Isolation and Structure of Bergofungin, a New Antifungal Peptaibol from *Emericellopsis donezkii* HKI 0059

A. BERG, M. RITZAU, W. IHN, B. SCHLEGEL,
W. F. FLECK, S. HEINZE and U. GRÄFE

Hans-Knöll-Institute of Natural Product Research e.V.,
D-07745 Jena, Beutenbergstr. 11, Germany

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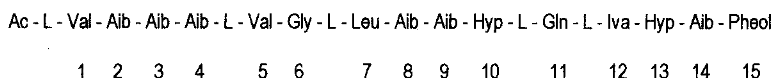
Previously we reported the isolation and structure of the chrysospermins as new peptaibol-type antifungal and antibacterial antibiotics from *Apiocrea chrysosperma* which display morphogenic activity against a small spectrum of fungal strains such as *Phoma destructiva*[1]. Peptaibols are linear amphiphilic peptide antibiotics[2] which are characterized by up to 20 amino acids linked in a linear order. They contain a high amount (up to 60%) of hydrophobic amino acids such as α-aminoisobutyric acid (Aib) and isovaline (Iva). The amino terminus is acetylated and the carboxyl terminus is reduced to the pertinent alcohols such as leucinol, phenylalaninol, valinol and tryptophanol[3]. Agents such as alamethicin, hypelcins, emerimicins, paracelsins, chrysospermins and others owe their antimicrobial effects to the formation of ion-conducting channels within biological membranes[4]. Our screening of other fungal strains for metabolites affecting fungal growth discovered *Emericellopsis donezkii* HKI 0059 as the producer of a new antifungal antibiotic which was given the name bergofungin.

Here, we report the isolation, structure and biological properties of this new representative of the peptaibols. Bergofungin (**1**) is produced as the main component by *Emericellopsis donezkii* HKI 0059, but occasionally, small amounts of homologues were detected varying in the composition of amino acids.

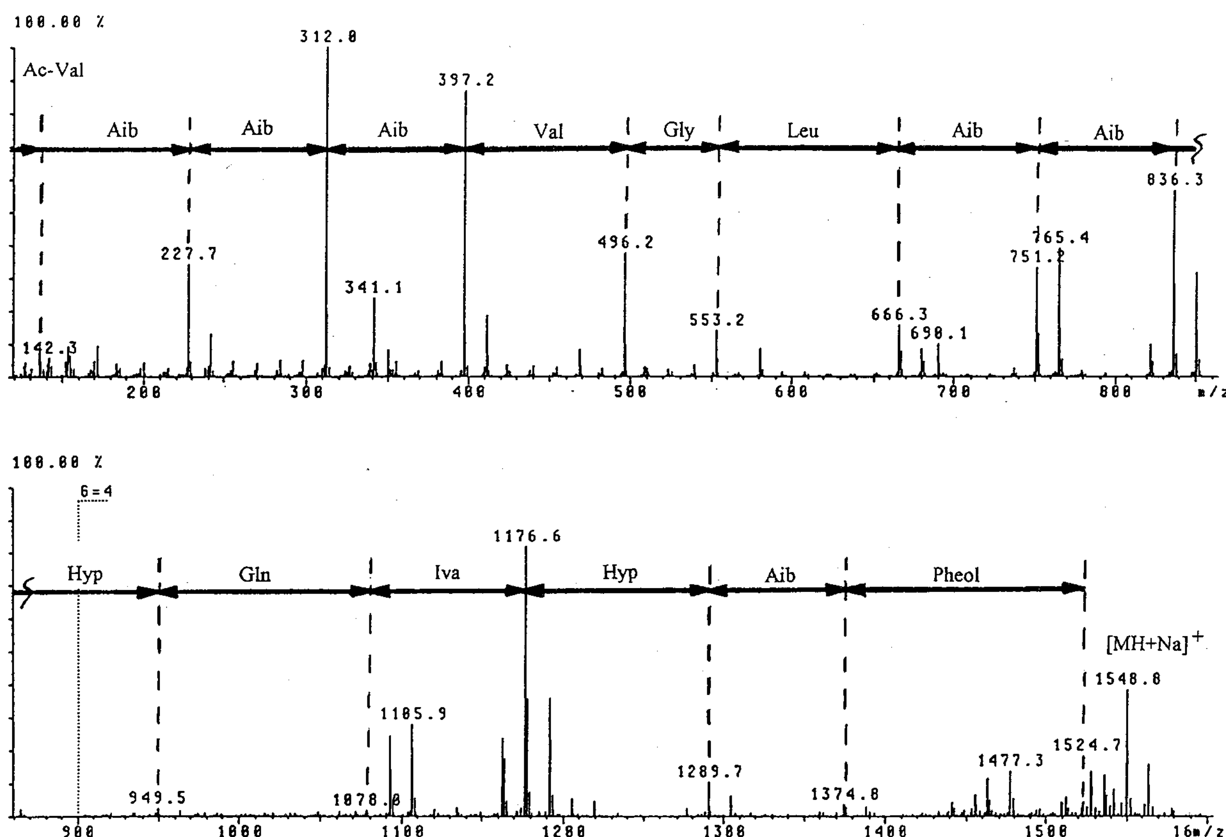
Bergofungin (**1**) was isolated from the fermentation broth of the producer strain (see Experimental) by a

Fig. 1. Structure of bergofungin (**1**).



(Ac-Val: *N*-acetyl-L-valine, Aib: α-aminoisobutyric acid, Hyp: 4-OH-L-proline, Pheol: phenylalaninol.

Fig. 2. Positive ion FAB mass spectrum of bergofungin (**1**).



series of chromatographic procedures. First, the whole culture liquid (40 liters) was extracted twice with 10 liters of ethyl acetate. The mycelium (800 g wet weight) was extracted overnight by 10 liters methanol. Thereafter, the methanol was evaporated *in vacuo* and the residue was extracted twice by 5 liters ethyl acetate. The combined extracts were dried by sodium sulphate. After evaporation the residue (4 g) was subjected to silica gel chromatography (Silica gel 60, 0.063∼0.1 mm, Merck, column 600×40 mm, $CHCl_3$-MeOH, 9:1, v/v). 10 ml fractions were collected and evaporated (yield 50 mg). Fractions containing bergofungin (**1**) were detected due to their antimicrobial effect against *Sporobolomyces salmonicolor* SBUG 549.

Final purification was achieved by isocratic preparative HPLC on silica gel RP_{18} column (acetonitrile - H_2O, 83:17, v/v). The retention time was 12.6 minutes, and 17 mg of pure bergofungin (**1**) was isolated. Bergofungin

Table 1. Assignment of ^1H and ^{13}C chemical shift data of bergofungin (**1**).

			δ_C	δ_H
1	Ac-Val	C-1	170.7	
		C-2	22.3	1.88 (s)
		N-H		8.07 (d)
		C-1	172.4	
		C-2	59.8	3.83 (dd)
		C-3	29.1	1.96 (m)
		C-4	19.2	0.91 (d)
		C-5	19.0	0.95 (d)
2	Aib	N-H		8.52 (s)
		C-1	175.1	
		C-2	55.89	
		C-3	23.7	1.34 (s)
		C-4	23.4	1.34 (s)
3	Aib	N-H		7.61 (s)
		C-1	174.9	
		C-2	55.9	
		C-3	24.9	1.33 (s)
		C-4	24.8	1.27 (s)
4	Aib	N-H		7.78 (s)
		C-1	175.6	
		C-2	53.9	
		C-3	25.3	1.39 (s)
		C-4	25.0	1.35 (s)
5	Val	N-H		7.41 (d)
		C-1	172.4	
		C-2	59.8	3.90 (dd)
		C-3	28.9	2.19 (m)
		C-4	19.0	0.95 (d)
		C-5	18.5	0.89 (d)
6	Gly	N-H		7.96 (t)
		C-1	170.0	
		C-2	42.8	3.71 (dd), 3.69 (dd)
7	Leu	N-H		7.71 (d)
		C-1	171.7	
		C-2	52.6	4.05 (m)
		C-3	39.4	1.52 (m)
		C-4	24.2	1.62 (m)
		C-5	22.6	0.88 (d)
		C-6	21.8	0.84 (d)
8	Aib	N-H		7.83 (s)
		C-1	176.0	
		C-2	56.1	
		C-3	25.9	1.44 (s)
		C-4	25.5	1.35 (s)
9	Aib	N-H		7.61(s)
		C-1	173.6	
		C-2	56.3	
		C-3	26.2	1.46 (s)
		C-4	24.8	1.34 (s)
10	Hyp	C-1	171.9	
		C-2	60.9	4.38 (dd)
		C-3	36.8	2.19 (m), 1.77 (m)
		C-4	69.0	5.08 (d) (OH), 4.28 (m)
		C-5	56.0	3.77 (m), 3.47 (m)
11	Gln	N-H		7.87 (d)
		C-1	172.1	
		C-2	52.6	4.11 (m)
		C-3	26.7	2.13 (m), 1.19 (m)
		C-4	31.6	2.12 (dd)
		C-5	173.1	
12	Iva	N-H		7.48 (s)
		C-1	173.8	
		C-2	58.2	
		C-3	23.4	1.41 (s)
		C-4	28.0	2.11 (m), 1.77 (m)
		C-5	7.0	0.75 (dd)
13	Hyp	C-1	171.5	
		C-2	61.8	4.19 (dd)
		C-3	36.6	2.11 (m), 1.65 (m)
		C-4	69.0	5.10 (d) (OH), 4.22 (m)
		C-5	56.8	3.65 (m), 3.39 (m)
14	Aib	N-H		7.51 (s)
		C-1	173.7	
		C-2	56.1	
		C-3	24.0	1.32 (s)
		C-4	23.9	1.16 (s)
15	Pheol	N-H		6.98 (d)
		C-1	63.3	4.28 (dd) (OH), 3.39 (m), 3.32 (m)
		C-2	59.8	3.88 (m)
		C-3	36.6	2.94 (dd), 2.54 (dd)
		C-4	139.2	
		C-5	129.1	
		C-6	127.8	7.19∼7.21 (m)
		C-7	125.7	
		C-8	127.8	
		C-9	129.1	

In DMSO, δ in ppm, multiplicity in parentheses (s: singlet; d: doublet, t: triplet, m: multiplet).
For abbreviations of the amino acids see Fig. 1.

Table 2. Antimicrobial activity of bergofungin (**1**).

Microorganisms	Diameter of inhibition zone (mm)
Bacillus subtilis ATCC 6633[a]	18
Sporobolomyces salmonicolor[b]	23
Penicillium notatum JP 36[c]	13

Each well contained 50 μg bergofungin (1) dissolved in 50 μl methanol.
[a] Standard I nutrient agar (SERVA, Germany).
[b] Sabouraud-2%-glucose agar (DIFCO).
[c] Malt agar (malt extract 4%, yeast extract 0.4%, agar 1.5%).

(**1**) is a white powder, which is soluble in methanol and chloroform; it melts at 239°C. The optical rotation was determined to amount to $[\alpha]_D^{25}$ +1.4° (*c* 0.5, MeOH).

Hydrolysis of bergofungin (**1**), derivatization of the amino acids by dabsyl chloride and Marfey's reagent and HPLC analysis of the obtained derivatives (see below) showed the presence of L-valine, L-leucine, α-aminoisobutyric acid (aib), glycine, 4-hydroxy-L-proline and L-glutamine or L-glutamic acid. The absolute stereochemistry of the terminal phenylalaninol has not yet been determined but could be suggested to be L (s) as it was shown for all of the phenylalaninol-containing peptaibols[3)]. Analytical HPLC-separations were accomplished with a reverse-phase column (Lichrospher 100 column RP 18, 250×4.5 mm).

For structure determination a series of MS-MS experiments using FAB ionisation recorded by B/E-, MIKES(E)-scan, ES ionisation and MS-MS triple quadropol techniques provided full evidence of the sequence of amino acids in Fig. 1.

The molecular weight of bergofungin (**1**) was determined by FAB high resolution mass measurements ((*m*/*z*) 1524.8962 (M^+), calculated for $C_{73}H_{120}N_{16}O_{19}$ (1524.8914).

Further conclusive structural evidence has been provided by the ^1H and ^{13}C NMR spectroscopic data (Table 1). Especially the constitution of the amino acids in positions 1, 5 and 12 (Val or Iva) could be assigned unambiguously. Moreover, the resolution of the 600 MHz spectra was sufficient to distinguish the overlapping signals. ^1H and ^{13}C signals gave evidence to six Aib-units in the molecule. The procedure was as follows: First, ^1H NMR assignments of different spin systems were derived from COSY and phase sensitive TOCSY experiments. By recording the phase-sensitive HSQC and HMBC spectra, the correlation between hydrogens and carbons as well as the assignment of the quaternary carbons and peptide carbonyls could be performed.

The latter experiment, together with phase sensitive NOESY data, fully confirmed the sequence of the amino acids as observed during the MS experiments.

Bergofungin (**1**) shows typical IR-absorptions at 3315, 2965, 2935, 1654, 1652, 1531, 1458, 1280, and 1080 cm^{-1}.

The antimicrobial activity (Table 2) was determined against several bacteria and fungi by the agar well diffusion assay. The method of the agar well diffusion assay was previously described[9,5,1)]. A characteristic property of bergofungin (**1**) is the comparably strong antimicrobial effect against the yeast *Sporobolomyces salmonicolor* SBUG 549. These results proved bergofungin (**1**) as a new representative of the peptaibol-type antibiotics. Its structure appears as related to emerimicin III. Hence, it could be suggested that bergofungin (**1**) forms ion channels in a similar manner but this property has to be determined in detail by our future investigations.

Experimental

Microorganisms and Cultivation

The strain *Emericellopsis donezkii* HKI 0059 from the strain collection of the Hans-Knöll-Institut für Naturstoff-Forschung Jena forms aerial mycelium and cleistothecia on malt agar. The hyaline cleistothecia (30~60 μm diam.) contain round asci and ellipsoidal, brown-pigmented ascospores (4~5×2~2.5 μm) with winged appendages (van Beyma)[8)]. It was propagated as inoculum on agar plates at 25°C for 15 days using a medium composed of (g/liter): malt extract 40, yeast extract 4, agar 15, deionized water pH 6.0. Thereafter 1~2 cm^2 areals of the agar plate cultures were used to inoculate 100 ml of a liquid medium in 500-ml Erlenmeyer bottles composed as follows (g/liter): malt extract 20, glucose 10, yeast extract 1; $(NH_4)_2SO_4$ 5, pH 6.0.

After 10 to 15 days breeding as emerged culture at 26°C a dense white lawn of mycelium was grown at the surface of the liquid medium. Thereafter, the whole mycelium and the culture medium were harvested and separated by filtration.

^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX 600 spectrometer operating at 600 MHz and 150 MHz, respectively.

Chemical shifts are given in δ (ppm) using as a reference standard the DMSO residual peak set at 2.49 ppm for ^1H, and set at 39.5 ppm for ^{13}C.

Mass spectra were obtained by a high resolution mass spectrometer AMD-402 (AMD Intectra; Harpstedt; Germany) and a quadropol mass spectrometer Quattro 400 (Fisons; VG Biotech; Altrincham, U.K.).

Specific optical rotation was determined with a Dr. Kernchen-model-Propol-automatic digital polarimeter.

IR spectra were recorded on a IR-470 Shimadzu spectrometer. HPLC chromatography was performed on a Gilson HPLC-System equiped with an UV-detector (210 nm). HPLC separation was achieved by the use of 83% acetonitrile (flow rate of 10 ml/minute) and a RP_{18} column (Spherisorb 550DS2; 250×25 mm, Promochem).

The constituting amino acids were determined by HPLC after hydrolysis[1)] of bergofungin (**1**). One mg of bergofungin (**1**) was hydrolysed by 1 ml of 6 N HCl for

24 hours at 120°C. The crude amino acids were derivatized by (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl). Separation of dabsylated amino acids was achieved using a gradient of sodium acetate buffer (pH 6.5) containing 4% DMF (Solvent A) and acetonitrile (Solvent B). The gradient was 15% B to 40% B for 20 minutes, 40% B to 70% B from 20 to 32 minutes, kept at 70% B from 32 to 34 minutes, then back to 15% B from 34 to 36 minutes. The cycle time from injection to injection was 44 minutes. The column temperature was 40°C. The flow rate was 1 ml/minute. The detector wavelength was adjusted to 436 nm[6].

Marfey's reagent (N^2-(5-fluoro-2,4-dinitrophenyl)-L-alanine-amide) was used to obtain the diasteromeric derivatives of chiral amino acids. For gradient elution in HPLC, buffer A was prepared by mixing 900 ml triethylammonium-phosphate (TEAP) buffer, 100 ml acetonitrile and buffer B by mixing 500 ml TEAP with 500 ml acetonitrile. A linear gradient was established from 0% B to 100% B in 45 minutes; flow rate, 1.5 ml/minute; column temperature 28°C. The UV detection was changed to 340 nm[7].

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