Helioferins; Novel Antifungal Lipopeptides From *Mycogone rosea*: Screening, Isolation, Structures and Biological Properties

U. GRÄFE, W. IHN, M. RITZAU, W. SCHADE,
C. STENGEL, B. SCHLEGEL, W. F. FLECK, W. KÜNKEL,
A. HÄRTL and W. GUTSCHE

Hans-Knöll-Institute of Natural Product Research,
Beutenbergstraße 11, D-07745 Jena, Germany

(Received for publication August 8, 1994)

Helioferins A and B were detected as novel aminolipopeptides in cultures of *Mycogone rosea* DSM 8822 in the course of a screening for mediators of helianthate anion transfer from aqueous to toluene phases. Their structures as novel antibiotics and cytotoxic agents were elucidated by mass spectrometry and NMR spectroscopic methods. Antimicrobial activity was estimated against *Candida albicans* and Gram-positive bacteria including *Mycobacterium* spp.

Formation of lipophilic ion pairs with lipophilic counter ions is well known to enhance the penetration of hydrophilic drug ions through biological membranes and thus to alter their pharmacokinetic properties[1]. Recently we have shown that macrodiolides carrying a dimethylamino function (see *e.g.* pamamycin-621[2~4]) are capable of conveying water-soluble dye anions such as helianthate (methylorange) from aqueous to toluene phases[5]. In screening for similar helianthate-conveying metabolites we used mycelium extracts of more than 300 *Streptomyces* strains, but among them we detected only two new producers of pamamycins. Our subsequent efforts employing fungal extracts disclosed a strain of *M. rosea* as the producer of novel aminolipopeptide antibiotics which strongly promoted the transfer of water-soluble anions to organic solvents.

Here we report on screening, isolation, structure elucidation and biological properties of the helioferins A and B (Fig. 1, **1** and **2**, respectively) as new representatives of the leucinostatin-trichopolyn group of aminolipopeptide antibiotics[6~10].

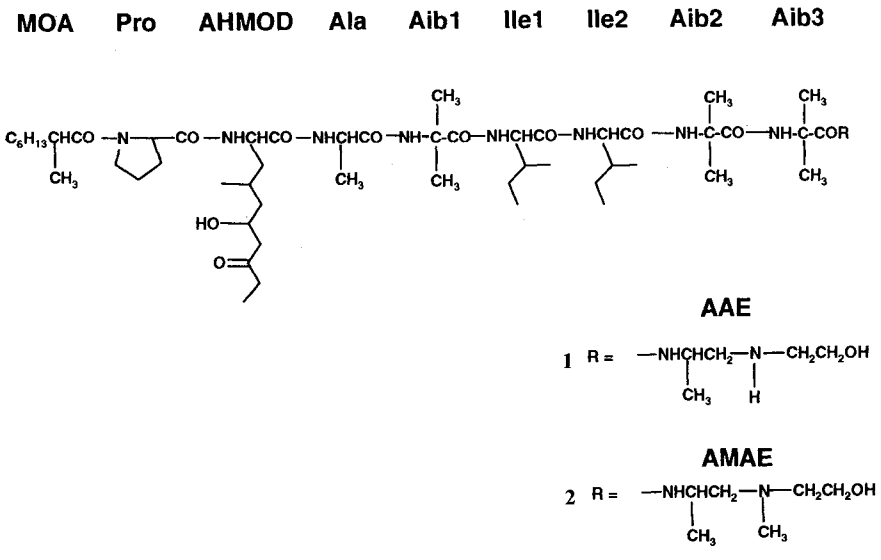
Results and Discussion

Screening for Anion-conveying Metabolites

The search for production of helianthate-conveying metabolites by more than 300 strains of *Fungi Imperfecti* was carried out by use of a modified two-phase assay as was described previously[3] (see Experimental).

Culture extracts of *M. rosea* DSM 8822 (My 299) and *Mycogone* sp. My 116 promoted helianthate transfer from buffer to organic solvent in a manner similar to

Fig. 1. Structures of helioferins A (**1**) and B (**2**).



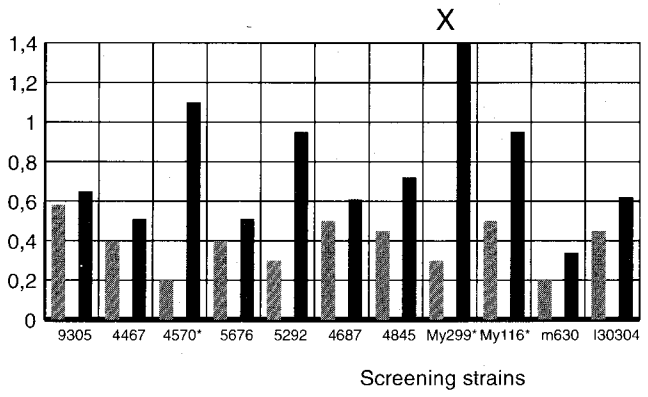
that demonstrated for the pamamycin-producing strains *Streptomyces aurantiacus* JA 4570 and *Streptomyces* sp. JA 5292[3]. Due to this property the active principle has been named helioferin (Fig. 2).

Isolation and Physico-chemical Properties

The isolation of helioferins from surface cultures of *M. rosea* DSM 8822 was carried out by culture extraction with ethylacetate, chromatographic purification, and separation of components **1** and **2** by preparative HPLC (Fig. 3). Active fractions were recognized either by helianthate or antimicrobial assays (see above). Usually, the strain produces **1** and **2** in the ratio of 3:7. Due to the strongly basic and lipophilic properties of the helioferins, HPLC chromatographic separation on reverse phase silica gel (*e.g.* Nucleosil 7 μm RP_{18}, Macherey & Nagel Düren, column 25 × 250 mm; flow rate 10 ml/minute; detection at 210 nm) requires admixture of a tertiary amine to the eluent, preferably triethylamine (Fig. 3). The physico-chemical properties of **1** and **2** are shown in Table 1. They convey helianthate anions very effectively from the aqueous to the toluene phases: 0.5 μmol of 2 dissolved in 2 ml toluene and 2 ml 0.1 M phosphate buffer (pH 6.0, containing 0.2% methyl-orange) causes an increase of O.D. of 1.6 units.

Fig. 2. Screening for mediators of helianthate transfer by measurement of the optical density of the upper toluene phase at 430 nm.

Half shaded columns: blank, no helianthate in the lower phase; full shaded columns: increase of OD 430 nm after the addition of helianthate to the aqueous phase.



Screening strains such as 9305, 4467, 5676, 4687 and 4845 as *Streptomycetes* sp. were obtained from our strain collection. Strains 4570 (*Streptomyces aurantiacus* JA 4570) and 5292 (*Streptomyces* sp. JA 5292) are new producers of pamamycins. My 299 and My 116 are strains of *M. rosea* DSM 8822 (My 299) and *Mycogone* sp. My 116. M 634 and I 30304 are synonymes for *Apiocrea chrysosperma* M 634 and *Ascodesmus* I 30304 *M. rosea* DSM 8822 (My 299 marked by "×") was used for detailed investigations.

Fig. 3. Isolation procedure of helioferins from the mycelium of *M. rosea* DSM 8822.

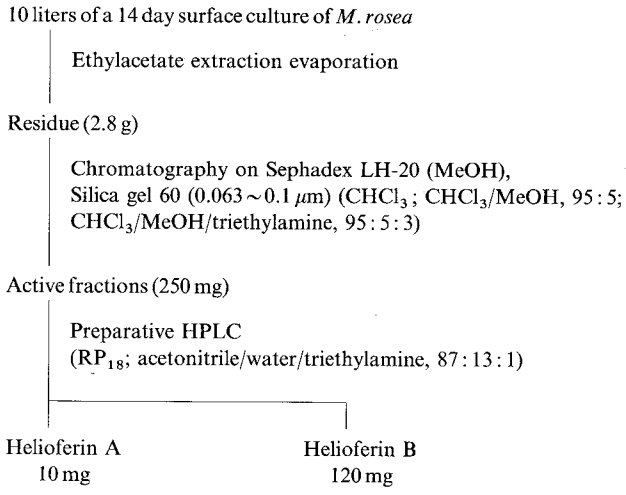


Table 1. Physico-chemical properties of **1** and **2**.

	1	**2**
Appearance	White microcrystalline powder	White microcrystalline powder
Melting point	200~205°C (decomposition)	200~205°C (decomposition)
HRFAB-MS	$(M+H)^+$: 1121.7912 (Calcd: 1121.7913) for $C_{57}H_{105}N_{10}O_{12}$	$(M+H)^+$: 1135.7960 (Calcd: 1135.7990) for $C_{58}H_{107}N_{10}O_{12}$
Molecular weight	1121	1135
Molecular formula	$C_{57}H_{104}N_{10}O_{12}$	$C_{58}H_{106}N_{10}O_{12}$
UV (MeOH)	End absorption	End absorption
Color reaction on TLC	Positive reaction with I_2 vapour or 2% vanillin in conc. H_2SO_4	
Rf on TLC (silica gel sheets Merck Kieselgel 60 F_{254};CH_2Cl_2 + 5% Triethylamine)	0.2	0.1
HPLC: retention time (minute)(Spherisorb RP_{18}; 5 μm; 4 × 250 mm; 1 ml/minute, 83:17:1; 210 nm, 23°C acetonitril/H_2O/triethylamine, 83:17:1)	1.85	2.0
Retention time on preparative HPLC (Nucleosil RP_{18}, 7 μm, 25 × 250 mm, 10 ml/minute acetonitril/H_2O/triethylamine, 83:17:1)	10.8	12.0

Structure Elucidation

The structures of **1** and **2** as shown in Fig. 1 were elucidated by mass-spectrometric investigations, NMR spectroscopic measurements, chemical hydrolysis and HPLC analysis of the constitutive amino acids.

The molecular compositions of $C_{57}H_{104}N_{10}O_{12}$ for **1** and $C_{58}H_{106}N_{10}O_{12}$ for **2** were readily inferred from the HRFAB mass spectra and elemental analysis.

The peptidic nature of **1** and **2** suggested by λ_{max} 1660 and 3400 cm^{-1} in the IR spectrum was confirmed by acidic hydrolysis. After derivatization with dabsyl chloride[13] Ala, Pro, Aib (α-aminoisobutyric acid) and Ile were found in the ratio of 1 : 1 : 3 : 2 in the hydrolysates of both helioferins. As was confirmed by derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine-amide (Marfey's reagent)[14] the chiral amino acids were found to be of the *S*-configuration.

Moreover, direct electrospray MS analysis of the hydrolysates revealed the above $(M+H)^+$ ions of the amino acids and the substituents at the nitrogen and carbon terminal ends of the peptide backbone. Among them, *m/z* 214 corresponded to $(M+H)^+$ of 4-methyl-6-(2-oxobutyl)-2-piperidine-carboxylic acid suggesting that helioferins contain the same 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid moiety (AHMOD) as the related leucinostatins[12,15,16] and trichopolyns[10].

FAB mass spectra of the helioferins using direct inlet system (Fig. 4) suggested the bonding sequence of the amino acids and the C- and N-terminal substituents. However, additional proof for the proposed structure was required for reasons related to the background level due to the NBA matrix, the fragment ions resulting from B-type cleavages, and many C-terminal ions arising from several types of cleavage.

A series of MS-MS experiments using FAB ionisation and linked B/E-, MIKES(E)-scan, ES ionisation and MS-MS triple quadropol techniques provided full evidence of the sequence of amino acids in **1** and **2**. As the result of these experiments, B-type fragmentation was predominant for N-terminal ions and all possible fragments from B1 to B9 were observed. The molecular composition of the N-terminal ions was finally determined by exact mass measurements using HRFAB-MS.

Conclusive evidence for the chemical constitution of helioferins **1** and **2** as depicted in Fig. 1 was obtained from high-field NMR spectroscopy (Fig. 5, Table 2). The ^{13}C NMR spectra of **1** and **2** showed 57 and 58 carbons, respectively. They were classified as seventeen (sixteen in 2) methyls, seventeen methylenes, 11 methines, three quaternary carbons, 9 amide carbonyles and one ketone carbonyl due to the DEPT spectra.

By the use of various homo- and heteronuclear 2D NMR techniques, full assignation of ^1H and ^{13}C chemical shift data could be achieved. Particularly, the nitrogen and carbon terminus and the unusual amino

Fig. 4. FAB-MS fragmentation pattern of **2**.

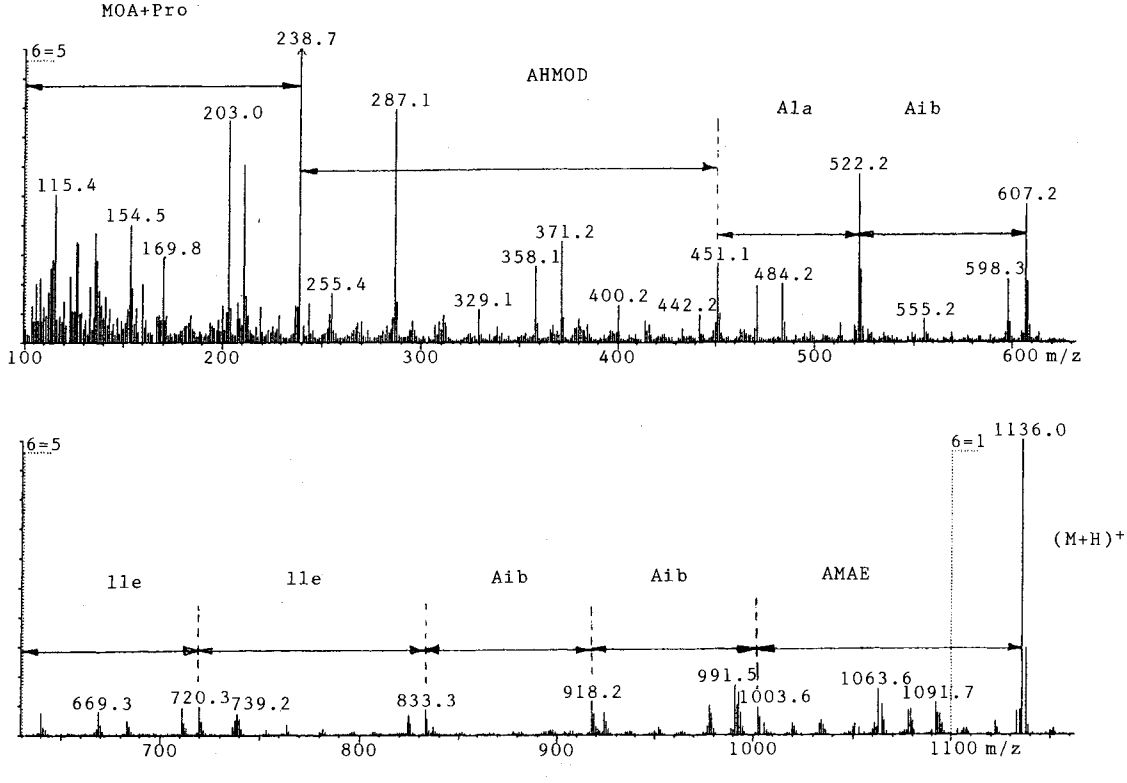


Table 2. Assignation of ^{1}H and ^{13}C chemical shift data of the **1** and **2** (in $CDCl_3$; δ in ppm relative to internal TMS).

AS	No.	1		2	
		^{13}C	^{1}H	^{13}C	^{1}H
MOA[a]	1	177.9 s	—	177.9 s	—
	2	38.0 d	2.67 (m)	37.9 d	2.68 (m)
	3	33.9 t	1.39, 1.60 (m)	33.8 t	1.38, 1.59 (m)
	4	27.3 t	1.21, 1.33 (m)	27.3 t	1.22, 1.34 (m)
	5	29.3 t	1.27 (m)	29.2 t	1.26 (m)
	6	31.6 t	1.24 (m)	31.6 t	1.22 (m)
	7	22.5 t	1.26 (m)	22.5 t	1.27 (m)
	8	14.0 q	0.84 (t, 6.9)	13.9 q	0.85 (t, 6.9)
	9	17.0 q	1.14 (d, 7.0)	17.1 q	1.14 (d, 7.0)
Pro	1	173.9 s	—	173.8 s	—
	2	62.3 d	4.26 (dd, 8.2, 6.0)	62.3 d	4.26 (dd, 8.2, 6.0)
	3	29.5 t	1.98, 2.33 (m)	29.5 t	1.97, 2.32 (m)
	4	25.3 t	1.98, 2.11 (m)	25.3 t	1.97, 2.11 (m)
	5	47.6 t	3.62 (m), 3.78 (m)	47.6 t	3.62 (m), 3.77 (m)
AHMOD[b]	NH	—	7.74 (br)	—	7.72 (br)
	1	175.0 s	—	173.8 s	—
	2	55.2 d	3.98 (m)	55.3 d	3.96 (m)
	3	35.4 t	1.56, 1.79 (m)	35.5 t	1.57, 1.77 (m)
	4	27.3 d	1.84 (m)	27.4 d	1.84 (m)
	5	42.1 t	1.24, 1.53 (m)	41.9 t	1.27, 1.54 (m)
	6	64.9 d	4.16 (m)	64.9 d	4.16 (m)
	7	48.9 t	2.49, 2.59 (m)	48.9 t	2.51, 2.57 (m)
	8	213.8 s	—	213.2 s	—
	9	37.0 t	2.47 m	37.0 t	2.48 m
	10	7.5 q	1.03 (t, 6.7)	7.4 q	1.04 (t, 6.7)
	11	20.7 q	0.96 (d, ~7)	20.7 q	0.97 (d, ~7)
Ala	NH	—	7.77 (br d, 5.3)	—	7.77 (br d, 5.3)
	1	174.2 s	—	174.1 s	—
	2	52.0 d	3.96 (m)	51.8 d	3.97 (m)
	3	16.3 q	1.47 (d, ~7)	16.2 q	1.46 (d, ~7)
Aib 1	1	176.2 s	—	176.2 s	—
	2	56.6 s	—	56.5 s	—
	3	23.0 q	1.47 (s)	22.9 q	1.47 (s)
	3′	27.3 q	1.49 (s)	27.2 q	1.50 (s)
Ile 1	NH	—	7.16 (br d, 4.5)	—	7.16 (br d, 4.5)
	1	173.1 s	—	172.8 s	—
	2	60.6 d	3.83 (t, 6.0)	60.8 d	3.82 (m)
	3	35.9 d	1.93 (m)	35.8 d	1.94 (m)
	4	25.8 t	1.32, 1.56 (m)	25.6 t	1.33, 1.55 (m)
	5	11.4 q	0.85 (t, ~7)	11.5 q	0.86 (t, ~7)
	6	15.6 q	0.95 (d, ~7)	15.6 q	0.96 (d, ~7)
Ile 2	NH	—	7.38 (br)	—	7.37 (br)
	1	173.0 s	—	172.5 s	—
	2	60.6 d	3.80 (m)	60.5 d	3.80 (m)
	3	35.4 d	1.92 (m)	35.4 d	1.93 (m)
	4	26.2 t	1.22, 1.63 (m)	25.9 t	1.23, 1.63 (m)
	5	11.1 q	0.84 (t, ~7)	11.3 q	0.85 (t, ~7)
	6	15.3 q	0.92 (d, ~7)	15.4 q	0.92 (d, ~7)
Aib 2	1	174.0 s	—	174.1 s	—
	2	56.5 s	—	56.8 s	—
	3	22.9 q	1.49 (s)	23.6 q	1.49 (s)
	3′	26.4 q	1.46 (s)	26.3 q	1.46 (s)
Aib 3	1	175.1 s	—	174.8 s	—
	2	56.7 s	—	56.9 s	—
	3	22.5 q	1.49 (s)	24.1 q	1.48 (s)
	3′	26.8 q	1.47 (s)	27.0 q	1.46 (s)
AMAE[c]/AAE[d]	1	60.7 t	3.68 (m)	58.7 t	3.52, 3.56 (m)
	2	51.2 t	2.90, 2.70 (m)	59.3 t	2.48 (q, 7.0), 2.64 (m)
	1′	54.0 t	2.72, 2.82 (m)	63.1 t	2.35, 2.66 (m)
	2′	44.3 d	4.18 (m)	43.5 d	4.03 (m)
	3′	18.7 q	1.13 (d, 6.5)	18.6 q	1.12 (d, 6.5)
	$N-CH_3$	—	—	42.5 q	2.29 (s)

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

[a] MOA = 2-methyloctanic acid.

[b] AHMOD = 2-amino-4-methyl-6-hydroxy-8-oxo-decanoic acid.

[c] AMAE = 2-[(2′-aminopropyl)-methylamino]-ethanol.

[d] AAE = 2-(2′-aminopropyl)-aminoethanol.

Fig. 5. 600 MHz ^{1}H NMR spectrum of **2** (in $CDCl_3$; δ in ppm relative to internal TMS).

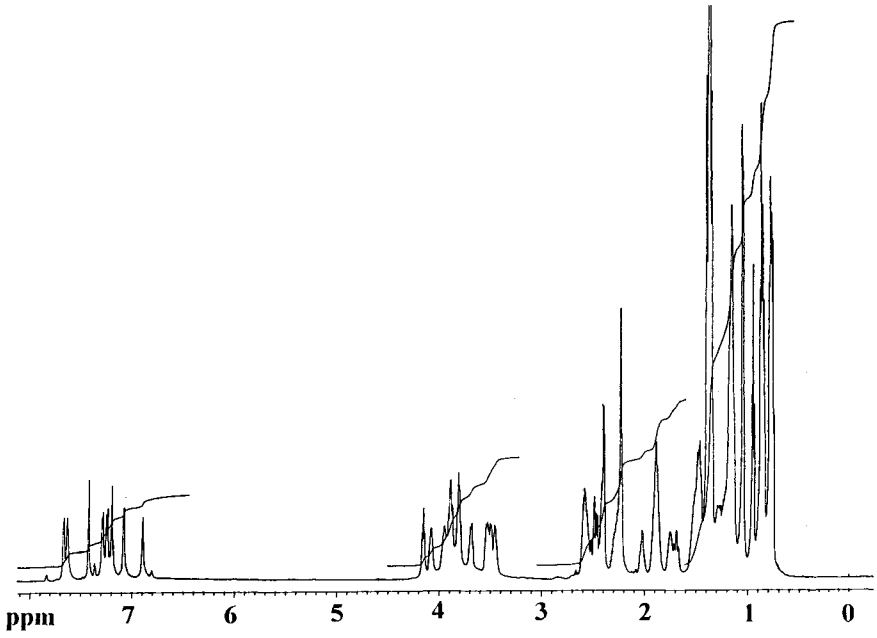
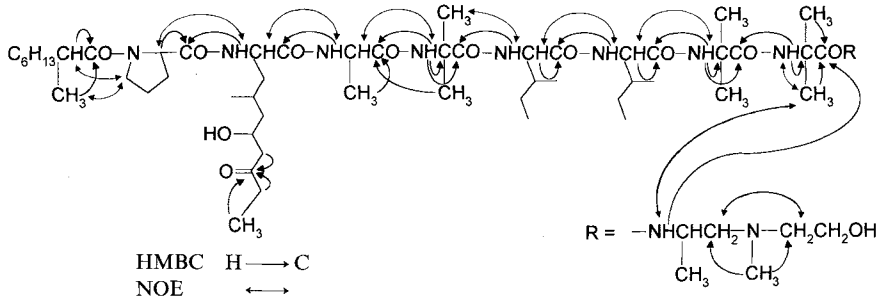


Fig. 6. Diagnostic heteronuclear long-range couplings and nuclear Overhauser effects in the subunits of **2**.



acid AHMOD (2-amino-4-methyl-6-hydroxy-8-oxo-*n*-decanoic acid) could be confirmed by COSY, NOESY, HMQC and HMBC spectra.

For instance heteronuclear long range couplings were observed in the HMBC spectrum of **2** between the ketone carbonyle of AHMOD at δ 213.2 and the two adjacent methylenes at δ 2.48 (9-CH_2) and δ 2.51/2.57 (7-$H_{\alpha/\beta}$) (Fig. 6). The structure of the N-terminus in **2** was elucidated by combining the two spin systems of this moiety (obtained by the COSY spectrum) due to $^3J_{(C,H)}$ couplings between the N–CH_3 at δ 2.29 and the methylene carbons at δ 59.3 and 63.1, respectively. Thus, the N-terminus was assigned as 2-[(2-aminopropyl)-methyl-amino]-ethanol (AMAE).

In the case of **1** a NH proton at δ 3.00 was present in this moiety instead of the N–CH_3 group, leading to 2-(2-aminopropylamino)-ethanol (AAE) as N-terminus. Accordingly the adjacent proton and carbon signals were shifted as expected[17].

The function of 2-methyloctanoic acid (MOA) as C-terminus was clearified because of the strong NOE crosspeak between 2-H of MOA (δ 2.68) and 5-H_a/H_b of Pro (δ 3.62/3.77) in the NOESY spectrum of **2**. All heteronuclear long range couplings and nuclear Overhauser effects which are important for the connectivity of the individual amino acids are summarized in Fig. 6. for **2**.

Biological Activities of the Helioferins

The helioferins A and B (as a natural 3 : 7 mixture or as single components) display strong antibacterial and antifungal activities, *e.g.* against a series of filamentous fungi and yeasts such as *e.g. C. albicans* (MIC 5 μg/ml; Table 3). The antibacterial effect is mainly directed against Gram-positive bacteria and mycobacteria with essentially no activity *vs.* Gram-negative bacteria. Moreover, helioferins are toxic to chicken embryos at concentrations >0.5 mg/kg egg and cause hemolysis at concentrations >100 μg/ml. As was also demonstrated for the leucinostatins[18~22], representatives of the same

structural group of antibiotics, the helioferins display cytotoxic activities in concentrations >0.01 5 μg/ml (IC_{50} for L 1210 leukaemia and L-929 mouse fibroblast cell lines: 0.01 ~ 0.04 μg/ml). Despite of the inhibition the cells remained viable in presence of helioferin concentrations up to 0.625 μg/ml suggesting that **1** and **2** possess mainly a cytostatic activity.

Preliminary investigations of ion transport across artificial membranes (P. GRIGORIEV *et al.*, to be published) suggested that helioferins are protonophoric antibiotics disturbing the cellular ion flux and energy metabolism. This interpretation is in accord with the uncoupling properties of the leucinostatins as chemically similar lipophilic agents[19,20,25].

Experimental

The Strain *M. rosea* and Culture Conditions

M. rosea My 299 LINK as an anamorphic representative of the Hypomyces[24], was isolated by Dr. H. DÖRFELT (University Jena, Institute of Special Botany) as a parasitic microorganism from the fruiting body of *Macrolepiota sp.* and was deposited in Deutsche Sammlung Mikroorganismen (DSM, Braunschweig/FRG) with the accession number DSM 8822 (Fig. 7). Morphological characteristics of the anamorphe form are a mycelium rich in septations, hyaline hyphae of 5 to 7 μm diameter, macroconidia of 23 ~ 33 μm diameter, conidia with ellipsoid cylindrical shape. The strain forms aerial mycelium and conidia on malt agar (g/liter: malt extract 40, yeast extract 4, agar 15, deionized water). 2 ~ 4 cm^2 pieces of malt agar cultures of *M. rosea* DSM 8822 (14 days) were inoculated into 500 ml Erlenmeyer flasks containing 100 ml of a medium composed of (g/liter) malt extract 20, glucose 10, yeast extract 2, $(NH_4)_2HPO_4$ 0.5 (tap water, pH 6.0). Cultivation was carried out as static surface cultures for 14 days at 28°C.

Table 3. Antimicrobial activities of helioferins (3 : 7 mixture of **1** and **2** in the microtiter dilution assay and agar diffusion assay*.

Microbial strain	MIC (μg/ml)
Bacillus subtilis ATCC 6633	3.0
Staphylococcus aureus	1.5
Micrococcus luteus SG 125	1.5
Escherichia coli S6 458	>100
Serratia marcescens SG 621	>100
Pseudomonas aeruginosa K 799/61	>100
Mycobacterium phlei SG 346	0.78
Mycobacterium smegmatis SG 987	3.12
Rhodotorula rubra IMET 25030*	0.78
Kloeckera brevis IH 3*	3.12
Fusarium culmorum JP 15*	6.25
Penicillium notatum JP 36*	1.56
Candida albicans ATCC 18804*	5.0

* The antifungal activities of helioferins were estimate in the agar diffusion assay (description see Ref 27).

Screening for Mediators of Helianthate Transfer

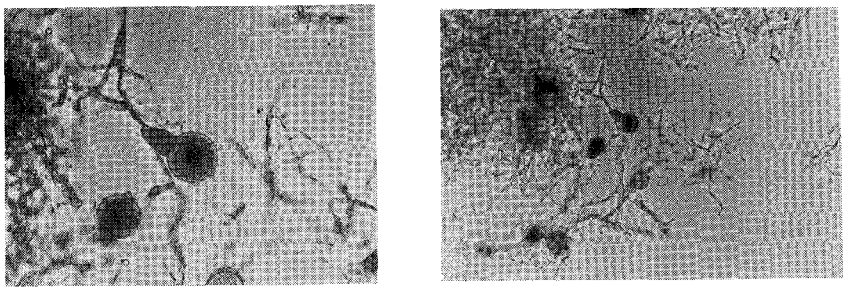
The assay system[3,5] contained 2 ml toluene and 2 ml 0.1 M potassium phosphate buffer (pH 6.0) in 10 ml tubes. The toluene phase was supplied with 100 to 500 μl of concentrated microbial extract dissolved in toluene. The mixture was well shaken, centrifuged and the optical density of the upper toluene phase measured at 430 nm. Thereafter, 200 μl of a methylorange (2 g/liter H_2O) were added. The mixture was well shaken, centrifuged and the toluene layer measured, again, to determine the O.D. at 430 nm. The difference in O.D. in comparison to the blank containing no helianthate signifies the anion conveying activity of metabolites.

Instruments

Mass-spectrometric investigations were carried out by the use of a high-resolution mass spectrometer AMD-402 (AMD Intectra, Harpstedt, Germany) and a quadrupol mass spectrometer Quattro (Fisons, VG Biotech; Altrincham, U.K.). NMR measurements were carried out using a Bruker AMX 600 instrument.

NMR measurements were carried out using a Bruker AMX 600 spectrometer with 17 and 36 mM solutions of **1** and **2**, respectively. NOESY spectra were measured in the phase sensitive mode (TIPPI) with a sweep width of 5494 Hz in F2. The mixing time was 400 ms, 256 experiments of 48 scans were recorded in F1 (zero-filled to 512 prior to Fourier transformation) with 2K

Fig. 7. Aerial mycelium and macroconidia of *M. rosea* DSM 8822 growing on malt extract agar (light micrograph, × 1500; stained by safranine) (in case the black/white photograph will be used instead of the colored ones, please note that amplification of the micrograph will be × 400!).



acquisition in F2. The HMBC spectrum was optimized for $^{2/3}J_{C,H}=7$ Hz and a low pass J-filter was used to supress ^{1}J-couplings. 256 experiments of 64 scans were sampled in F1 (zerofilling to 512) with a 2K data acquisition in F2 (sweep width 4808 Hz).

Analysis of the Amino Acids

Ten mg of the helioferins were hydrolysed by 10 ml 6 N HCl for 24 hours at 120°C. The crude amino acids were derivatized by dabsylation. Separation was achived using a gradient of sodium acetate buffer (pH 6.5) and acetonitril[14]. However, Marfey's reagent[13] was used to obtain the diasteromeric derivatives of chiral amino acids. These were subjected to HPLC (Lichrospher C_{18}, 5 μm, 125×4 mm, 40°C; elution by a gradient of triethylamine-H_3PO_4 (pH 3.0) and acetonitril[14].

Cell Cultivation and Estimation of the Cytotoxic Properties

Cytotoxicity was estimated after seeding 10^4 L1210 or L-929 cells in Eagle's minimum essential medium into 96-wells microplates (COSTAR)[26]. A helioferin stock solution (a 3 : 7 mixture of **1** and **2**, 5 mg/ml ethanol) was diluted stepwise (1 : 2, 1 : 4, 1 : 8, *etc.*) by culture medium to yield final antibiotic concentrations of >4 μg/ml. Incubation occurred at 37°C for 48, 72 and 96 hours (5% CO_2, humidified atmosphere). After fixation by glutaraldehyde, at the end of each experiment the cells were stained by methylene blue. Subsequently, the dye was dissolved by 0.33 N HCl in isopropanol (0.2 ml) followed by a gentle washing procedure. Optical density was measured by a microplate reader (Dynatech 7000) at 630 nm in single modus against one well containing solely the medium but stained in the same manner. The IC_{50} values of adherent cells (L-929) were estimated graphically from the diagram of helioferin concentration (Fig. 6) versus inhibition of cell growth. A vertical line was drawn from the point of insertion between half maximum O.D. 630 value and concentration-dependent curve of growth inhibition (see. Fig. 6). The suspension culture of L-1210 cells was analysed by the CASY1 system (Schärpe System, Reutlingen, Germany).

Agar diffusion assay of antifungal activities: 60 ml of yeast morphology agar (Difco) inoculated by 5×10^6/ cells were used to obtain test plates of 130 mm diameter. Samples were filled into 12 wells (9 mm diameter) and the plates were breeded at 28°C for 20 hours.

Acknowledgments

We are gratefully indepted to Dr. H. DÖRFELT (Institute of Special Botany, University of Jena, Germany for the strain *M. rosea* and to Dr. V. HAVLICEK (Insitute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic) for kind recording linked B/E scan mass spectra of helioferins.

References

1) AMLACHER, R.; A. HÄRTL, R. NEUBERT, U. STÖCKEL & K. WENZEL: Influence of ion-pair formation on the pharmacokinetic properties of drugs. J. Pharm. Pharmacol. 43: 794~797, 1991
2) KONDO, S.; K. YASUI, M. NATSUME, M. KATAYAMA & S. MARUMO: Isolation, physico-chemical properties and biological activity of pamamycin-607, an aerial mycelium-inducing substance from *Streptomyces alboniger*. J. Antibiotics 41: 1196~1204, 1988
3) GRÄFE, U.; C. STENGEL, U. MÖLLMANN & L. HEINISCH: Formation of lipophilic ion pairs by derivatives of pamamycin. Pharmazie 49: 343~346, 1994
4) GRÄFE, U.; R. SCHLEGEL, K. DORNBERGER, W. IHN, M. RITZAU, C. STENGEL & M. Beran: Isolation and structure elucidation of pamamycin-621, a new macrodiolide antibiotic from *Streptomyces aurantiacus*. Nat. Prod. Lett. 3 (4): 265~271, 1994
5) STENGEL, C.; G. REINHARDT & U. GRÄFE: A simple screening procedure for microbial phase-transfer mediators conveying anions. J. Basic Microbiol. 32: 339~345, 1992
6) MORI, Y.; M. TSUBAI, M. SUZUKI, K. FUKUSHIMA & T. ARATI: Isolation of leucinostatin A and one of its constituents, the new amino acid, 4-methyl-6-(2-oxobutyl)-2-piperidinecarboxylic acid, from *Paecilomyces lilacinus* A-267. J. Antibiotics 35: 543~544, 1982
7) ISOGAI, A.; A. SUZUKI, S. HIGASHIKAWA, S. KUYAMA & S. TAMURA: Constituents of a peptidal antibiotic P168 produced by *Paecilomyces lilacinus* (Thom). Samson. Agric. Biol. Chem. 44: 3029~3031, 1980
8) FUKUSHIMA, K.; T. ARAI, Y. MORI, M. TSUBAI & M. SUZUKI: Studies on peptide antibiotics, leucinostatins II. The structure of leucinostatins A and B. J. Antibiotics 36: 1613~1630, 1983
9) ARAI, T.; Y. MIKAMI, K. FUKUSTIMA, F. UTSUMI & K. YAZAWA: A new antibiotic, leucinostatin, derived from *Penicillium liliaceum*. J. Antibiotics 26: 157~161, 1973
10) FUJITA, T.; Y. TAKAISHI, A. OKAMURA, E. FUJITA, K. FUJI, N. HIRATSUKA, M. KOMATSU & J. ARITA: New peptide antibiotics, trichopolyns I and II, from *Trichoderma polysporum*. J. Chem. Soc. Chem. Commun. 1981: 585~587, 1981
11) SCOBLE, H. A.; J. E. BILLER & K. BIEMANN: A graphics display-oriented strategy for the amino acid sequencing of peptides by tandem mass spectrometry. Fresenius Z. Anal. Chem. 327: 239~245, 1987
12) ROEPSTORFF, P.; P. HOJRUP & J. MOLLER: Evaluation of fast atom bombardment mass spectrometry for sequence determination of peptides. Biomedical Mass Spectrometry 12: 181~188, 1985
13) KNECHT, R. & J. Y. CHANG: Liquid Chromatographic determination of amino acids after gas-phase hydrolysis and derivatization with (dimethylamino)-azobenzenesulfonyl-chloride. Analyt. Chem. 58: 2375~2379, 1986
14) BRÜCKNER, H. & C. KELLER-HOEHL: HPLC separation of DL-amino acids derivatized with N^2-(5-fluoro-2,4-dinitrophenyl)-L-amino acid amides. Chromatographia 30: 621~629, 1990
15) ISOGAI, A.; J. NAKAYAMA, S. TAKAYAMA, A. KUSAI & A. SUZUKI: Structural elucidation of minor components of peptidyl antibiotic P168s (Leucinostatins) by tandem mass spectrometry. Biosci. Biotech. Biochem. 56: 1079~1085, 1992
16) ISOGAI, A.; A. SUZUKI, S. TAMURA, S. HIGASHIKAWA &

S. KUYAMA: Structure of a peptidal antibiotic P168 by *Paecilomyces lilacinus* (Thom) Samson. J. Chem. Soc. Perkin Trans. 1: 1405~1411, 1984

17) KALINOWSKI, H.; S. BERGER & S. BRAUN: ^{13}C NMR Spektroskopie. G. Thieme Verlag Stuttgart, pp. 200, 1984

18) STROH, J. G.; K. L. RINEHART Jr. J. CARTER COOK, T. KIKANA, M. SUZUKI & T. ARAI: Identification and structure assignment of components of Leucinostatin and CC-1014 by directed coupled liquid chromatography/fast atom bombardment mass spectrometry. J. Amer. Chem. Soc. 108: 858~859, 1986

19) ISOGAI, A.; A. SUZUKI, S. HIGASHIKAWA, S. KUYAMA & S. TAMURA: Isolation and biological activity of a peptidal antibiotic P168. Agric. Biol. Chem. 45: 1023~1024, 1981

20) MORI, Y.; M. SUZUKI, K. FUKUSHIMA, & T. ARAI: Struture of leucinostatin B, an uncoupler of mitochondria. J. Antibiotics 36: 1084~1086, 1983

21) ROSSI, C.; L. TUTTOBELLO, M. RICCI, C. G. CASINOVI & L. RADICS: Leucinostatin D, a novel peptide antibiotic from *Paecilomyces marquandii*. J. Antibiotics 40: 130~132, 1987

22) SATO, M.; T. BEPPU & K. ARIMA: Properties and Structure of a novel peptide antibiotic no. 1907. Agric. Biol. Chem. 44: 3037~3040, 1980

23) RADICS, L.; M. KAJTAR-PEREDY, C. G. CASINOVI, C. ROSSI, M. RICCI & L. TUTTOBELLO: Leucinostatins H and K, two novel peptide antibiotics with tertiary amine-oxide terminal group from *Paecilomyces marquandii*. Isolation, structure, and biological activity. J. Antibiotics 40: 714~716, 1987

24) ARX VON, J. A. (*Ed.*): The genera of fungi sporulating in pure culture, Ganthner, K. G., Cramer, J. FL-9490 Vaduz, Liechtenstein, 1981

25) CERRINI, S.; P. LAMBA, A. SCATTURIN, C. ROSSI & G. UGHETTO: The crystal and molecular structure of the α-helical nonapeptide antibiotic Leucinostatin A. Biopolymers 28: 409~420, 1989

26) WINKELMEIER, P.; B. GLANNER & T. LINDL: Quantification of cytotoxicity by cell volume and cell proliferation. ATCA 21: 269~280, 1993

27) "Deutsches Arzneibuch", 9th edition 1986, Deutscher Apotheker Verlag Stuttgart, pp. 47~48 and 424~430).