolysis conditions and the resulting amino acids were analyzed by standard HPLC analysis with post-column ninhydrin detection. This showed principally three residues, LEU, ILE and *allo*-ILE. Another sample of deprotected pentapeptide **7** was adhered to a second filter and this time subjected to three cycles of Edman degradation. Again, the filter was removed and amino acids liberated by 6N HCl treatment. HPLC analysis of this gave principally LEU and *allo*-ILE, although a small amount of ILE still remained. This finding supported a PHE-LEU-ILE-*allo*-ILE-LEU sequence which was confirmed by the following. HPLC of the peptides resulting from partial hydrolysis of hormothamnin A gave tripeptide **8** which analyzed by traditional amino acid analysis for PHE, LEU and ILE, and tetrapeptide **9** which analyzed for PHE, LEU, ILE and *allo*-ILE. The structures of **8** and **9** were confirmed by ^{1}H NMR and FAB MS analyses. Complete acid hydrolysis of peptides **8** and **9** followed by derivatization with Marfey's reagent and gradient HPLC analysis gave for **8** *D*-PHE, *D*-LEU and *L*-ILE while **9** analyzed for *D*-PHE, *D*-LEU, *L*-ILE, and *D-allo*-ILE. Implicit in this data set is the answer to the second question, that is, that *D*-LEU is adjacent to *D*-PHE, and hence, the *L*-LEU must reside between the *D-allo*-ILE and GLY residues. The location of *D*- and *L*-LEU residues in hormothamnin A (**1**) was additionally confirmed by HPLC isolation of a small amount of the dipeptide

PHE-LEU (**10**) from partial hydrolysis of hormothamnin A. Complete acid hydrolysis of this fragment followed by derivatization with Marfey's reagent and HPLC analysis gave only *D*-PHE and *D*-LEU. Hence, the complete structure of key pentapeptide fragment **7** obtained from hormothamnin A (**1**) was determined to be *D*-PHE-*D*-LEU-*L*-ILE-*D-allo*-ILE-*L*-LEU.

Incorporation of these stereochemical features into the planar structure of hormothamnin A (**1**) yields its complete structure as cyclo-[*D*-PHE-*D*-LEU-*L*-ILE-*D-allo*-ILE-*L*-LEU-GLY-*D*-BAOA-*L*-HSER-DHHA-*L*-HYPRO-*L*-HSER].[22] It is interesting to note the segregation of hydrophobic and hydrophilic residues in this novel cyclic peptide. The location of the dehydro-amino acid residue (DHHA), which is sandwiched between the three hydrophiiic residues in the molecule, may be important to the cytotoxic, antimicrobial and possible chemical defense properties of hormothamnin A (**1**).[12,16] A closely related cyclic peptide, laxaphycin A, possessing the same overall constituitive structure as hormothamnin A but differing in several stereochemical features, has recently been obtained from a terrestrial isolate of the related blue-green alga *Anabaena laxa* (R.E. Moore, U. Hawaii, personal communication). By direct comparisons, laxaphycin A was shown to minimally differ from hormothamnin A in the stereochemistry of the DHHA unit [laxaphycin A (see table 1 for hormothamnin A) ^{1}H NMR (DMSO-*d*-6) δ 10.88, 5.60, 1.71; ^{13}C NMR δ 166.8, 130.7, 119.3, 13.9; NOE irrad. NH (δ10.88), 17% enhancement of H3 (δ5.60); NOE irrad. H3, 6% enhancement of NH].

Experimental

General Instrumentation. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AM 400 and AC 300, and Varian VXR 500 spectrometers. All shifts are reported relative to an internal tetramethylsilane (TMS) standard. Mass spectra were recorded on Kratos MS 50TC and Finnigan 4023 mass spectrometers. Ultraviolet spectra were recorded on a Beckman DB-GT UV-VIS spectrophotometer and infrared spectra on Nicolet 5 DXB FT 15 and Nicolet 510 spectrophotometers. High performance liquid chromatography (HPLC) was performed with Waters M-6000 and M-45 pumps, U6K injectors, and either a R401 differential refractometer or a Waters lambda-Max 480 lc spectrophotometer. Amino acid analyses were performed at the Protein Structure Laboratory at U. California, Davis on a Beckman 6300 with detection at 440 nm and 570 nm. Partial peptide sequence data were obtained using an Applied Microsystem 475 Gas Phase Protein Sequencer followed by HPLC detection using a Beckman System Gold (0.3 mL/min) with post-column ninhydrin detection. Merck aluminum-backed thin-layer chromatography (TLC) sheets were used for TLC, and all solvents were distilled prior to use.

Collection, extraction and isolation of hormothamnin A (1). Small attached tufts of *Hormothamnion enteromorphoides* were collected from -1 to -3 meters at Vega Baja on the North Coast of Puerto Rico in 1986 and 1987 and stored in IPA until workup. Peptides were extracted by repetitive steeping in $CHCl_3$/MeOH (2:1) and purified by vacuum chromatography followed by repetitive reverse phase HPLC as previously detailed.[12] Pure hormothamnin A (**1**) showed spectral characteristics as previously reported[12] and as found in table 1; amino acid analysis (concentration in nMoles, retention time) HYPRO (1.50, 12.55 min), HSER (not calc., 19.95 min), GLY (1.77, 30.30 min), *allo*-ILE (ca. 1.28, 49.48 min), ILE (1.24, 50.62 min), LEU (3.55, 51.58 min), PHE (1.64, 56.96 min); LR FAB MS (relative intensity) *m/z* 1218.7 (89), 1198.7 (15), 1197.7 (35.0), 1196.7 (52.7), 1174.5 (4.4), 970 (1.3), 899.5 (1.3), 897.5 (1.3), 857.4 (1.4), 835.5 (1.3), 814.4 (1.4), 784.4 (2), 752.4 (1.5), 722.4 (1.6), 701.4 (2.7), 671.3 (2.3), 639.4 (2.3), 609.3 (2.7), 588.3 (5.4), 558.3 (3.9), 526.3 (2.9), 496.3 (4), 475.2 (7.3), 445.2 (5.4), 413.2 (3.1), 383.2 (9.8), 362.1 (22), 326.2 (4.7), 300.2 (10), 298.1 (6), 282.1 (6.9), 261.1 (9.5), 227.1 (18), 215.1 (31), 199.1 (34), 197.1 (17), 195.1 (10), 187 (10), 185 (16), 182 (17), 169 (21), 167 (12), 157 (12), 154 (37), 152 (13), 140(22), 138 (21), 136 (36), 131 (17), 125 (33), 120 (92), 114 (31).

Partial acid hydrolysis of hormothamnin A (1). Hormothamnin A (66.7 mg) was dissolved in one drop of methanol and then 10 ml of 2 N HCl was added. After refluxing for 24 hours, the solution was neutralized by the addition of saturated $NaHCO_3$ (pH 9.5) and evaporated *in vacuo* to give a salty white powder. The powder was acetylated (Ac_2O/pyridine, 1:1) with stirring overnight at room temperature. The acetylation was stopped by pouring the reaction products into acidified saturated NaCl (pH 2). The solution was then extracted with $CHCl_3$ (2x). The $CHCl_3$ layer was washed with acidic NaCl solution and evaporated in vacuum to yield a residue. The residue was methylated twice with CH_2N_2 in ether to give 42 mg of material which was then separated by HPLC [Lichrosorb RP-18 (7μm) 250 x 10 mm, 60% MeOH/H_2O)] to yield seven fractions. The materials retained in the column were eluted with 100% MeOH. Fraction 6 was further purified [Versapack Silica 10 μ, 2 x (300 x 4.1 mm), 50% EtOAc/hexanes] resulting in pure methyl 3-D-acetamidooctanoate (**3**, 0.8 mg). Fraction 7 contained the derivatized dipeptide **10**. The materials eluting with 100% methanol were further separated by HPLC (linear gradient of CH_3CN in H_2O, 40% to 70% in 40 minutes, 1.5 ml/min, Phenomenex Ultracarb RP-ODS (20), 30 cm x 4.1 mm) to yield the derivatized tripeptide (**9**) and tetrapeptide (**8**).

Hormothamnin A triacetate (2). ^{1}H NMR δ (300 MHz, $CDCL_3$): 9.4 (1H, D_2O exch.), 7.65 (1H, D_2O exch.), 7.32 (2 H, bd, J = 6 Hz), 7.24 (2H, t, J = 6 Hz), 7.19 (1H, m), 6.88 (1H, D_2O exch.), 6.6 (1H, D_2O exch.), 5.52 (1H, m), 5.26 (1H, m), 4.69 (1H, m), 4.60 (1H, t, J = 5 Hz), 4.50 (2H, m), 4.35 (4H, m), 4.0 (2H, m), 3.87 (2H, m), 3.7 (2H, m), 3.18 (1H, bd, J = 12 Hz), 2.98 (1H, bt, J = 12 Hz), 2.57 (1H, m), 2.46 (1H, m), 2.33 (5H, m), 2.07 (3H, s), 2.06 (3H, s), 1.99 (3H, s), 1.90 (2H, m), 1.80 (3H, d, J = 7), 1.1 - 1.7 (22H, m), 0.7 - 1.05 (27H, m).

Methyl 3-D-acetamidooctanoate (3). Derivative **3** showed $[\alpha]_D$ = + 20.2° (c = 0.08, MeOH); ^{1}H NMR δ (300 MHz, $CDCL_3$): 6.02 (1H, m), 4.24 (1H, m), 3.69 (3H, s), 2.53 (2H, m), 1.97 (3H, s), 1.50 (2H, m), 1.28 (6H, m), 0.89 (3H, t, J = 7.8 Hz); ^{13}C NMR δ (75 MHz, $CDCl_3$): 172.5, 169.5, 51.6, 45.9, 38.1, 34.0, 31.5, 27.2, 25.9, 23.5, 22.5, 14.0; EIMS (rel. intensity %): 216 (M + H^+, 3), 215 (M^+,3), 184 (7), 172 (26), 144 (38), 102 (100), 70 (14), 60 (28), 43 (66); *N-(2',4'-dinitrophenyl)-p-methoxyanalide (4):* Derivative **3** was hydrolyzed in 6 N HCl by refluxing for 6 hours and the solution was passed through a small RP-18 column. After thoroughly washing with distilled water, the column was eluted with 100% MeOH. The MeOH eluent was evaporated to give the free amino acid which was derivatized directly according to Nagai et al.[20] to produce the N-(2',4'-dinitrophenyl)-p-methoxyanalidederivative (**4**, CD (MeOH), $[\theta]_{267}$ +3900, $[\theta]_{400}$ -1200).

Derivatization of amino acids with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) and HPLC analysis. For the FDAA (Marfey's reagent) derivatization procedure (Pierce Chemical Company), a small amount of sample in 100 μl of acetone was mixed with 200 μl of a 1% solution of FDAA in acetone. To this was added 40 μl of 1.0 M sodium bicarbonate solution, and the resultant solution was heated at 40°C for one hour and then allowed to cool. After addition of 20 μl of 2 M HCl, the resulting solution was degassed and then analyzed by HPLC. The HPLC analysis used the following conditions: solvent A, 0.05 M Et_3N in H_3PO_4 at pH 3; solvent B, acetonitrile; linear gradient with flow rate of A + B at 1 ml/minute, solvent B from 10% to 60% in 2 hours; column, Phenomenex Ultracarb RP(20), 30 cm x 4.1 mm; UV detector at 340 nm with 0.10 AUFS.

Synthesis of $\pm$ 3-amino octanoic acid. Malonic acid (12.5 g) was dissolved in 28 ml of dried pyridine and then 12.5 ml of hexanal and 1.2 ml of distilled pyrrolidine were added. After refluxing for 30 minutes, the products were poured into 300 ml of chilled dilute HCl solution. The solution was extracted with ether (3 x 100 ml) and the ether layer was washed with distilled water, dried with Na_2SO_4, and evaporated to give 12.9 g of octa-2(*E*)-enoic acid. The acid (0.26 g) was methylated with CH_2N_2 in ether and then mixed with 0.31 g of phthalimide in 10 ml of dried pyridine. The mixture was refluxed for 24 hours in the presence of EtONa catalyst. After cooling to room temperature, the reaction was poured into ice water and the resultant solution extracted with $CHCl_3$ (2 x 20 ml). The extract was fractionated by vacuum silica gel column chromatography to yield 124 mg of methyl 3-phthalimidooctanoate. ^{1}H NMR δ ($CDCl_3$, 300 MHz): 7.70 (2H, m), 7.6 (2H, m), 4.59 (1H, m), 3.53 (3H, s), 3.10 (1H, ddd, J = 1.8, 9.8, 16.0 Hz), 2.72 (1H, ddd, J = 1.5, 5.4, 16.0), 2.0 (1H, m), 1.66 (1H, m), 1.19 (6H, m), 0.77 (3H, m). ^{13}C NMR δ ($CDCl_3$, 75 MHz): 171.3, 168.2, 133.8, 131.6, 123.1, 51.6, 47.9, 36.6, 32.1, 31.1, 25.8, 22.2, 13.8. The methyl 3-phthalimidooctanoate product was hydrolyzed (6 N NaOH,

100°C, 2 h) and the resultant solution acidified and passed through a pipet column containing reverse phase silica gel (ODS). The product was eluted from the ODS column with MeOH, the MeOH evaporated and then redissolved in acetone, to give 3-amino octanoic acid contaminated by a small amount of phthalic acid: ^{1}H NMR δ ($(CD_3)_2CO$, 300 MHz): 4.4 (1H, m), 2.75 (1H, dd, J = 17, 6 Hz), 2.55 (1H, dd, J = 17, 9 Hz), 1.65 (2H, m), 1.5 (2H, m), 1.35 (4H, m), 0.9 (3H, t, J = 6). This 3-amino octanoic acid was reacted directly with FDAA for HPLC analysis.

Synthesis of methyl 3-D-acetamidoheptanoate (5). *D*-norleucine (1 g) and N-ethoxycarbonylphthalimide (1.7 g) were dissolved in 25 ml of tetrahydrofuran and 2 ml of triethylamine and then refluxed for 24 hr. The reaction solution was filtered and the solvents removed *in vacuo* to give a residue which was dissolved in 30 ml of $CHCl_3$. The $CHCl_3$ solution was extracted with 10% Na_2CO_3 (3 x 30 ml). The Na_2CO_3 solution was then acidified to pH 2 with 6 N HCl and then extracted with $CHCl_3$ (3 x 50 ml). The $CHCl_3$ layer was washed with water and then evaporated to give pure N-phthaloyl D-norleucine (1.63 g). N-phthaloyl D-norleucine (0.4 g) was mixed with 1 ml of thionyl chloride and refluxed for 2 hr. Excess thionyl chloride was removed by evaporation and the residue was dissolved in anhydrous ether and then slowly dropped into an excess of diazomethane (Et_2O-MeOH) in an ice bath. After the addition was completed, the reaction was allowed to warm to room temperature for 1 h and then excess reagent and solvents were removed *in vacuo* to give a residue (0.4 g) which contained about 85% 1-diazo-3-phthalimidoheptan-2-one and 15% 1-chloro-3-phthalimidoheptan-2-one by ^{1}H NMR analysis. The mixture (0.13 g) in 100 ml of dioxane was treated with 100 ml of aqueous Ag_2O-$Na_2S_2O_3$ (pH ca. 7.5) prepared by dissolving Ag_2O (formed by precipitation of 6.0 g of $AgNO_3$ with 1.7 g of NaOH in H_2O) and $Na_2S_2O_3$ (12 g) in 100 ml dioxane. The solution was refluxed (1 h), acidified to pH 2, extracted with ether (3 x 150 ml), dried over Na_2SO_4, concentrated *in vacuo*, methylated (CH_2N_2 in ether), and purified by silica gel chromatography to give methyl 3-*D*-phthalimidoheptanoate (57 mg). This 3-*D*-phthalimidoheptanoate was refluxed in 6 N HCl (8 h), concentrated *in vacuo*, and then methylated (CH_2N_2) and acetylated (Ac_2O/pyridine, 1:1, overnight). The acetylation was quenched with the addition of methanol, diluted with 20 ml of $CHCl_3$, washed with 15% HOAc (aq), and the chloroform layer was evaporated to give an oily material which was further purified by HPLC [Lichrosorb RP-18 (7 μm), 250 x 10 mm, 60% MeOH in H_2O] to yield 7.5 mg of methyl 3-*D*-acetamidoheptanoate. ^{1}H NMR δ ($CDCl_3$, 300 MHz): 6.04 (1H, br. d, J = 8.9 Hz), 4.21 (1H, m), 3.69 (3H, s), 2.58 (1H, dd, J = 5.0, 16.0 Hz), 2.50 (1H, dd, J = 5.0, 16.0 Hz), 1.98 (3H, s), 1.51 (2H, m), 1.30 (4H, m), 0.88 (3H, t, J = 6.7 Hz); ^{13}C NMR δ ($CDCl_3$, 75 MHz): 172.4, 169.4, 51.6, 45.9, 38.1, 33.7, 28.3, 23.4, 22.3, 13.9; FAB MS m/z (rel. intensity): 224 ($M+Na^+$, 70), 202 (MH^+, 100), 176 (32), 170 (81), 160 (40), 128 (72), 111 (49), 102 (44), 86 (72), 60 (32); $[\alpha]_D$ = +9.0° (c = 0.5, MeOH).

Formation and isolation of derivatized pentapeptide 6: Hormothamnin A (21 mg) was dissolved in one drop of methanol and then 0.5 ml of 20% trifluoroacetic acid was added. The reaction tube was sealed and heated in a 100° C oven for one hour. The reaction solution was repetitively extracted with $CHCl_3$ and the solvent evaporated *in vacuo* to give a residue which was methylated by (CH_2N_2 in ether) and acetylated (Ac_2O/pyridine, 1:1, 24 hr). Excess Ac_2O and pyridine were evaporated *in vacuo* and the resultant material purified by HPLC (Lichrosorb RP-18 (7μm) RP-18, 250 x 10 mm, 80% MeOH/H_2O) to yield derivatized pentapeptide **6** (ca. 2 mg) which showed the following: ^{1}H NMR δ (300 MHz, MeOH-d_4): 7.25 (5H, m), 4.60 (1H, dd J = 4.7, 9.6 Hz), 4.4 (3H, m), 4.24 (1H, d, J = 6.7 Hz), 3.66 (3H, s), 3.13 (1H, dd, J = 4.8, 14.0 Hz), 2.87 (1H, dd, J = 9.7, 13.9 Hz), 1.0 - 2.0 (12H, m), 1.88 (3H, s), 0.8 - 1.0 (24H, m); ^{13}C NMR δ (75 MHz, MeOH-d_4): 174.9, 174.4, 174.0, 173.9, 173.8, 173.4, 138.5, 130.2, 129.5, 127.8, 59.8, 58.0, 56.1, 53.5, 52.6, 52.2, 41.5, 41.2, 38.6, 38.0, 37.5, 27.4, 25.9, 23.4, 23.3, 22.4, 22.1, 21.8, 16.1, 15.0, 11.9, 11.6; FAB MS (rel. intensity %): 674(MH^+, 23), 529 (15), 485 (3), 416 (16), 372 (4), 259 (8), 190 (4), 162 (8), 120 (19), 86 (100); high resolution FAB MS: obs. M^+ at *m/z* 674.4493 gives $C_{36}H_{60}N_5O_7$ (0.1 mmu deviation).

Formation and sequencing of pentapeptide 7. Pentapeptide **6** (1 mg) was dissolved in 100 μl of MeOH and then 120 μl of trifluoroacetic acid and 380 μl of water were added. The solution was sealed in a vial and heated for 1 hr at 100°C with periodic shaking. The solution was evaporated *in vacuo* and the residue purified by HPLC [Partisil PXS ODS (5 μm), 250 x 5 mm, 80% MeOH/H_2O] to give the deprotected pentapeptide **7**: FAB MS (% rel. intensity): 640 ($M+Na^+$, 100), 618 ($M+H^+$, 39), 519 (19),

487 (19), 485 (67), 471 (15), 374 (13), 329 (25), 261 (27), 245 (13), 227 (25). Sequencing of the peptide was carried out on an immobilon membrane filter using an automated gas phase protein sequencer. After two cycles of Edman degradation, the immobilon membrane was hydrolyzed in 6N HCl at 110°C for 20 hours. Amino acid analysis of this acid hydrolysate showed 98 pmol of LEU, 58 pmol of ILE and 20 pmol of *allo*-ILE. The same experiment was repeated but with 3 cycles of Edman degradation and the resultant hydrolysate was found to contain 58 pmol of LEU, 25 pmol of ILE and 28 pmol of *allo*-ILE.

Derivatized tetrapeptide ***8***. ^{1}H NMR δ (300 MHz, MeOH-d_4): 7.2 (5H, m), 4.59 (1H, dd, J = 5.6, 11.9 Hz), 4.50 (1H, d, J = 5.6 Hz), 4.40 (1H, t, J = 7.7 Hz), 4.35 (1H, d, J = 7.7 Hz), 3.63 (3H, s), 3.1 (1H, m), 2.82 (1H, dd, J = 10.5, 14.0 Hz), 1.0 - 2.0 (9H, m), 1.88 (3H, s), 1.0 - 2.0 (18H, m); FAB MS (% rel. intensity): 561 (MH^+, 37), 416 (21), 303 (26), 258 (20), 255 (22), 176 (10), 146 (14). Standard amino acid analysis indicated one molar equivalent each of PHE, LEU, ILE and *allo*-ILE. HPLC analysis of the FDAA derivatives from acid hydrolysis showed four residues co-eluted with FDAA derivative standards of *D*-PHE, *D*-LEU, *L*-ILE and *D-allo*-ILE.

Derivatized tripeptide ***9***: ^{1}H NMR δ (300 MHz, MeOH-d_4): 7.35 (5H, m), 4.6 (1H, m), 4.48 (1H, m), 4.35 (1H, m), 3.70 (3H, s), 3.1 (1H, m), 2.82 (1H, dd, J = 8.4, 12.3 Hz), 1.0 - 2.0 (6H, m), 1.89 (3H, s), 0.8 - 1.0 (12H, m); FAB MS (% rel. intensity): 448 (MH^+, 44), 433 (16), 303 (10), 259 (26), 255 (100), 176 (30), 146 (16). Standard amino acid analysis indicated one molar equivalent each of PHE, LEU and ILE. HPLC analysis of the FDAA derivatives obtained from acid hydrolysis gave *D*-PHE, *D*-LEU and *L*-ILE.

Derivatized dipeptide ***10***. ^{1}H NMR δ (300 MHz, $CDCl_3$): 7.3 (5H, m), 6.08 (1H, br.d, J =7.6 Hz), 6.2 (1H, br. d, J = 7.4 Hz), 4.65 (1H, m), 4.51 (1H, m), 3.70 (3H, s), 3.07 (2H, m), 1.98 (3H, s), 1.5 (3H, m), 0.89 (6H, d, J = 5.9 Hz); FAB MS (% rel. intensity): 335 ($[M + H]^+$, 50), 277 (74), 255 (100), 176 (16), 146 (40). HPLC analysis of the FDAA derivatives obtained from acid hydrolysis gave *D*-PHE and *D*-LEU.

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