Ampullosporin, a New Peptaibol-type Antibiotic from *Sepedonium ampullosporum* HKI-0053 with Neuroleptic Activity in Mice

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Ampullosporin (**I**; Ac-Trp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol) was isolated from the mycelium of *Sepedonium ampullosporum* as a new 15-membered peptaibol-type antibiotic. The structure was determined by mass spectrometric and two-dimensional NMR experiments. Ampullosporin displays narrow-spectrum antibacterial and antifungal activity, induces pigment formation by *Phoma destructiva*, causes hypothermia and decreased spontaneous locomotor activity in mice in dosages >1 mg/kg.

Microbial metabolites inducing the morphogenesis and cytodifferentiation of other microbes have been shown to display interesting pharmacological activities[1]. Recently we described a fungal strain, *Phoma destructiva*, which responded during surface cultivation to small amounts of cyclosporin A by the formation of a blackish pigment[2]. In a screening for similar inducers of pigment formation we discovered the chrysospermins[2,3] as new peptaibol-type antibiotics[4] from *Apiocrea chrysosperma* which showed the same effect as this immunomodulatory compound. But several other peptaibols such as alamethicin[4] and bergofungin[5] failed to afford this phenomenon.

In order to obtain more information about structure-activity relationships of the peptaibol antibiotics as inducers of pigment formation, we searched for other peptides from fungal cultures that could affect *Phoma destructiva*. Here, we report on a new peptaibol-type antibiotic, ampullosporin (**I**) from *Sepedonium ampullosporum* HKI-0053, which is active in the same manner as described for cyclosporin A and chrysospermins[2]. Moreover, ampullosporin was found to induce hypothermia in mice suggesting a neuroleptic activity.

Materials and Methods

Microorganism and Cultivation

Sepedonium ampullosporum HKI-0053 was obtained from the culture collection of the Hans-Knöll-Institute of Natural Product Research Jena (Germany). The strain was deposited in the DSMZ culture collection (Braunschweig, Germany) under the registry number DSM 10602. Fifteen days agar-plate cultures (25°C) were prepared as seed medium composed of malt extract 4%, yeast extract 0.4%, agar 1.5% and deionized water, pH 6.0. Four to five cm^2 areas of the agar-plate cultures were used to inoculate a liquid medium composed of glycerol 3%, glucose 1%, peptone 0.5%, NaCl 0.2%, molecular sieve (0.5 nm, Merck) 0.1% and agar 0.1%, pH 7.0. The surface cultivation was carried out at 25°C in 1 liter Erlenmeyer flasks containing 100 ml of the above medium for two weeks. Subsequently, the whole culture broth was extracted twice with two volumes of ethyl acetate.

Instruments and Analytical Methods

HPLC was carried out using a Gilson binary gradient HPLC system equipped with a UV detector (210 nm). Positive ion FAB mass spectra were recorded on an AMD 402 double-focussing mass-spectrometer with BE geometry (AMD, Intectra, Harpstedt, Germany). Ions were produced by Cs^+ ion bombardment generated by a Cs^+ gun (liquid SIMS system, AMD Intectra). Peptide solutions were mixed with 3-nitrobenzyl alcohol as matrix on the FAB probe tip. High-resolution mass measurements were carried out using the peak matching technique.

Positive ion electrospray-ionization and collision-induced MS/MS mass spectra (CID-MS/MS) were recorded on a triple quadrupole instrument Quattro

(Fisons Biotech VG; Altrincham, England). Samples dissolved in acetonitrile/water (1:1; 0.2 mg/ml) were directly applied to a nebulizer-assisted electrospray ion source. Ammonium acetate was added to the solution of sample to promote the formation of $[M+H]^+$ ions.

NMR spectra were recorded at 300 K in DMSO-d_6 on an Avance DRX 500 spectrometer (Bruker, Rheinstetten, Germany) with a sample concentration of 16 mM. Chemical shifts (Table 1) are given in ppm relative to internal TMS. The HMBC spectrum was optimized to a long range coupling constant of 7 Hz and the mixing time of the NOESY spectrum was 300 milliseconds. TOCSY and HSQC-TOCSY experiments were run with a spin lock time of 80 milliseconds. DQF-COSY, HSQC, HSQC-TOCSY and HMBC spectra were recorded using pulsed field gradients rather than phase cycling for coherence pathway selection. In F1 one order of zero filling was performed, and in the case of HSQC and HMBC a foreward linear prediction[6,7] was applied.

Analysis of Amino Acids

Total hydrolysis of **I** was carried out in sealed tubes with 6 N HCl at 110°C for 18 hours. MARFEY's reagent[8] was used to obtain the diastereomeric derivatives of chiral amino acids and leucinol. These were subjected to HPLC (Lichrospher C_{18}, 5 μm, 250 × 4 mm, 40°C, elution by a gradient of pH 3.0 triethylamine-H_3PO_4 buffer and acetonitrile)[2,8].

Determination of Activities of I against Microorganisms

The antibiotic activity of **I** was determined as the diameter of inhibition zone caused by 150 μg ampullosporin (**I**) added to agar wells (9 mm diameter) in a standardized agar plate diffusion assay[9]. All the microbial strains used for testing were obtained from the culture collection of the Hans-Knöll-Institute of Natural Products Research Jena (Germany). The inducing effect on pigment formation by *Phoma destructiva* was determined as reported earlier[2]. Thereby samples of microbial extracts or chromatographic fractions dissolved in methanol (50 μg/50 μl) were given to agar wells. The following media were used for strain cultivation and agar diffusion assay: malt agar medium (malt extract 4%, yeast extract 0.4%, agar 1.5%), standard I nutrient agar (Serva) and Sabouraud-2% glucose agar (Difco).

Fig. 1. Amino acid sequence of ampullosporin (**I**).

Acetyl-L-Trp[1]-L-Ala[2]-Aib[3]-Aib[4]-L-Leu[5]-Aib[6]-L-Gln[7]-Aib[8]-Aib[9]-Aib[10]-L-Gln[11]-L-Leu[12]-Aib[13]-L-Gln[14]-L-Leucinol[15]

Aib: α-aminoisobutyric acid.

In Vivo Determination of Neuroleptic Activity

Neuroleptic activity of several peptaibols was determined according to THOMPSON[10]. Solutions with different concentrations of peptaibols were administered intraperitoneally in male and female mice (strain NMRI from the Central Breeding Laboratory for Animals, Beutenberg campus Jena, dosages 1, 10, 20, 40, and 100 mg/kg body weight (b.w)). Basal body temperature was measured by a thermistor probe into the rectum of the animal until a stable temperature was shown on the thermometer. Records of the colonic temperature occured 0.5, 1, 3, 5, 8, 24, 48, 96 hours after administration of peptaibols. Hypothermic potency peak-time of hypothermic response and duration was recorded by use of a single dose response. Past it, the animals behaviour was observed using a scoring sheet.

Results and Discussion

Screening

In the primary screening for peptaibols stimulating the pigment formation by *Phoma destructiva* a series of fungal extracts were subjected to the pigment formation assay using agar plate cultures of *Phoma destructiva*[2]. As was shown in our preceding work, electrospray mass-spectrometry (ESI-MS) could be used as a powerful tool for the discovery of new microbial peptides such as bergofungin[5], even in crude culture extracts[12]. Hence, positive extracts from a primary microbiological screening were subjected to a secondary screening involving ESI-MS and, subsequently, CID-MS/MS of the suggested quasi-molecular ions to record the daughter-ion spectra. In this way, the extract of a fungal strain,

Fig. 2. Isolation of ampullosporin from the culture broth of *Sepedonium ampullosporum* HKI-0053.

Culture broth (40 liters)
↓
Ethyl acetate extract (7 g)
| silica gel chromatography, *n*-hexane, *n*-hexane-ethyl acetate, ethyl acetate, methanol
↓
Methanol eluate fraction (380 mg)
| Sephadex LH-20 (MeOH)
↓
Crude **I**
| HPLC (RP_{18}) (gradient H_2O to acetonitrile 30 minutes)
↓
Pure ampullosporin (**I**) (130 mg)

Sepedonium ampullosporum HKI-0053, was found to contain a new peptide detectable due to *m/z* 1645.4 ($[M+Na]^+$). Moreover, ESI-CID-MS/MS experiments displayed the occurrence of several fragment peaks differing by 85 mass units (see also the FAB mass spectrum in Fig. 4). This difference is attributable to α-aminoisobutyric acid moiety as the most frequently occurring amino acid constituent of the peptaibol-type family of antibiotics[2].

Isolation

Ampullosporin (**I**, Fig. 1) was isolated from the ethyl acetate extract of 40 liters of a surface culture of *Sepedonium ampullosporum* HKI-0053 as shown in Fig. 2. The crude extract (7 g) was subjected to column chromatography on silica gel 60 (Merck, 3 × 40 cm). The column was eluted sequentially with 500-ml portions of *n*-hexane, *n*-hexane - ethyl acetate (8 : 2, 7 : 3, 1 : 1, 1 : 3,

Table 1. ^1H and ^{13}C NMR assignments for ampullosporin (**I**) in DMSO-d_6.

Residue		δ_H (*J* (Hz))	δ_C	Residue		δ_H (*J* (Hz))	δ_C
Ac	CO		170.65 s	Aib[9]	NH	7.91 (br, s)	
	CH_3	1.84 (s)			α		55.85 s
Trp[1]	NH	8.21 (d, 7.2)			β_1	1.38 (s)	26.48 q
	α	4.38 (ddd, 7.2, 5.5, 4.8)	54.75 d		β_2	1.35 (s)	22.11 q
	β_1	2.97 (dd, 15.2, 5.5)	27.19 t		CO		175.21 s
	β_2	3.11 (dd, 15.2, 4.8)		Aib[10]	NH	7.55 (br, s)	
	1′	10.82 (d, 2.4)			α		55.74 s
	2′	7.21 (d, 2.4)	123.70 d		β_1	1.48 (s)	26.64 q
	4′	7.53 (d, 7.9)	118.19 d		β_2	1.36 (s)	22.72 q
	5′	6.94 (t, 7.9)	118.11 d		CO		176.13 s
	6′	7.04 (t, 7.9)	120.88 d	Gln[11]	NH	7.77 (br, d, 5.7)	
	7′	7.31 (d, 7.9)	111.32 d		α	3.91 (dd, 7.0, 5.7)	55.57 d
	CO		173.11 s		β	1.96 (m), 2.04 (m)	26.48 t
Ala[2]	NH	8.16 (br, d, 5.5)			γ	2.15 (m), 2.37 (m)	31.98 t
	α	4.01 (dq, 6.8, 5.5)	50.60 d		δ		173.58 s
	β	1.22 (d, 6.8)	16.00 q		NH_2	6.71 (br, s), 7.17 (br, s)	
	CO		174.21 s		CO		173.88 s
Aib[3]	NH	8.23 (br, s)		Leu[12]	NH	7.79 (d, 7.0)	
	α		55.77 s		α	4.05 (m)	53.16 d
	β_1	1.36 (s)	25.73 q		β	1.58 (m), 1.70 (m)	38.78 t
	β_2	1.33 (s)	22.34 q		γ	1.74 (m)	24.17 d
	CO		174.91 s		δ_1	0.83 (d, 6.6)	20.96 q
Aib[4]	NH	7.99 (br, s)			δ_2	0.85 (d, 6.4)	22.70 q
	α		55.67 s		CO		173.40 s
	β_1	1.38 (s)	26.82 q	Aib[13]	NH	7.49 (br, s)	
	β_2	1.33 (s)	22.51 q		α		56.29 s
	CO		176.64 s		β_1	1.40 (s)	25.98 q
Leu[5]	NH	7.66 (d, 5.6)			β_2	1.36 (s)	24.01 q
	α	3.87 (ddd, 7.1, 5.6, 4.8)	54.55 d		CO		173.98 s
	β	1.56 (m), 1.78 (m)	38.90 t	Gln[14]	NH	7.16 (br, d, 7.7)	
	γ	1.71 (m)	24.56 d		α	3.97 (m)	53.44 d
	δ_1	0.83 (d, 6.5)	21.42 q		β	1.92 (m), 2.04 (m)	27.11 t
	δ_2	0.91 (d, 6.5)	22.40 q		γ	2.09 (m), 2.18 (m)	31.71 t
	CO		174.09 s		δ		173.98 s
Aib[6]	NH	7.82 (br, s)			NH_2	6.69 (br, s), 7.12 (br, s)	
	α		55.85 s		CO		170.98 s
	β_1	1.46 (s)	26.64 q	Leuol[15]	NH	7.05 (d, 7.5)	
	β_2	1.34 (s)	22.45 q		α	3.75 (m)	48.76 d
	CO		175.83 s		β	1.34 (m), 1.36 (m)	39.73 t
Gln[7]	NH	7.38 (br, d, 5.5)			β′	3.18 (m), 3.30 (m)	64.02 t
	α	3.82 (dt, 7.0, 5.5)	56.02 d		γ	1.63 (m)	24.00 d
	β	1.95 (m), 2.00 (m)	26.07 t		δ_1	0.79 (d, 7.0)	21.76 q
	γ	2.11 (m), 2.21 (m)	31.13 t		δ_2	0.84 (d, 6.5)	23.58 q
	δ		173.18 s				
	NH_2	6.72 (br, s), 7.19 (br, s)					
	CO		173.35 s				
Aib[8]	NH	7.95 (br, s)					
	α		55.85 s				
	β_1	1.41 (s)	25.73 q				
	β_2	1.34 (s)	22.67 q				
	CO		175.26 s				

Abbreviations: s; singlet, d; doublet, t; triplet, q; quartet, br; broad, m; multiplet.

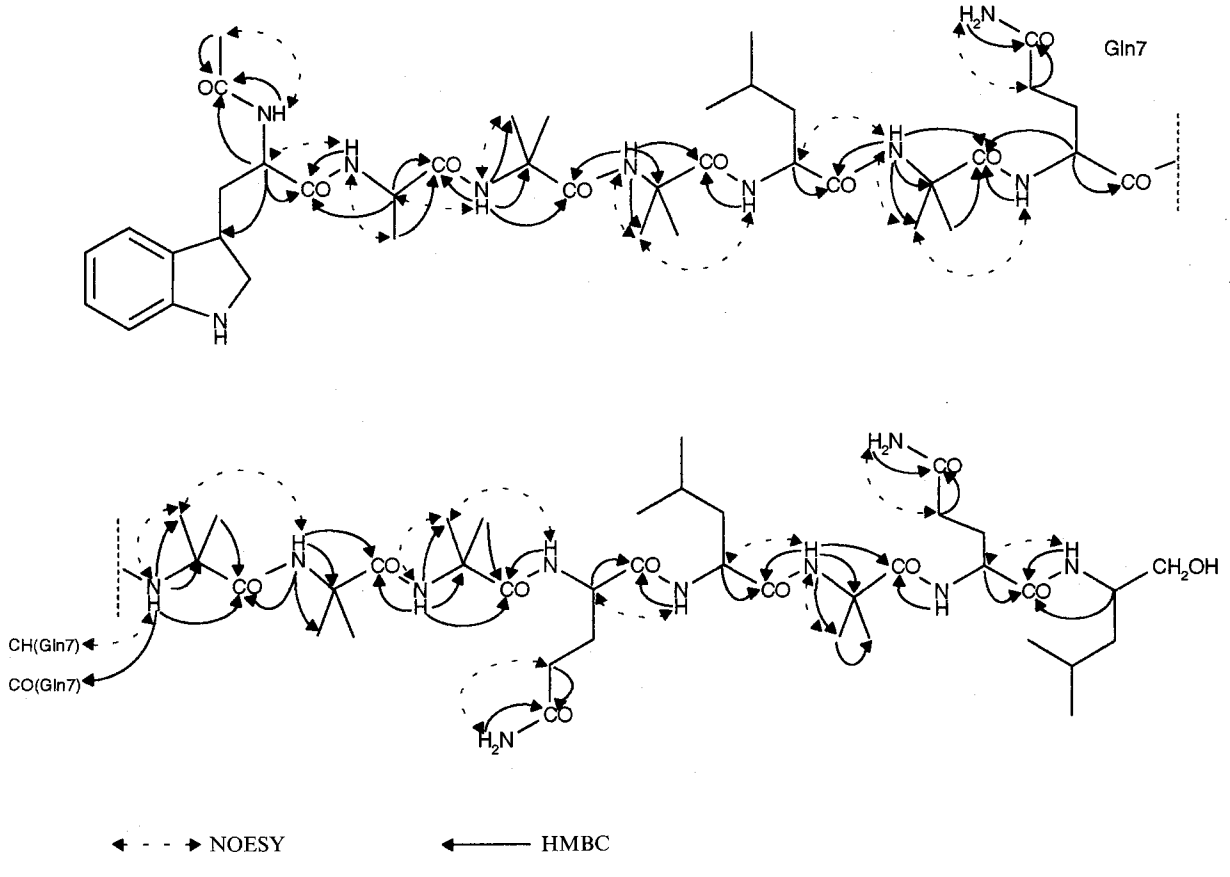
0:1) and finally compound **I** was obtained by elution with methanol. The residue of the evaporated methanol eluate (380 mg) was subjected to chromatography on Sephadex LH-20 (5 × 30 cm, methanol as eluent). The isolation procedure was checked by bioassay of the fractions using *Phoma destructiva* as the assay organism[2] and monitoring of the quasi-molecular ion *m/z* 1645 by electrospray mass spectrometry. Final purification was carried out by preparative HPLC on silica gel RP_{18} (Spherisorb ODS-2, 5 μm, 2.5 × 25 cm) using a binary gradient of water/acetonitrile ranging from 95% water at 0 min to 95% acetonitrile at 30 minutes (flow rate 10 ml/minute, detection at 210 nm). Ampullosporin eluted at 15 minutes as a single component. By repeated preparative HPLC, 130 mg of pure ampullosporin was isolated as a colorless mass melting at ≥230°C (decomposition). **I** dissolves well in nonpolar and polar organic solvents such as chloroform, methanol and dimethylsulfoxide. The chiral nature of ampullosporin was confirmed by optical rotation $[a]_D^{25}$ −30.6° (0.5 cm cuvette, 2.03 mg/ml methanol).

Structure Elucidation

The FT-IR spectrum showed diagnostic bands at (ν_{max}; KBr) 1204, 1288, 1361, 1393, 1456, 1532, 1654 (CO), 2950 and 3315 cm^{-1} (NH). Analysis of the ^1H and ^{13}C NMR spectra (Table 1) indicated a peptide structure for **I**. Seven Aib units were easily detected due to the presence of seven quaternary α-carbons between $\delta = 55.57$ and 56.29. Unambiguous assignments of the ^1H and ^{13}C chemical shifts of Trp-1, Ala-2, Leu-5 and -12, Gln-7, -11 and -14, and leucinol-15 were achieved by use of DQF-COSY, TOSCY, HSQC and HSQC-TOCSY 2D NMR data. The backbone structure as well as the chemical shift assignments of the seven Aib units were determined by HMBC and NOESY experiments (Fig. 3). Due to the shift dispersion of the Aib-NH resonances it was possible to assign all the overlapping α, β_1 and β_2 carbons and protons of these units. The only ambiguous connectivity after the detailed analysis of ^1H, ^{13}C long range couplings and nuclear Overhauser effects occured between Aib-9 and Aib-10 due to the severe overlap of the carbonyls of Aib-8 (175.26 ppm) and Aib-9 (175.23 pm) and the lack of chemical shift separation of the corresponding β_1 and β_2 methyls. But there is no alternative to a bond between Aib 9 and Aib 10 for connecting the two remaining subunits.

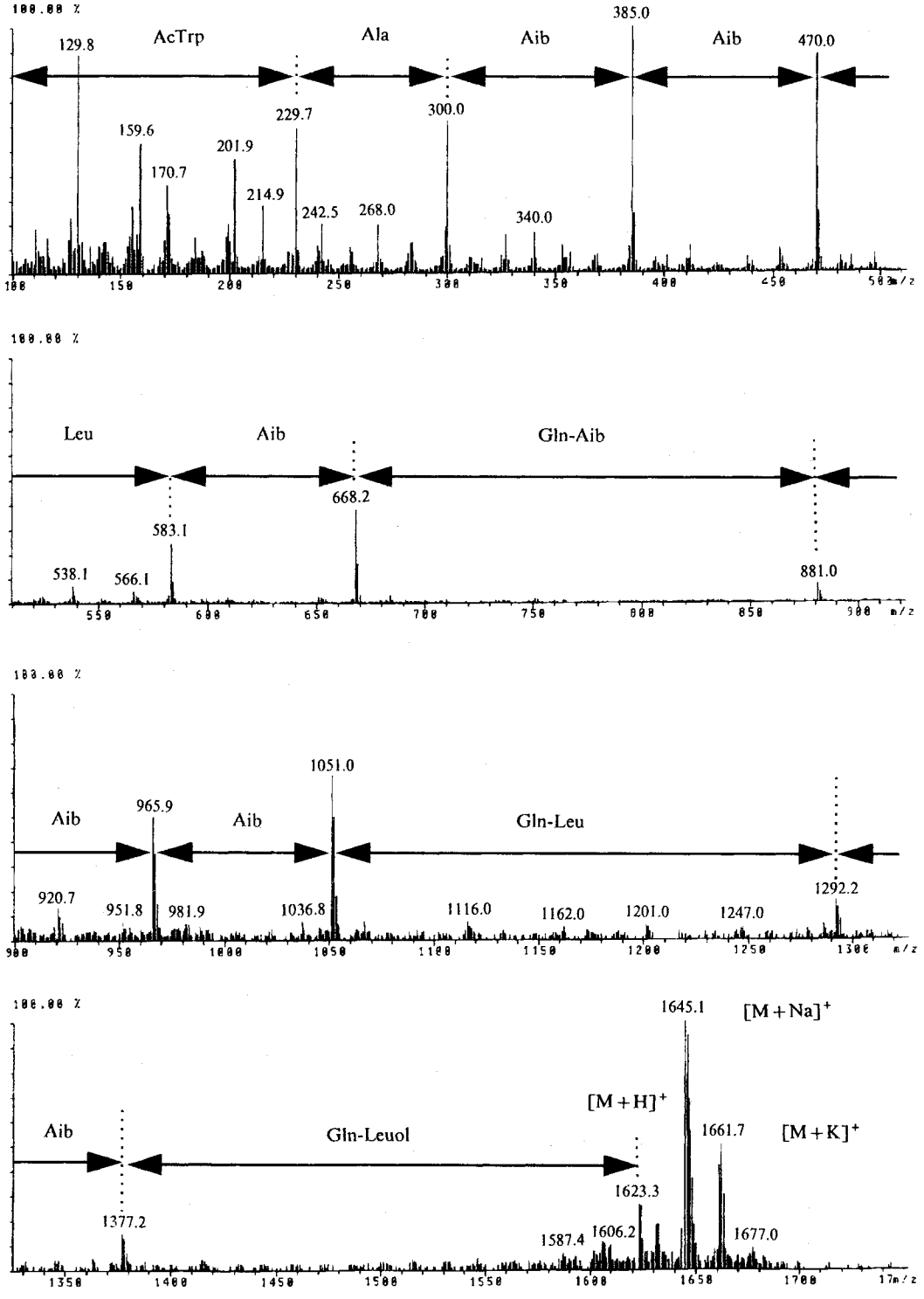
These conclusions were supported by the mass spec-

Fig. 3. Important NOE's and ^1H, ^{13}C long range couplings derived from NOESY and HMBC experiments of **I**.



trometric data. The HRFAB mass spectrum (Fig. 4; 3-nitrobenzyl alcohol matrix) displayed *m/z* 1622.9657 ($[M+H]^+$, calcd. 1622.9653 for $C_{77}H_{128}N_{19}O_{19}$), *m/z* 1644.9476 ($[M+Na]^+$, calcd. 1644.9448 for $C_{77}H_{127}N_{19}O_{19}Na$) and *m/z* 1661.7 $[M+K]^+$. During the high-resolution FAB experiments the admixture of bergofungin and reference to its quasi-molecular ion (*m/z* 1549.1; $[M+Na]^+$) with a known nominal mass[5] was of particular advantage. Moreover, the diagnostic B-type fragments[12,13] (see Fig. 4) gave full support to the structure of **I** as shown in Fig. 1. The most important diagnostic fragments occured at *m/z* = 965.9 and 1051.0,

Fig. 4. FAB mass spectrum of ampullosporin I.



Abbreviations: Aib; α-aminoisobutyric acid, Gln; L-glutamine; Leu: L-leucine, Ala; L-alanine, Trp; L-tryptophane, Leuol; L-leucinol.

proving the connection between Aib 9 and Aib 10. Further confirmation was obtained from the electrospray mass spectrum. In addition to *m/z* 1645.4 $[M+Na]^+$, positive ion peaks with *m/z* 812.6 ($[M+2H]^{2+}$, 823.4 $[M+H+Na]^{2+}$, and *m/z* 834.1 $[M+2Na]^{2+}$ attested to the nominal molecular mass of 1622 for **I**. CID-MS/MS of the single ion peak *m/z* 1622.2, which was observed after the addition of ammonium acetate to the sample, afforded the same series of fragments as visible in the FAB mass spectrum.

Finally, the absolute stereochemistry of the amino acids (Trp, Ala, Leu, Gln) and leucinol in ampullosporin was determined to be *S* after hydrolysis of **I**, derivatization by MARFEY's reagent[8] and HPLC analysis of the diastereomeric products in comparison to chiral reference compounds[2,5,14]. As in the other peptaibols containing leucinol (Leuol)[4] this moiety of **I** also possesses the *S*-configuration[2,14]. The above data show ampullosporin **I** as a new representative of the peptaibol family of antibiotics.

Antimicrobial Activity

Ampullosporin displays moderate narrow-spectrum antibacterial and antifungal activity. During the common agar-well diffusion assay[9] 150 μg **I** per agar well (9 mm diameter) caused 28 mm diameter of inhibition zone with *Bacillus subtilis* ATCC 6633, 23 mm with *Staphylococcus aureus* SG 511, 16 mm with *Pseudomonas aeruginosa* K599/WT, and 18.5 mm with *Escherichia coli* SG458 (standard nutrient agar Serva). The same amount of **I** afforded 21 mm diameter of inhibiton zone with *Sporobolomyces salmonicolor* SBUG 549 and 14 mm with *Rhodotorula rubra* IMET 25030 as test organisms (Sabouraud-2% glucose agar DIFCO). No activity was found against a series of fungi and yeasts such as *Candida albicans*. On agar-plate cultures of *Phoma destructiva*[2], 30 μg of **I** (50 μl methanol) per agar well was the minimum concentration affording pigment formation by this organism. This is a much lesser amount as was needed in the case of the chrysospermins[2].

Neuroleptic (Antipsychotic) Acitivity of I

Despite of the similarity of cyclosporin A and chrysospermins with regard to their inducing effect on pigment formation by *Phoma destructiva* the latter displayed no immunosuppressory activity (unpublished results). In order to establish correlations of the above effect on *Phoma destructiva* to pharmacological activities of drugs we checked a series of pharmacological agents for their activity as inducers of pigment formation. Surprisingly, chlorpromazin as a known antipsychotic (neuroleptic) agent was found to be active in the same manner. This finding intended us to investigate *vice versa* the antipsychotic effects of ampullosporin in small laboratory animals (mice). In dosages of 1 to 100 mg/kg b.w. a dose-dependent decrease of body temperature down to room temperature and decreased spontaneous locomotor activity were observed. The earliest decrease of body temperature was recorded 30 minures after administration and lasted up to 4 days after 40 mg/kg b.w. The effect was in the same order of magnitude as was described for chlorpromazin[11] both with regard to dose and duration. Chrysospermins (as 1 : 1 mixtures of A/B and C/D components)[2] displayed a comparable neuroleptic activity, but bergofungin[5] which is incapable of inducing pigment formation also failed to afford a chlorpromazin-like effect on mice. Obviously there is a correlation between the induction of pigment formation by *Phoma destructiva* and the capacity of reduction of bodies temperature in mice. It appears as promising to study structure-activity relationships of peptaibol-type antibiotics concerning the pigment formation by *Phoma destructiva* and the potential neuroleptic activity more in detail.

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