Cicadapeptins I and II: New Aib-Containing Peptides from the Entomopathogenic Fungus Cordyceps h eteropoda

ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · FEBRUARY 2005

Impact Factor: 3.8 · DOI: 10.1021/np0497189 · Source: PubMed

Cicadapeptins I and II: New Aib-Containing Peptides from the Entomopathogenic Fungus *Cordyceps heteropoda*

Stuart B. Krasnoff,*,† Ricardo F. Reátegui,‡ Melissa M. Wagenaar,‡,§ James B. Gloer,‡ and Donna M. Gibson⊥

Department of Plant Pathology, Cornell University, Ithaca, New York 14853, Department of Chemistry, University of Iowa, Iowa City, Iowa 52242, and USDA-ARS-Plant Protection Research Unit, Ithaca, New York 14853

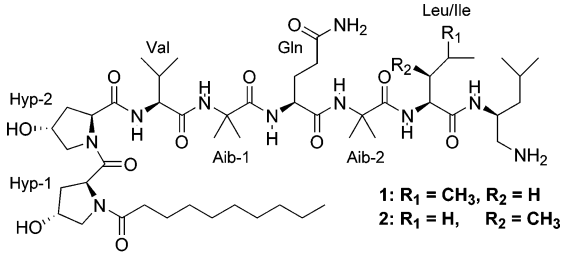
Received August 27, 2004

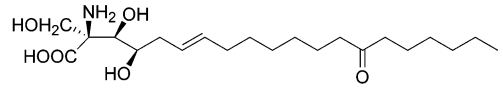
Fermentation extracts of *Cordyceps heteropoda* (ARSEF #1880), an entomopathogenic fungus isolated from an Australian cicada, yielded a known antifungal compound, myriocin, and a complex microheterogeneous family of novel nonribosomal peptides, each containing two residues of α-aminoisobutyric acid (Aib). Structure elucidation of two major components of the peptide mixture, cicadapeptins I and II (**1** and **2**), was accomplished by amino acid analysis and various MS, 1-D NMR, and 2-D NMR experiments. Both compounds are acylated at the N-terminus by *n*-decanoic acid and amidated at the C-terminus by 1,2-diamino-4-methylpentane. The amino acid sequence of cicadapeptin I is N-terminus-Hyp-Hyp-Val-Aib-Gln-Aib-Leu-C-terminus. Ile substitutes for Leu in cicadapeptin II. To our knowledge, this is the first report from fungi of consecutive Hyp or Pro residues in a nonribosomal linear peptide. ROESY data indicated that the cicadapeptins adopt a helical conformation. Cicadapeptins I and II displayed antibacterial activity and limited antifungal activity.

As part of an ongoing search for biologically active secondary metabolites from entomopathogenic fungi,[1−4] we detected antifungal and antibacterial activity in fermentation extracts of a fungus that was isolated from an Australian cicada, *Cicadetta puer* (Homoptera: Cicadidae) and accessioned by the USDA-Agricultural Research Service Collection of Entomopathogenic Fungi as *Cordyceps heteropoda* (ARSEF #1880; Clavicipitaceae). In our hands, this fungus grew only in the conidial state. Entomopathogenic *Cordyceps* anamorphs in the genera *Beauveria*, *Metarhizium*, *Paecilomyces (Isaria)*, and *Tolypocladium* produce a variety of biologically active peptides, including the immunosuppressant cyclosporins,[5] the mitochondrial ATPase-inhibitory efrapeptins[2] and leucinostatins,[6] and the cytotoxic peptaibols LP237-F5, -F7, and -F8.[7] Nonproteinogenic α,α-dialkylated amino acids, the most common of which is α-aminoisobutyric acid (Aib), occur in the efrapeptins, the leucinostatins, and the LP237 peptaibols. Here we report the isolation and identification of the antibacterial metabolites from ARSEF #1880 as a group of novel linear heptapeptides containing two Aib residues and present details of the structural elucidation by NMR and MS methods of two of these peptides, which we have named cicadapeptins I (**1**) and II (**2**). In addition, we report a known compound, myriocin (**3**), as the fungicidal principle.

Results and Discussion

Dichloromethane extracts of culture broth and mycelia from fermentation of ARSEF #1880 demonstrated antifungal and antibacterial activity. Silica gel fractionation of active culture extracts afforded separate antifungal and antibacterial fractions. Analysis of the antibacterial fraction by reversed-phase HPLC revealed four major components, I−IV, as well as an abundance of minor peaks. Preparative HPLC separation of these components, followed by ESIMS and ^{1}H NMR analysis, suggested a complex mixture of related peptides, each containing at least two residues of Aib. Components III and IV each appeared to be mixtures of at least two peptide analogues, while components I and II were purified sufficiently to support their identification as **1** and **2**, respectively, following detailed analysis of MS and NMR data for the natural products and their hydrolysis products. The antifungal fraction yielded a white precipitate, which, upon recrystallization from MeOH, afforded the known compound myriocin (**3**).





3

HRESIMS data were consistent with the molecular formula $C_{50}H_{90}N_{10}O_{11}$ for both **1** and **2**. ^{13}C and DEPT NMR spectra of each compound confirmed the presence of 50 carbons, including nine ester or amide carbonyls, two upfield quaternary carbons, 11 methyls, 17 methylenes, and 11 methines. Hydrolysis, followed by amino acid analysis of **1**, indicated the presence of seven amino acid units: two Hyp, two Aib, one Gln/Glu, one Leu, and one Val. Results for **2** were identical except that Leu was replaced by Ile. Low-resolution ESIMS data for **1** and **2** were virtually identical, showing pseudomolecular $[M + H]^+$ and $[M + Na]^+$ ions at *m/z* 1007 and 1029, respectively, as well as MS-MS peaks consistent with peptidal N-terminal A and B fragment ions[8] at *m/z* 891(B_7), 778 (B_6), 693 (B_5), 565 (B_4), 480 (B_3), 452 (A_3), 381(B_2) and 268 (B_1),

* Corresponding author. Tel: (607) 255-2026. Fax: (607) 255-1132. E-mail: sbk1@cornell.edu.
† Cornell University.
‡ University of Iowa.
§ Current address: Wyeth Research, 401 N. Middletown Rd., Bldg 205/422, Pearl River, NY 10965.
⊥ USDA-ARS-PPRU at Ithaca.

10.1021/np0497189 CCC: $30.25
Published on Web 12/30/2004

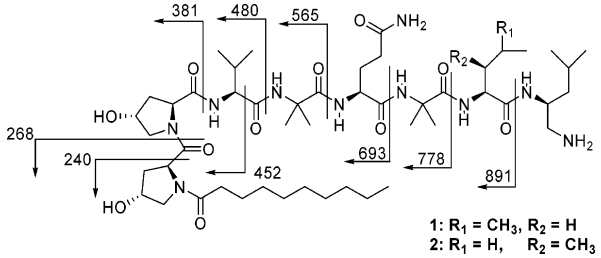


Figure 1. ESIMS-MS fragmentation of cicadapeptins.

and 240 (A_1) (Figure 1). Following deuterium exchange (in CD_3OD or CH_3OD), pseudomolecular ions were observed at *m/z* 1020 $[M + D]^+$ and 1041 $[M + Na]^+$, and fragments were observed at *m/z* 900 (B_7 with nine D), 786 (B_6 with eight D), 700 (B_5 with seven D), 569 (B_4 with four D), 483 (B_3 with three D), 455 (A_3 with three D), 383 (B_2 with two D), 269 (B_1 with one D), and 241 (A_1 with one D). These data indicated a peptide with an N-terminal unit of 268 Da containing one exchangeable proton, followed in order by amino acids with internal residual masses of 113, 99, 85, 128, 85, and 113 Da (each containing one exchangeable proton except for *m/z* 128, which contained three), and a C-terminal unit of mass 115 Da containing three exchangeable protons. This narrowed the possible peptide sequences to N-terminal unit-(Hyp or Leu/Ile)-Val-Aib-Gln-Aib-(Hyp or Leu/Ile)-C-terminal unit.

The N-terminal blocking group for both compounds was isolated from the hydrolyzate and identified by GC-EIMS as *n*-decanoic (capric) acid by comparison of its GC retention time and EI mass spectrum with those of an authentic standard. Subtracting the mass of the decanoyl moiety (155 Da) from the N-terminal acylium ion fragment representing the blocking group plus one amino acid residue (268 daltons) left 113 Da, which indicated either Ile/Leu or Hyp as the first amino acid. Thus, at this stage, the positions of the Ile/Leu and the two Hyp units, as well as the structure of the C-terminal blocking group, remained to be determined.

A core sequence, consisting of the four residues Val-Aib-Gln-Aib, was assigned on the basis of the MS and amino acid analysis data. This substructure provided numerous points of entry for analyzing 2-D NMR spectral data in combination with ^1H, ^{13}C, and DEPT NMR data, to enable deduction of the complete peptide sequence, up to the C-terminal unit, of both **1** and **2**. The ^1H NMR spectra of **1** and **2** showed the presence in the amide region of two singlets and four doublets. The amide singlets in both **1** and **2** were consistent with the presence of two Aib residues in each compound, while three of the doublets were assigned to the Val, Gln, and Leu/Ile units on the basis of analysis of COSY experiments and confirmed by analysis of TOCSY, HMBC, and HMQC spectra. The fourth amide proton in **1** and **2** remained unassigned, although dqCOSY, HMBC, and HMQC spectra indicated that this amide proton is vicinal to a putative α-proton, whose carbon resonates somewhat upfield of the range for a Leu α-carbon in CD_3OH,[9,10] and is attached to an isobutyl group.

The two Aib carbonyl carbon signals in **1** were identified by HMBC correlations to their respective sets of β-methyl singlet proton signals (Table 1). The Aib amide proton singlets each showed three-bond correlations with the corresponding Aib-unit carbonyl, as well as two-bond correlations with the carbonyls of their acylating units (Gln and Val). The carbonyl of the Aib acylating the Gln was identified by a correlation to the Gln amide proton. Thus, the sequence Val-Aib-Gln-Aib was verified, and the two Aibs could be assigned as Aib-1 (the unit acylated by Val) and Aib-2 (the unit acylated by Gln). The carbonyl carbon signal for Leu in **1** was identified by observation of an HMBC correlation with the Leu α-proton. Further HMBC correlations between the Aib-2 carbonyl carbon and the amide proton of Leu, and between the carbonyl carbon of Leu and the unassigned (C-terminal unit) amide doublet, extended the sequence to Val-Aib-Gln-Aib-Leu-C-terminus in **1**. An analogous set of correlations was observed for **2**, leading to assignment of the same sequence with Ile in place of Leu.

Since the residual mass of the C-terminal unit is 115 Da and it also contains an amide proton, it could not contain Hyp, and thus the Hyp units were constrained to the first and second positions, establishing the sequence of **1** as *n*-decanoyl-Hyp-Hyp-Val-Aib-Gln-Aib-Leu-C-terminal and the analogous sequence for **2** with Ile in place of Leu.

The signals for the Hyp residues were assigned by analysis of 2-D NMR spectra, which readily revealed the spin systems of the pyrrolidine rings. For example, in **1** HMBC correlations between a Hyp carbonyl (δ_C 176.7) and the amide and α-protons of Val as well as with the intraresidual α-proton at δ 4.53 identified the Hyp residue (Hyp-2) that acylates the Val unit, and thus also permitted assignment of the Hyp residue (Hyp-1) acylated by the N-terminal *n*-decanoyl unit. A virtually identical set of correlations supported analogous assignments for **2**. ROESY correlations between the δ-protons of Hyp-1 and Hyp-2, and between the δ-protons of Hyp-2 and the Val amide proton, as well as the observation of all possible sequential NH−NH correlations for both **1** and **2** also supported the deduced sequences and assignments (Tables 1 and 2).

Once the above units had been assigned, the HRESIMS and NMR data indicated that the remaining C-terminal unit in both **1** and **2** must have the molecular formula $C_6H_{15}N_2$ and must contain three exchangeable protons. As noted above, analysis of 2-D NMR data in **1** indicated that this unit contains the remaining amide doublet NH linked to a methine that bears a downfield-shifted proton (δ 4.29) and is connected to an isobutyl group. Only one additional methylene unit (diastereotopic doublets of doublets at δ_H 3.08 and 3.14 with δ_C 44.6) and two exchangeable protons remained to be assigned. The latter must be combined with the final nitrogen atom to form a primary amine. The two diastereotopic methylene proton signals showed COSY correlations to the methine proton at δ 4.29, and the shifts of these signals were consistent with attachment of this methylene to the primary amine group. HMBC, dqCOSY, TOCSY, and HMQC data were fully consistent with the presence of this unit in **2** as well as **1**. On the basis of these results, the C-terminal blocking group was established as a 1,2-diamino-4-methylpentane unit, which is acylated by the neighboring Leu residue in **1** (or Ile in **2**). The chemical shifts for the C-terminal unit in **1** and **2** closely match those reported for C-terminal hydrolysis products of the efrapeptins in which an isobutyl group is similarly attached to the carbon that is positioned alpha both to the last amide nitrogen and to a methylene carbon that is, in turn, bonded to another nitrogen atom[2] (albeit the pyrimido nitrogen in a 1,5-diazabicyclo[4.3.0]non-5-ene ring system, rather than a primary amino nitrogen).

Efforts were made to isolate the corresponding free 1,2-diamine from the peptide hydrolyzates by making the aqueous solutions basic and extracting directly with deuterated solvent, followed by NMR analysis to minimize the potential for sample loss due to volatility. However, analy-

Table 1. NMR Spectral Data for Cicadapeptin I

residue	position	δ_H[a] (*J* in Hz) [$-\Delta\delta/\Delta T$, ppb/K]	δ_C[b]	HMBC[c]	dqCOSY	ROESY[d,e]
n-decanoyl	C10	0.88 (t, 7.1)	14.6		H_2-9	
	C9	1.30 (m)[f]	23.9	H_3-10	H_3-10	
	C8	1.28 (m)[f]	33.2	H_3-10		
	C7	1.31 (m)[g]	30.9[h]			
	C6	1.31 (m)[g]	30.8[h]			
	C5	1.31 (m)[g]	30.6[h]			
	C4	1.40 (m)	30.6[h]	H_2-2	H_2-3	
	C3	1.65 (m)	26.0	H_2-2	H_2-4	
	C2	2.48 (t, 7.3)	35.1		H_2-3	H_2-3, H_2-4, Hyp1-$\delta_{a,b}$, Hyp2-δ_b
	C=O		176.0	H_2-2, Hyp1-α, δ_a		
Hyp-1	α	4.65 (dd, 11.3, 7.4)	62.6	β_a, γ, δ_a	$\beta_{a,b}$	$\beta_{a,}$**$_b$**, Hyp2-δ_b, Aib1-β2
	β_a (*pro*-S)	2.17 (m)	37.2	α, δ_a	α, β_b, γ	α, δ_b, Hyp2-δ_b,
	β_b (*pro*-R)	2.32 (m)			α, β_a, γ	α, γ
	γ	4.58 (m)	71.0	β_b, δ_b	$\beta_{a,b}$, $\delta_{a,b}$	$\beta_{a,b}$, $\delta_{a,}$**$_b$**
	δ_a (*pro*-R)	3.70 (m)	57.1	γ	δ_b, γ	$H_2$2
	δ_b (*pro*-S)	3.82 (m)			δ_a, γ	β_a, Hyp2-δ_b, $H_2$2
	C=O		173.2	α, β_a, Hyp2-α, δ_a		
Hyp-2	α	4.53 (m) (dd, 11.3, 7.2)	63.5	β_a, δ_b	$\beta_{a,b}$	$\beta_{a,}$**$_b$**, δ_b, Val-NH, Gln-NH
	β_a (*pro*-S)	1.92 (m)	38.6	γ, δ_b	α, β_b, γ	δ_a, Val-NH
	β_b (*pro*-R)	2.36 (m)			α, β_a, γ	α, γ
	γ	4.52 (m)	71.8	β_b, δ_b	$\beta_{a,b}$, $\delta_{a,\ b}$	$\beta_{a,b}$, $\delta_{a,}$**$_b$**
	δ_a (*pro*-S)	3.49 (m)	56.4		δ_b, γ	β_a, γ, $H_2$2, Val-NH
	δ_b (*pro*-R)	3.53 (m)			δ_a, γ	Hyp1-α, β_a, δ_b, Aib2-β2
	C=O		176.7	α, β_a, Val-NH, α		
Val	NH	7.51 (d, 7.7) [0.0]			α	α, β, γ1, Hyp2-α, β_a,γ,δ_a, Aib1-NH
	α	3.62 (dd, 10.3, 7.7)	64.6	NH, β, γ1, γ2	NH, β	NH, α, β, γ1,2 Aib1-NH, Aib2-NH
	β	2.26 (m)	30.8	NH, α, γ1, γ2	α, γ1, 2	NH, Aib1-NH
	γ1 (*pro*-R)	0.97 (d, 6.7)	19.7	α, β, γ2	β	α, Aib1-NH
	γ2 (*pro*-S)	1.02 (d, 6.6)	20.9	α, γ1	β	NH, α
	C=O		174.7	α, Aib1-NH		
Aib-1	NH	7.76 (s) [2.6]				β2, Val-NH, α, β, γ1, Gln-NH
	α		57.5	NH, β1, 2		
	β1 (*pro*-R)	1.47 (s)	23.3	β2		
	β2 (*pro*-S)	1.51 (s)	27.0	NH, β1		NH, Hyp1-α, Gln-NH
	C=O		178.6	NH, β1, 2, Gln-α, NH		
Gln	NH	8.16 (d, 4.1) [2.0]			α	α, β_b, $\gamma_{a,\ b}$, Hyp2-γ, Aib1-NH, Aib2-NH, Leu-NH
	α	3.82 (m)	59.0	NH, $\gamma_{a,b}$	NH, $\beta_{a,b}$	NH, α, β_b, γ_a, Cterm-NH, NH_2
	β_a	2.05 (m)	27.7	NH, $\gamma_{a,b}$	α, β_b, $\gamma_{a,b}$	
	β_b	2.12 (m)			α, β_a, $\gamma_{a,b}$	NH, α, $\epsilon_{\alpha,b}$
	γ_a	2.32 (m)	32.5	α, $\beta_{a,b}$, $\epsilon_{\ a}$	γ_b, $\beta_{a,b}$	α, $\epsilon_{\alpha,b}$
	γ_b	2.37 (m)			γ_a, $\beta_{a,b}$	NH
	δ C=O		177.3	$\beta_{a,b}$, $\gamma_{a,b}$		
	ϵ_a NH_2	6.78 (br s) *syn*				$\epsilon_{b,}$ $\gamma_{a,b}$
	ϵ_b NH_2	7.47 (br s) *anti*				ϵ_a, $\gamma_{a,b}$
	C=O		175.9	α, β_a, β_b, Aib2-NH		
Aib-2	NH	7.90 (s) [2.6]				β1, 2, Val-α, Gln-NH, Leu-NH
	α		58.0	NH, β1, 2		
	β1 (*pro*-R)	1.49 (s)	23.5	β2		NH, Leu-NH
	β2	1.58 (s)	27.1	NH, β1		NH, Leu-α, NH
	C=O		179.2	NH, β1, 2, Leu- NH, α		
Leu	NH	7.94 (d, 6.1) [4.0]			a	Aib2-NH, β1, 2, Cterm-NH
	α	4.19 (m)	56.0	NH, β_b, Aib-NH	NH, $\beta_{a,b}$	$\beta_{a,b}$, $\beta_{a,}$**$_b$**, γ, δ1, 2
	β_a CH_2	1.55 (m)	41.4	NH, α, δ1, 2	α, β_b, γ	β_b, α
	β_b CH_2	1.80 (ddd, 13.6, 4.7, 2.1)			α, β_a, γ	β_a, α
	γ	1.91 (m)	25.9[i]	$\beta_{a,b}$, δ1, 2	$\beta_{a,b}$, δ1,2	a
	δ1	0.87 (d, 6.6)	21.1	$\beta_{a,b}$, δ2	γ	α
	δ2	0.89 (d, 6.5)	23.7	$\beta_{a,b}$, δ1	γ	α
	C=O		176.6	α, C-term-NH, α		
C-terminal	NH	7.49 (d, 9.2) [2.8]			a	β1$_b$, β2$_b$, Leu-NH, β_b
	α	4.29 (m)	46.9	β1$_{a,b}$, β2$_{a,b}$, NH	NH, β1$_{a,b}$, β2$_{a,b}$	NH, β1$_{a,b}$, β2$_{a,b}$, γ, δ1, 2
	β1$_a$ CH_2	1.23 (m)	42.3	β2$_{a,b}$, γ, δ1, 2	α, β1$_b$, γ	γ, β2$_{a,}$**$_b$**
	β1$_b$ CH_2	1.67 (m)			α, β1$_a$	NH, α, β2$_{a,}$**$_b$**
	γ	1.72 (m)	25.8[i]	β1$_{a,b}$, δ1, 2	δ1, 2, β1$_a$	NH, α, β1$_a$, β2$_a$
	δ1	0.91 (d, 6.5)	23.8	β1$_{a,b}$, δ2	γ	α, β2$_b$
	δ2	0.93 (d, 6.5)	22.1	β1$_{a,b}$, δ1	γ,	α, β2$_a$
	β2$_a$ CH_2(NH_2)	3.08 (dd, 12.8, 3.8)	44.6	α, β1$_{a,b}$	α, β2$_b$	β1$_{a,b}$, γ, δ2, NH_2
	β2$_b$ CH_2(NH_2)	3.15 (dd, 12.8, 9.9)			α, β2$_a$	β1$_{a,}$**$_b$**, γ, δ1, NH_2
	NH2	7.70 (br s)				β2$_{a,b}$

[a] Recorded at 600 MHz in CD_3OH. [b] Recorded at 125 MHz in CD_3OD. [c] Protons showing long-range correlation with indicated carbon. [d] Geminal correlations omitted except where necessary to support assignment. [e] Stronger correlation to one member of a geminal pair indicated in bold. [f–i] Interchangeable with assignments marked with same letter.

Table 2. NMR Spectral Data for Cicadapeptin II[a]

residue	position	δ_H[b] (J in Hz) [$-\Delta\delta/\Delta T$, ppb/K]	δ_C[c]	HMBC[d]	dqCOSY	ROESY[e,f]
Ile	NH	7.85 (d, 6.5) [4.6]			α	α, β,$\gamma1_{a,b}$, $\gamma2$, δ, Gln-NH, Aib2-NH, $\beta1$, 2, Cterm-NH
	α	3.88 (dd, 6.5, 9.7)	62.8	NH, β, $\gamma2$	NH, β	NH, β, $\gamma1_{a,b}$, $\gamma2$, δ, Aib2−$\beta2$
	β	1.92 (m)	36.9	NH, α, $\gamma2$, δ	α, $\gamma1_{a,b}$, $\gamma2$	NH, $\gamma1_{a,b}$
	$\gamma1_a$ CH_2	1.32 (m)	27.3	α, $\gamma2$, δ	β, $\gamma1_b$, δ	NH, α, β
	$\gamma1_b$ CH_2	1.73 (m)			β, $\gamma1_a$, δ	NH, α, β
	$\gamma2$ CH_3	0.94 (d, 6.8)	15.8	α	β	NH, α
	δ	0.84 (t, 7.6)	10.9	$\gamma1_{a,b}$, $\gamma2$	$\gamma1_{a,b}$	NH, α
	C=O		175.4	α, C-term-NH, C-term-α		
C-terminal	NH	7.58 (d, 9.0) [4.0]			α	α, $\beta1_b$, γ, $\delta1,2$, $\beta2_b$, Ile-NH, α, β,
	α	4.32 (m)	46.7	NH, $\beta1_{a,b}$, $\beta2_{a,b}$	NH, $\beta1_{a,b}$, $\beta2_{a,b}$	NH, $\beta1_{a,b}$, $\beta2_{a,b}$, γ, $\delta1$
	$\beta1_a$ CH_2	1.23 (m)	41.9	$\beta2_{a,b}$, $\delta1$, 2	α, $\beta1_b$, γ	γ, $\beta2_{a,b}$
	$\beta1_b$ CH_2	1.68 (m)			α, $\beta1_a$	NH, α, $\beta2_{a,b}$
	γ	1.74 (m)	25.8	$\beta1_{a,b}$, $\delta1$, 2	$\delta1$, 2, $\beta1_a$	NH, α, $\beta1_a$, $\beta2_a$
	$\delta1$	0.92 (d, 6.6)	23.9	$\delta2$	γ	α, $\beta2_b$
	$\delta2$	0.93 (d, 6.6)	21.9	$\beta1_{a,b}$, $\delta1$	γ	α, $\beta2_a$
	$\beta2_a$ $CH_2(NH_2)$	3.10 (dd, 12.7, 3.4)	44.8	α, $\beta1_a$	α, $\beta2_b$	$\beta1_b$, γ, $\delta2$, NH_2
	$\beta2_b$ $CH_2(NH_2)$	3.15 (dd, 12.7, 9.5)			α, $\beta2_a$	$\beta1_{a,b}$, γ, $\delta1$, NH_2
	NH_2	7.67 (br s)				$\beta2_{a,b}$

[a] Data presented only for residues in which assignments and correlations differed substantially from data for **1** (see Supporting Information for complete assignment and correlation data). [b] Recorded at 600 MHz in CD_3OH. [c] Recorded at 125 MHz in CD_3OD. [d] Protons showing long-range correlation with indicated carbon. [e] Geminal correlations omitted except where necessary to support assignment. [f] Stronger correlation to one member of a geminal pair indicated in bold.

sis of ^{1}H NMR data for the $CDCl_3$ extract from the hydrolyzates of **1** and **2** suggested a mixture of the expected C-terminal diamine together with a second hydrolytic fragment consisting of the penultimate Ile/Leu residues and the C-terminal diamine unit, even upon extending the hydrolysis time to 48 h. This unexpected resistance of the Ile/Leu-C-terminal fragment to hydrolysis, together with limitations of scale, prevented isolation of the free diamine product in pure form in these experiments.

The 1,2-diamino-4-methylpentane unit seems most likely to arise biogenically from Leu in which the carbonyl carbon has been aminated and reduced. This hypothesis is supported by the occurrence in other Aib-containing fungal peptides of similar C-terminal diamines that also appear to arise from amino acids, e.g., Ala in the leucinostatins,[6] trichopolyns,[11] and helioferins;[12] Phe in aibellin;[13] and Leu in the efrapeptins.[2]

Chiral amino acid analysis showed that all of the chiral amino acids in **1** and **2** have the L-configuration at the α-carbon. The Hyp obtained from both **1** and **2** coeluted with *trans*-4-L-hydroxyproline (*S*, *R*), and the Ile in **2** coeluted with L-Ile (*S*, *S*). The only remaining chiral center is the α-carbon of the C-terminal diamine, which we postulate as L-configured on the basis of the likelihood that the diamine derives biogenically from L-Leu. This hypothesis is further supported by the observation that all of the previously reported C-terminal-blocking diamines in Aib-containing peptides from fungi are L-configured.[2,6,11,13]

NMR spectra of **1** and **2** acquired in CD_3OH revealed the labile ϵ-NH_2 amide protons of the Gln unit and the NH_2 amine protons of the C-terminal 1,2-diamino-4-methylpentane unit. In the ROESY spectra of both **1** and **2**, the Gln ϵ-amide protons produced strong antiphase exchange cross-peaks. In the spectrum of **1**, this was the only clear evidence of the presence of the more downfield signal as it overlapped with and was masked by both the C-terminal and Val amide signals at ca. δ 7.50. The NH_2 amine proton signals of the C-terminal diamine of **1** and **2** were identified and assigned as a broad peak that integrated as two protons in ^{1}H NMR spectra taken in CD_3OH and correlated with the C-terminal β2-protons in ROESY spectra.

Multiple Aib residues induce the formation of either α- or 3_{10} helices or helices of mixed α/3_{10} character in peptides.[14,15] Key interresidual ROEs, particularly the observation of all possible $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ correlations, as well as other conformationally relevant NMR data for both **1** and **2** indicate that these compounds adopt a helical conformation in solution.[16] The evidence is especially compelling for the core Aib-1-Gln-Aib-2 segment shared by the two peptides (Tables 1 and 2) in which the Gln $^3J_{HN\alpha}$ (4.1 Hz) is intermediate between the values for an α- (3.9 Hz) and a 3_{10} helix (4.2 Hz).[16] The L-configuration of all chiral amino acids in **1** and **2** indicates a right-handed helix.[15]

ROESY data were also used to make stereospecific assignments for the ϵ-NH_2 amide protons of Gln, the β- and δ-protons of the Hyp residues, and the geminal methyl signals for the Aib and Val units (Tables 1 and 2) according to published approaches.[9,10,17,18]

Myriocin (**3**), purified by crystallization from MeOH, was identified by comparison of its ^{1}H NMR spectrum (CD_3OD) with published data.[19,20]

In agar disk/diffusion assays using 100 μg/disk, **1** and **2** produced clear kill zones against *Bacillus cereus* (13 and 12 mm, respectively), *B. subtilis* (13 and 11 mm, respectively), and *Escherichia coli* (16 mm for both peptides). In comparison, a standard antibiotic, tetracycline, tested at 30 μg/disk against *B. cereus* and *B. subtilis* only, produced larger, but hazier zones of inhibition (29 and 18 mm, respectively). Compound **3** showed no activity against any of the bacterial targets, but was inhibitory to all the filamentous fungi tested, producing inhibition zones against *Botrytis cinerea*, *Colletotrichum fragariae*, *C. gloeosporioides*, and *Fusarium oxysporum*, of 28, 14, 8, and 17 mm, respectively. Activity against a range of other fungi has been previously reported for this compound.[21] Compounds **1** and **2** showed inhibitory activity only against *B. cinerea* among the fungi tested, both producing 11 mm zones.

Among the known peptaibols,[22] where Pro or Hyp occur they are always preceded by either Aib or Iva. Pro and MePro are acylated by N-terminal blocking groups in the leucinostatins,[23] the related trichopolyns,[11,24] and the helioferins.[12] However, to our knowledge, the Hyp-Hyp se-

quence motif reported here for cicadapeptins (as well as Hyp-Pro, Pro-Hyp, and Pro-Pro sequences deduced for other minor cicadapeptin analogues from ESIMS data not shown) has not been previously reported from nonribosomal linear fungal peptides. Hyp-Hyp sequences associated with glycosylation sites occur in plant glycoproteins[25] and also occur in some of the conotoxins,[26] as well as in collagen.[27]

We are currently exploring the possibility that the occurrence of these tandem sequences in the cicadapeptins may be related to the biological function that they perform for the producing organism. The modified N- and C-terminal blocking groups and nonproteinogenic amino acids (Aib) in the cicadapeptins are hallmarks of peptides of nonribosomal origin.[28] We anticipate using the recently published gene sequence for the nonribosomal peptide synthetase from an Aib-containing fungal peptide[29] to construct nucleic acid probes for identifying the cicadapeptin synthetase, with the ultimate goal of developing knock-out mutants for evaluating the role of these compounds in the pathogenicity of the producing organisms to their insect hosts.

Experimental Section

General Experimental Procedures. ^{1}H NMR spectra were acquired at 400, 500, or 600 MHz on Varian Inova 400, Unity 500, Inova 500 (HCN inverse detection probe for 2-D/DBG broad band decoupler probe for 1-D ^{13}C NMR and DEPT), Inova 600, or Bruker AMX-600 spectrometers. ^{13}C NMR and DEPT spectra were acquired at 125 (Inova 500) or 100 MHz (Bruker DPX-400). HMBC and HMQC spectra were acquired at 600 or 500 MHz (^{1}H-dimension), and experiments were optimized for $^nJ_{CH} = 8.0$ and $^1J_{CH} = 150$ Hz, respectively. TOCSY and ROESY spectra were acquired with a spin lock and mixing time of 120 and 250 ms, respectively. Amide thermal chemical shift coefficients ($-\Delta\delta/\Delta T$, ppb/K) were derived from curves for values at 5 K steps in the range from 293 to 313 K. NMR data for intact peptides were recorded in CD_3OH or CD_3OD, and chemical shifts were referenced using the centers of the residual CHD_2OH (CHD_2OD) quintuplet at δ 3.31 and $^{13}CD_3OD$ septuplet at δ_C 49.15. ROESY (**1** and **2**) and TOCSY (**1**) spectra acquired in CD_3OH employed solvent suppression using the WET modification of the pulse sequence. Solvent signal suppression in dqCOSY and HMBC spectra acquired in CD_3OH was accomplished by modifying the pulse sequence with a presaturation delay of 1 s. Spectra of C-terminal groups were obtained in $CDCl_3$ or CD_2Cl_2 and referenced to solvent signals for residual protiated solvent at δ 7.24 and 5.32, respectively. Low-resolution ESI mass spectra were acquired in the positive ion mode by infusion of methanolic solutions at 5 μL/min via a syringe pump (Harvard Apparatus) into a Micromass ZMD 4000 spectrometer (capillary voltage of 4 kV; sample cone voltage of 120 V), and a ABI-Sciex Q-trap 2000 spectrometer (needle voltage 5.5 kV, declustering potential 130 V; collision energy 50 V). High-resolution ESI mass spectra were similarly acquired using a Micromass Autospec spectrometer. GC/MS data were acquired using a ThermoQuest Finnigan Voyager GC/MS.

Fungal Material and Culture Conditions. Cryogenically preserved mycelial material of *Cordyceps heteropoda* ARSEF #1880 was used to inoculate stock cultures maintained on plates of Sabouraud's dextrose agar plus yeast extract (1.0%). Plugs from 4- to 6-week-old solid cultures were used to inoculate liquid cultures in 2 L Fernbach flasks each containing 1 L of Sabouraud's dextrose broth plus yeast extract (1.0%). Cultures were grown for 4−6 weeks on a rotary shaker (160 rpm, 20 ± 1 °C) and harvested by separating mycelium from broth by filtration though cheesecloth.

Isolation. The filtered culture broth from three 1 L batches was extracted with three 500 mL portions of CH_2Cl_2 per liter of broth. The organic layers were combined and dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo to afford an oily brown residue (167 mg). The mycelial mats from each liter batch were homogenized in 300 mL of EtOH and allowed to stand >24 h at 20 °C. The ethanolic extract was then filtered and dried in vacuo. The residue was taken up in H_2O (60 mL) and extracted with three 150 mL portions of CH_2Cl_2. The organic layers were concentrated as with the broth extracts. The crude mycelial extract (1134 mg) was combined with the broth extract, redissolved in CH_2Cl_2, applied to a silica gel column (Baker 40-μm; 250 × 25 mm), and chromatographed by elution with 100% CH_2Cl_2 (1 L), 500 mL each of 10, 20, 30, 40, and 50% MeOH in CH_2Cl_2, 100% MeOH, and 1% NH_4OH (28%) in MeOH. The fraction eluting with 100% MeOH was dried in vacuo. The residue (565 mg) displayed antibacterial activity and was further fractionated by repeated semipreparative reversed-phase HPLC (5 μm Metachem Polaris C18 column; 250 × 10 mm; 80:20 MeCN−H_2O, 0.3% TFA; 4 mL/min) to furnish **1** (6.7 mg) and **2** (16.0 mg) of sufficient purity for spectral analysis. Because of the complexity of the peptide mixture and the difficulty of achieving complete separation of the major components by chromatographic methods, it was not possible to obtain compounds of absolute purity. On the basis of analysis of the amide regions of NMR spectra, samples of **1** and **2** were estimated to be ca. 95% pure with contamination from other major components or coeluting minor unidentified analogues.

The open-column silica fraction eluting with 60% MeOH was dried in vacuo. The residue, which displayed antifungal activity, was concentrated to dryness in vacuo (109 mg). Following resuspension in MeOH, a white precipitate formed. The solvent was decanted, and the precipitate was repeatedly recrystallized from MeOH to afford **3** (38 mg).

Amino Acid Analysis. A sample of 2 mg of **1** was hydrolyzed in 2.5 mL of 6 N HCl in a sealed hydrolysis tube in vacuo at 110 ± 1 °C for 24 h. The hydrolysis mixture was then partitioned three times with CH_2Cl_2 (2.5 mL each) and the aqueous layer made basic (ca. pH 10) using concentrated NH_4OH. This solution was partitioned again with three portions of 2.5 mL of CH_2Cl_2 and the aqueous layer neutralized with 0.5 N HCl and evaporated in vacuo. The residue was combined with 3 mL of 3 N HCl in *n*-butanol (Regis Chemical Co.), maintained at 100 °C for 30 min, and evaporated to dryness under air. The resulting solid was combined with 1 mL of TFA and 1 mL of TFAA in a sealed tube in vacuo and heated at 130 °C for 5 min. The residue was dried under N_2 until nearly all the solvent had evaporated and then mixed with 1 mL of CH_2Cl_2 and diluted 1:10 with CH_2Cl_2. A sample of 1 μL of this solution was analyzed by GC/MS using a helium flow of 2.5 mL/min through a ZB-5 Phenomenex capillary column (15 m × 0.32 mm i.d., 0.25 μm film thickness) with a temperature program of 50 °C for 1 min, 50−250 °C at 10 °C/min, and 250 °C for 10 min. This process was repeated for compound **2**.

Chiral Amino Acid Analysis. Samples of **1** (4.0 mg) and **2** (5.1 mg) were hydrolyzed in 1 mL of 6 N HCl at 120 °C for 24 h. The acidic hydrolyzate was diluted by adding 1 mL of H_2O and extracted with three 1 mL portions of CH_2Cl_2. The organic layers were combined, dried under a stream of dry N_2, and redissolved in CH_2Cl_2 for GC/MS analysis. The extracted acidic aqueous portion was dried in vacuo and redissolved in 1 mL of H_2O. A 5 μL aliquot of this fraction was subjected to analysis on a 250 × 4.6 mm Phenomenex Chirex column using a mobile phase of 2-propanol/2.5 mM aqueous $CuSO_4$ (1:9) at 1 mL/min with detection by UV absorption at 254 nm. Amino acids were identified and chirality was established by matching retention times with those of standards for D- and L-isomers of all candidate amino acids plus Aib (Sigma). The remainder of the aqueous fraction was made basic (to pH 10) with 30% NH_4OH and extracted with $CDCl_3$. The organic layer was collected, dried over anhydrous Na_2SO_4, filtered, and transferred to an NMR tube for analysis.

Bioassay. Effects of fungal fermentation extracts, chromatographic fractions, and purified compounds were tested against bacterial target strains grown on nutrient agar plates (9 cm) and against fungal target strains grown on potato

dextrose agar plates. Plates spread with 0.6 mL aliquots of a liquid bacterial culture grown for 24 h in nutrient broth or with 0.6 mL of a fungal spore suspension (1×10^6 spores/mL) were allowed to dry for 1 h. Test samples were then applied to 4 mm filter paper disks in 10 μL of MeOH. Disks were air-dried for 1 h and then placed on test plates. Zones of inhibition were measured after 48 h.

Cicadapeptin I (1): colorless oil; HPLC $t_R = 6.1$ min; ^{1}H, ^{13}C NMR, HMBC, dqCOSY, ROESY data, see Table 1; TOCSY data, see the Supporting Information; ESIMS/MS (for M + H at *m/z* 1007) *m/z* 891 (<1), 778 (9), 693 (2), 565 (100), 480 (69), 452 (24), 381 (55), 268 (79), 240 (17); 86 (5); HRESIMS obsd *m/z* 1007.6904 $[M + H]^+$; calcd for $C_{50}H_{90}N_{10}O_{11}$ + H 1007.6869.

Cicadapeptin II (2): colorless oil; HPLC $t_R = 6.3$ min; ^{1}H, ^{13}C NMR, HMBC, dqCOSY, ROESY data, see Table 2; TOCSY data, see the Supporting Information; ESIMS/MS (for M + H at *m/z* 1007) *m/z* 891 (<1), 778(12), 693 (2), 565 (100), 480 (73), 452 (25), 381 (51), 268 (70), 240 (15); 86 (6); HRESIMS obsd *m/z* 1007.6869 $[M + H]^+$; calcd for $C_{50}H_{90}N_{10}O_{11}$ + H 1007.6869.

Myriocin (3): rosettes of minute white crystals (MeOH); ESIMS *m/z* 402 $[M + H]^+$; 424 $[M + Na]^+$ (^{1}H NMR data have been reported previously).[19,20]

Acknowledgment. We thank Dr. I. Keresztes, Mr. A. M. Condo, Jr., and Dr. F. C. Schroeder (Cornell University) for help in acquiring NMR spectra. We thank L. Gallo, J. D. Leehr, W. T. Lester, M. Devinney, and J. E. Williams for technical assistance. We also thank the NMR and MS facilities at University of Iowa for technical support. Support from the National Science Foundation (CHE-0315591; University of Iowa) is gratefully acknowledged.

Supporting Information Available: Table of complete NMR assignments for **2**; TOCSY correlation data for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Gupta, S.; Krasnoff, S. B.; Renwick, J. A. A.; Roberts, D. W.; Steiner, J. R.; Clardy, J. *J. Org. Chem.* **1993**, *58*, 1062−1067.
(2) Gupta, S.; Krasnoff, S. B.; Roberts, D. W.; Renwick, J. A. A.; Brinen, L. S.; Clardy, J. *J. Org. Chem.* **1992**, *57*, 2306−2313.
(3) Krasnoff, S. B.; Gibson, D. M.; Belofsky, G. N.; Gloer, K. B.; Gloer, J. B. *J. Nat. Prod.* **1996**, *59*, 485−489.
(4) Wagenaar, M. M.; Gibson, D. M.; Clardy, J. *Org. Lett.* **2002**, *4*, 671−673.
(5) Wenger, R. M.; Payne, T. G.; Shreier, M. H. *Prog. Clin. Biochem. Med.* **1986**, *3*, 157−191.
(6) Cerrini, S.; Lamba, D.; Scatturin, A.; Rossi, C.; Ughetto, G. *Biopolymers* **1989**, *28*, 409−420.
(7) Tsantrizos, Y. S.; Pischos, S.; Sauriol, F.; Widden, P. *Can. J. Chem.* **1995**, *74*, 165−172.
(8) Roepstorff, P.; Højrup, P.; Møller, J. *Biomed. Mass Spectrom.* **1985**, *12*, 181−189.
(9) Rebuffat, S.; Hlimi, S.; Prigent, Y.; Goulard, C.; Bodo, B. *J. Chem Soc., Perkin Trans. 1* **1996**, 2021−2027.
(10) Rebuffat, S.; Goulard, C.; Hlimi, S.; Bodo, B. *J. Pept. Sci.* **2000**, *6*, 519−533.
(11) Fujita, T.; Takaishi, Y.; Okamura, A.; Fujita, E.; Fuji, K.; Hiratsuka, N.; Komatsu, M.; Arita, I. *J. Chem. Soc., Chem. Commun.* **1981**, 585−587.
(12) Gräfe, U.; Ihn, W.; Ritzau, M.; Schade, W.; Stengel, C.; Schlegel, B.; Fleck, W. F.; Künkel, W.; Härtl, A.; Gutsche, W. *J. Antibiot.* **1995**, *48*, 126−133.
(13) Kumazawa, S.; Kanda, M.; Aoyama, H.; Utagawa, M.; Kondo, J.; Sakamoto, S.; Ohtani, H.; Mikawa, T.; Chiga, I.; Hayase, T.; Hino, T.; Takao, T.; Shimonishi, Y. *J. Antibiot.* **1994**, *47*, 1136−1144.
(14) Marshall, G. R.; Hodgkin, E. E.; Langs, D. A.; Smith, G. D.; Zabrocki, J.; Leplawy, M. T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 487−491.
(15) Karle, I. L.; Balarams, P. *Biochemistry* **1990**, *29*, 6747−6756.
(16) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley and Sons: New York, 1986.
(17) Esposito, G.; Carver, J. A.; Boyd, J.; Campbell, I. D. *Biochemistry* **1987**, *26*, 1043−1050.
(18) McIntosh, L. P.; Brun, E.; Kay, L. E. *J. Biomol. NMR* **1997**, *9*, 306−312.
(19) Sasaki, S.; Hashimoto, R.; Kiuchi, M.; Inoue, K.; Ikumoto, T.; Hirose, R.; Chiba, K.; Hoshino, Y.; Okumoto, T.; Fujita, T. *J. Antibiot.* **1994**, *47*, 420−433.
(20) Fujita, T.; Inoue, K.; Yamamoto, S.; Ikumoto, T.; Sasaki, S.; Toyama, R.; Chiba, K.; Hoshino, Y.; Okumoto, T. *J. Antibiot.* **1994**, *47*, 208−215.
(21) Craveri, R.; Manachini, P. L.; Aragozzini, F. *Experientia* **1972**, *28*, 867−868.
(22) Whitmore, L.; Wallace, B. A. *Nucleic Acids Res.* **2004**, *32*, D593−D594.
(23) Isogai, A.; Nakayama, J.; Takayama, S.; Kusai, A.; Suzuki, A. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1079−1085.
(24) Iida, A.; Mihara, T.; Fujita, T.; Takaishi, Y. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3393−3396.
(25) Shpak, E.; Leykam, J. F.; Kieliszewski, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14736−14741.
(26) Lancelin, J.-M.; Kohda, D.; Tate, S.-i.; Yanagawa, Y.; Abe, T.; Satake, M.; Inagaki, F. *Biochemistry* **1991**, *30*, 6908−6916.
(27) Vitagliano, L.; Berisio, R.; Mastrangelo, A.; Mazzarella, L.; Zagari, A. *Protein Sci.* **2001**, *10*, 2627−2632.
(28) Marahiel, M. A.; Stachelhaus, T.; Mootz, H. D. *Chem. Rev.* **1997**, *97*, 2651−2673.
(29) Wiest, A.; Grzegorski, D.; Xu, B.-W.; Goulard, C.; Rebuffat, S.; Ebbole, D. J.; Bodo, B.; Kenerley, C. *J. Biol. Chem.* **2002**, *277*, 20862−20868.

NP0497189