New Peptaibols from *Mycogone cervina*†

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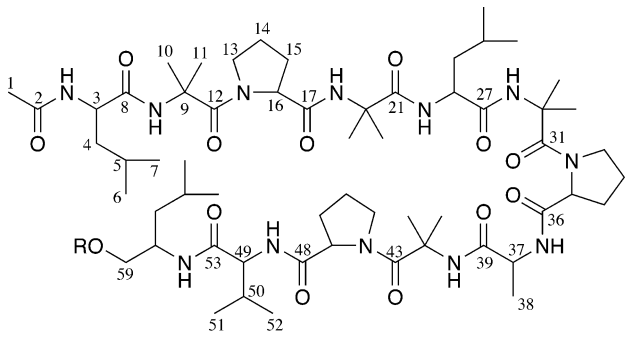
Received June 24, 2003

From cultures of *Mycogone cervina*, a mycophilic fungus growing on the fruit bodies of ascomycete *Paxina acetabulum*, two new peptaibols (cervinin, *N*-Ac-Leu−Aib−Pro−Aib−Leu−Aib−Pro−Ala−Aib−Pro−Val−*red*-Leu (**1**) and the *red*-Leu *O*-acetylated derivative **2**) were isolated. The structures of **1** and **2** were elucidated by spectroscopic techniques. They exhibited weak antibacterial as well as cytotoxic activities. They are the first secondary metabolites described from *M. cervina*.

Mycophilic fungi are widespread in nature, and their host range comprises higher and lower fungi.[1,2] Among mycoparasites, nectrotrophic fungi, fungi that kill their hosts, have been found to be a good source for antimicrobial metabolites.[3−7] This has led to speculation about the role of these compounds in the host−parasite relationship.[8] During our ongoing screening of mycophilic fungi for the production of cytotoxic and antimicrobial metabolites, cultures of *Mycogone cervina* were found to produce bioactive compounds. Other species of this genus (e.g., *M. rosea*) have previously been described as prolific sources of secondary metabolites, especially peptaibols.[9−11] Peptaibols are polypeptides isolated from fungi having typically between 15 and 20 residues, although smaller and larger peptaibols are known.[12,13] They have a high proportion of nonstandard amino acids, in particular aminoisobutyric acid (Aib), and while the N terminus is acylated, the C terminus is reduced to a primary alcohol. They possess antimicrobial activity due to their membrane activity and ability to form pores in lipid membranes, and that in turn depends on the presence of Aib, which has a strong tendency to stabilize a helical structure.[13] As no secondary metabolites were known from *M. cervina*, the antimicrobial and cytotoxic compounds were isolated and their structures elucidated in the present investigation.



1 R = H
2 R = $COCH_3$

The structures of **1** and **2** were determined by mass spectrometry and NMR spectroscopy. High-resolution MS measurements indicated the elemental composition of **1** and **2** as $C_{59}H_{102}N_{12}O_{13}$ and $C_{61}H_{104}N_{12}O_{14}$, respectively, suggesting that they differ by an acetyl group. This was confirmed by the 1D NMR data (see Table 1) of **1** and **2**, which indeed are very similar except for the region around a primary alcohol/acetoxy group. The free alcohol **1** was named cervinin and the acetylated metabolite consequently called cervinin 59-acetate (**2**).

Many of the NMR signals of **1** were overlapping, making a straightforward structure elucidation difficult. However, by recording the spectra in DMSO-d_6 and making use of the signals for the amide protons in both the COSY and the HMBC spectra, it was possible to assign all signals. In DMSO-d_6, the carbonyl signals in the ^{13}C NMR spectrum were well resolved, which facilitated the establishment of the peptide backbone. The H_3-1 and NH-2 signals both gave HMBC correlations to C-2, showing that this nitrogen is acetylated. The COSY correlations from NH-2 to H-3 and further on to H-4, H-5, and H_3-6/H_3-7 together with the HMBC correlations H-3 and NH-8 to C-8 identified the first amino acid as leucine in **1**. The NH-8 signal also showed a HMBC correlation to a quaternary carbon, which also is substituted by two methyl groups. The H_3-10 and H_3-11 also showed HMBC correlations to C-12. The H_2-13 and H-16 resonances gave weak HMBC correlations to C-12, and COSY correlations in the spin system H_2-13/H_2-14/H_2-15/H-16 established the proline ring. The HMBC correlations from both H-16 and NH-17 to C-17 connected the following amino acid. The corresponding correlations showed that this was another aminoisobutyric acid, which was followed by a leucine, a third aminoisobutyric acid, another proline, an alanine, a fourth aminoisobutyric acid, a third proline, a valine, and finally a reduced leucine which was acetylated in **2**. Thus, following the COSY and HMBC correlations made it possible to analyze the entire structure.

Compounds **1** and **2** contain two leucines, four aminoisobutyric acids, three prolines, one alanine, one valine, and one 2-amino-4-methylpentanol (or reduced leucine). The fact that the NMR data only differ significantly in the reduced leucine suggests that both compounds adopt the same 3D form. It was not possible to determine the stereostructure of the products by NMR, but as there are no reports of peptaibols containing D-amino acids, it is reasonable to assume all the asymmetric amino acids are L.

Contrary to the activities reported for the peptaibols from *M. rosea*,[9−11] the biological activities of the new peptaibols are weak. Compound **1** showed activity toward Gram-positive bacteria but not toward filamentous fungi. In the

† Dedicated to the memory of the late Dr. U. Gräfe.

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10.1021/np0302899 CCC: $27.50
Published on Web 01/14/2004

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts for **1** and **2** in DMSO-d_6

	1		**2**			**1**		**2**	
position	^{1}H (δ; mult.; J)	^{13}C (δ; mult.)	^{1}H (δ; mult.; J)	^{13}C (δ; mult.)	position	^{1}H (δ; mult.; J)	^{13}C (δ; mult.)	^{1}H (δ; mult.; J)	^{13}C (δ; mult.)
1	1.86; s	22.4; q	1.86; s	22.4; q	32	3.75/3.45; m	48.2; t	3.75/3.44; m	48.2; t
2		169.5; s		169.5; s	33	1.83; m	25.3; t	1.82/1.81; m	25.3; t
NH-2	7.94; d; 7.7		8.02; d; 7.6		34	2.21/1.64; m	28.3; t	2.20/1.64; m	28.3; t
3	4.27; m	51.4; d	4.27; m	51.4; d	35	4.26; m	62.5; d	4.26; m	62.5; d
4	1.48; m	40.1; t	1.49; m	40.1; t	36		171.2; s		171.2; s
5	1.61; m	24.2; d	1.60; m	24.2; d	NH-36	7.72; d; 8.4		7.72; d; 8.6	
6	0.90; d; 6.6	22.6; q	0.90; d; 6.6	22.6; q	37	4.22; m	47.9; d	4.22; m	47.9; d
7	0.85; d; 6.6	21.6; q	0.85; d; 6.6	21.6; q	38	1.31; d; 7.3	16.2; q	1.31; d; 7.3	16.3; q
8		172.8; s		172.8; s	39		172.7; s		172.6; s
NH-8	8.48; s		8.54; s		NH-39	7.81; s		7.79; s	
9		55.8; s		55.8; s	40		55.8; s		55.8; s
10	1.38; s	23.4; q	1.38; s	23.4; q	41	1.45; s	23.1; q	1.45; s	23.2; q
11	1.34; s	25.3; q	1.34; s	25.2; q	42	1.34; s	25.6; q	1.35; s	25.6; q
12		173.1; s		173.1; s	43		172.1; s		172.1; s
13	3.68/3.43; m	48.0; t	3.67/3.42; m	48.0; t	44	3.62/3.46; m	47.9; t	3.62/3.46; m	47.9; t
14	1.91/1.85; m	25.5; t	1.92/1.83; m	25.5; t	45	1.82; m	25.5; t	1.83/1.81; m	25.5; t
15	2.19/1.64; m	28.0; t	2.19/1.64; m	28.0; t	46	2.16/1.64; m	28.8; t	2.15/1.64; m	28.8; t
16	4.12; m	63.6; d	4.11; m	63.6; d	47	4.25; m	62.4; d	4.26; m	62.3; d
17		173.2; s		173.2; s	48		171.7; s		171.7; s
NH-17	7.53; s		7.53; s		NH-48	7.42; d; 9.4		7.44; d; 9.3	
18		56.1; s		56.1; s	49	4.08; m	58.2; d	4.10; m	58.0; d
19	1.42; s	23.3; q	1.42; s	23.3; q	50	2.22; m	29.1; d	2.23; m	29.0; d
20	1.40: s	26.5; q	1.40: s	26.5; q	51	0.87; d; 7.0	17.7; q	0.88; d; 7.0	17.7; q
21		174.5; s		174.6; s	52	0.84; d; 7.0	19.4; q	0.85; d; 7.0	19.3; q
NH-21	7.41; d; 7.6		7.41; d; 8.2		53		170.1; s		170.4; s
22	4.14; m	51.5; d	4.13; m	51.4; d	NH-53	6.70; d; 9.0		6.90; d; 9.0	
23	1.57; m	39.6; t	1.54; m	39.7; t	54	3.87; m	45.1; d	4.11; m	45.1; d
24	1.65; m	24.1; d	1.65; m	24.1; d	55	1.28; m	39.9; t	1.35/1.18; m	39.6; t
25	0.81; d; 6.5	22.6; q	0.80; d; 6.6	22.7; q	56	1.65; m	23.5; d	1.66; m	23.5; d
26	0.74; d; 6.5	20.6; q	0.74; d; 6.6	20.5; q	57	0.80; d; 6.6	22.7; q	0.79; d; 6.6	23.2; q
27		173.4; s		173.4; s	58	0.78; d; 6.6	21.2; q	0.78; d; 6.6	21.0; q
NH-27	7.88; s		7.88; s		59a	3.28; m	64.2; t	3.91; dd; 5.7, 10.9	65.9; t
28		55.9; s		55.9; s	59b	3.17; ddd; 6, 6, 10		3.79; dd; 6.6, 10.9	
29	1.48; s	23.1; q	1.48; s	23.1; q	OH-59	4.44, dd, 6, 6			
30	1.40; s	25.8; q	1.40; s	25.8; q	60				170.2; s
31		173.0; s		173.0; s	61			1.98; s	20.5; q

standard disk assays using 100 μg/disk, **1** caused the following inhibition zones: *Bacillus subtilis* (ATCC 6633) 8 mm; *B. brevis* (ATCC 9999) 7 mm, and *Micrococcus luteus* (ATCC 21415) 15 mm. Under the same conditions, compound **2** caused the following inhibition zones: *B. subtilis* 11 mm, *B. brevis* 10 mm, and *M. luteus* 16 mm. Compound **2** was also active against *Paecilomyces variotii* (ETH 114646) and *Mucor miehei* (Tü 284); 100 μg/disk resulted in inhibition zones of 10 and 9 mm, respectively. Cytotoxic activities were also equivocal. Compound **1** had no effect on L1210 cells (ATCC CCL 219) at 100 μg/mL, while **2** affected the cells with an IC_{50} value of 32 μg/mL. The IC_{50} values of compounds **1** and **2** toward Colo-320 cells (DSMZ ACC-144) were 80 and 40 μg/mL, respectively.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 141 polarimeter at 22 °C. The UV and the IR spectra were recorded with a Perkin-Elmer λ 16 and a Bruker IFS 48 spectrometer, respectively. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probe head equipped with a shielded gradient coil. The spectra were recorded in DMSO-d_6, and its signals (2.50 and 39.51 ppm in ^{1}H and ^{13}C NMR, respectively) were used as reference. The chemical shifts (δ) are recorded in ppm, and the coupling constants (J) in Hz. COSY, HMQC, and HMBC experiments were recorded with gradient enhancements using sine-shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH} = 145$ Hz and $^nJ_{CH} = 10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra were recorded with a JEOL SX102 spectrometer.

Producing Organism. *Mycogone cervina* Ditm.[14] was isolated from fruit bodies of *Paxina acetabulum* (L. ex St. Amans) O. Kunze[15] growing on a meadow of the campus of the University of Kaiserslautern in April 2002. The strain (A09-02) is deposited at the Department of Biotechnology, University of Kaiserslautern. The strain was grown and kept on YMG medium.

Fermentation of *M. cervina* and Isolation of the Compounds. Fermentations were carried out in YMG medium consisting of glucose 1%, yeast extract 0.4%, and malt extract 1%, at pH 5.5, or in PD medium (potato-dextrose broth by DIFCO Laboratories, Detroit, MI) composed of 2.4% potato dextrose medium (DIFCO), at pH 5.1 in 5 L Erlenmeyer flasks containing 2 L of medium at 22 °C and shaking with 120 rpm or in still cultures in Fernbach flasks containing 500 mL of medium at 22 °C. The compounds were isolated from the culture broth (20 L) filtrate by adsorption onto HP 21 resin (Mitsubishi) and elution with MeOH (1.5 L). The crude extract (0.48 g) obtained by concentration was applied onto a silica gel (Merck 60, 0.063−0.2 mm) column (15 mm × 120 mm) and eluted with EtOAc followed by EtOAc−MeOH (3:1) and EtOAc−MeOH (1:1). The last fraction contained the active compounds (90 mg). Final separation and purification was achieved by preparative HPLC on a Merck LiChroSpher RP-18 (7 μm, 250 × 25 mm) column and elution with H_2O−MeOH (linear gradient 60 min, 60% MeOH−100% MeOH) and yielded 12 and 14 mg of cervinin (**1**) and cervinin 59-acetate (**2**), respectively. The cultures were harvested after 7 days. At this point the carbon source (glucose) was used up.

Cervinin (1): amorphous powder; $[\alpha]^{22}_D$ −14° (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ϵ) no maxima above 220 nm; ^{1}H and ^{13}C NMR data, see Table 1; FABMS *m*/*z* 1209 $[M + Na]^+$; HREIMS *m*/*z* 1209.7599 (calcd for $C_{59}H_{102}N_{12}O_{13}Na$, 1209.7587).

Cervinin 59-acetate (2): amorphous powder; $[\alpha]^{22}_D$ −16° (*c* 0.9, MeOH); UV (MeOH) λ_{max} (log ϵ) no maxima above 220 nm; ^{1}H and ^{13}C NMR data, see Table 1; FABMS *m*/*z* 1251 $[M + Na]^+$; HREIMS *m*/*z* 1251.7732 (calcd for $C_{61}H_{104}N_{12}O_{14}Na$, 1251.7693).

Antimicrobial Assays. Antibacterial activities were tested with *Bacillus brevis*, ATCC 9999, *B. subtilis*, ATCC 6633; *Micrococcus luteus*, ATCC 21415, *Enterobacter dissolvens*, LMG 2683, and *Proteus vulgaris*, DSM 30119; for antifungal activities *Nematospora corylii*, ATCC 10647, *Mucor miehei*, Tü 284, and *Paecilomyces variotii*, ETH 114646, were used. Bacteria were grown on nutrient agar (DIFCO) at 37 °C and fungi on YMG agar. Test plates were seeded with 5×10^5 cells or spores per mL. Filter disks (6 mm diameter) were loaded with the compounds to be tested and placed onto the agar plates. The diameters of inhibition zones were monitored after 24 h.

Cytotoxic Activities. L1210 cells (ARCC CCL 219) and Colo-320 (DSM ACC-144) were grown and assayed in RPMI 1640 medium as described before.[16]

Acknowledgment. Financial support from the Swedish Research Council is gratefully acknowledged. We thank R. Reiss for expert technical assistance.

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NP0302899