Roseoferin, a New Aminolipopeptide Antibiotic Complex from *Mycogone rosea* DSM 12973, Structures and Biological Activities

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In the course of microbiological screening aimed at new antifungal antibiotics promoting the transport of hydrophilic anions such as helianthate to organic solvents[1,2], we disclosed recently *Mycogone rosea* DSM 12973 as the producer of a strongly antifungal and cytotoxic agent. The complex of at least 16 closely related aminolipopeptide antibiotics named roseoferin (**I**, Table 1) was chromatographically inseparable but structural assignment of every of these components was possible on the basis of tandem mass spectrometry (ESI-triple quadrupol-CID-MS/MS and ESI-ion-trap-CID-MS^n), hydrolysis, and chromatographic analysis of the fatty and amino acids. The general building scheme of the roseoferin (**I**) is given in Fig. 2.

The producer strain *Mycogone rosea* DSM 12973 from the culture collection of the Hans-Knöll-Institute for Natural Products Research develops a mycelium which is rich in septation. The hyaline hyphae are five to seven micrometers in diameter. Chlamydospores (aleurioconidia) are typically two-celled: the warty apical cell is almost globose of (17.1×17.9) μm, the basal cell smooth of (9.0×11.3) μm. The average length of the chlamydospore was measured to be 26.1 μm in total. However, formation of a *Verticillium*-like stage, bearing 1-celled phialoconidia, which may eventually be present[3], has not been observed, thus far.

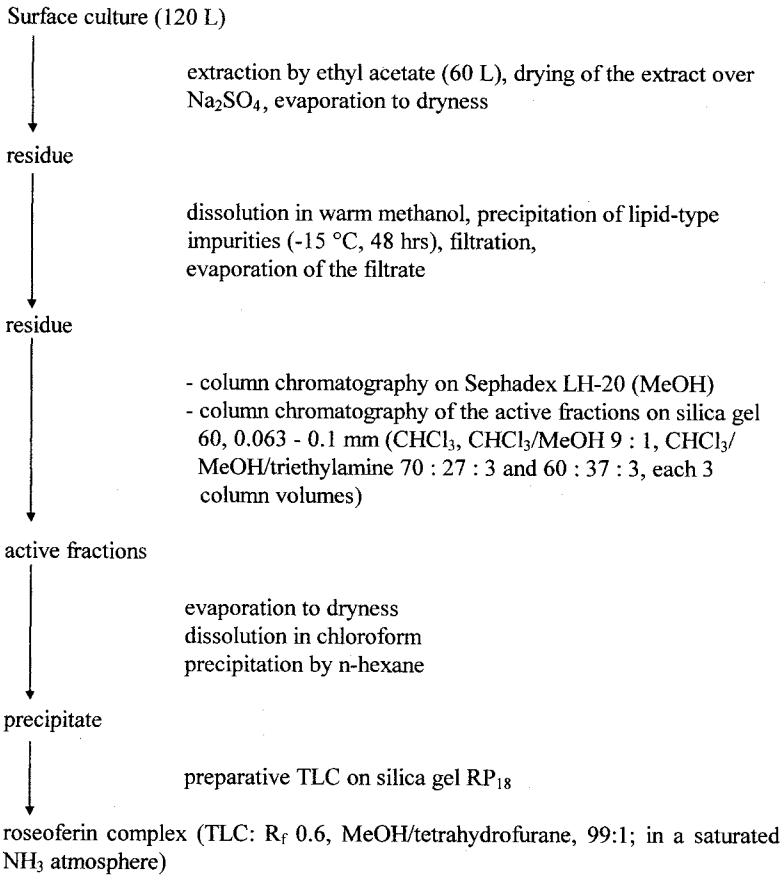
Roseoferin (**I**) was isolated from a three-week surface culture of *Mycogone rosea* DSM 12973 by extraction with ethyl acetate and several subsequent steps of chromatographic purification (Fig. 1). Fractions containing roseoferin were detected by their antifungal activity and the two-phase vertical stacking assay[1,2]. Moreover, electrospray (ESI)-MS was of pivotal importance due to the well detectable $[M+H]^+$ ions of the roseoferin components.

The roseoferin complex (**I**) thus isolated (Fig. 1) was inseparable by the commonly used HPLC methods on the basis of RP_{18} materials, solvents of different polarity and pH values. It was obtained as a slightly brownish microcrystalline solid. Supporting evidence for the peptide skeleton was obtained from the observable positive biuret reaction, whereas ninhydrin and Dragendorff's reagent failed to react. Further support was inferred from the IR spectrum (in KBr) due to λ_{max} 1,660 cm^{-1} (amide-I), 2,900 cm^{-1} and 3,300 cm^{-1} (amine/amide ν_{NH} and ν_{OH}).

Table 1. Molecular weights and chemical formulas of the six basic components of roseoferin as determined by HRESI-MS (Finnigan MAT 95XL).

Roseoferin	$[M+H]^+$	(relative intensity)	Chemical formula	
C_1, C_2	1163.8364	20	$C_{60}H_{111}N_{10}O_{12}$	calcd.: 1163.8383
A_1, A_2, A_3	1149.8211	100	$C_{59}H_{109}N_{10}O_{12}$	calcd.: 1149.8226
B_1, B_2, B_3	1135.8111	40	$C_{58}H_{107}N_{10}O_{12}$	calcd.: 1135.8072
D_1,D_2, D_3	1121.7950	10	$C_{57}H_{105}N_{10}O_{12}$	calcd.: 1121.7913
E_1, E_2, E_3	1107.7797	5	$C_{56}H_{103}N_{10}O_{12}$	calcd.: 1107.7757
F	1093.7618	1	$C_{55}H_{101}N_{10}O_{12}$	calcd.: 1093.7601

Fig. 1. Isolation of the roseoferin complex (**I**) from surface cultures of *Mycogone rosea* DSM 12973.



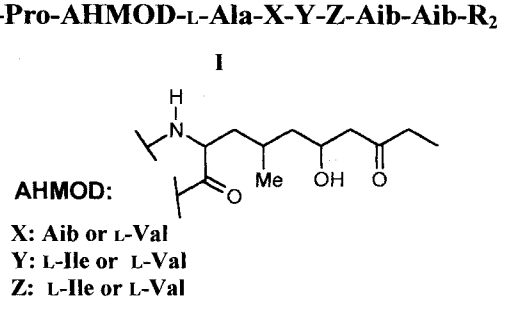
Acidic hydrolysis of the roseoferin complex (**I**), derivatization of the amino acids by 1-F-2,4-dinitrophenyl-5-L-alanine-amide (Marfey's reagent)[4] and HPLC analysis of the diastereomers thus formed suggested that all the constituting chiral amino acid of roseoferins possess the *S*-configuration.

Further conclusive evidence for the structures of the individual members of the roseoferin complex was furnished by ESI-CID-MS/MS and ESI-CID-MS^n experiments.

The molecular weight and the chemical formulas of the six basic components (A~F) were readily determined by HRESI-MS of the $[M+H]^+$ pseudomolecular ions (high-resolution sector-field instrument Finnigan MAT 95XL (Finnigan, Bremen, Germany) equipped with electrospray ion source; Table 2).

Electrospray ionization CID-MS/MS with a triple-quadrupole instrument (Quattro, VG Biotech, Altrincham, England; argon as collision gas) of any of the above pseudomolecular ions generated a series of diagnostic B-

Fig. 2. General building scheme of the antibiotics of the helioferin/roseoferin group.



R_1: 2-methyloctanoic acid (MOA) or 2-methyldecanoic acid (MDA)

AHMOD: 2-amino-4-methyl-6-hydroxy-8-oxo-decanoic acid.

R_2: 2-[(2′-aminopropyl)-methylamino]-ethanol (AMAE) or 2-(2′-aminopropyl)-aminoethanol (AAE).

Table 2. Sequences of fatty and amino acids in the individual components of roseoferin (a)) and corresponding $[M+H]^+$ and daughter-ions observed during ESI-CID-MS/MS and MS^n (b)).

a)

Roseoferin			3	4	5	6	7	8	9	10
G	MDA	Pro	AHMOD	Ala	Val	Ile	Ile	Aib	Aib	AMAE
C_1	MDA	Pro	AHMOD	Ala	Aib	Ile	Ile	Aib	Aib	AMAE
C_2	MDA	Pro	AHMOD	Ala	Val	Val	Ile	Aib	Aib	AMAE
A_1	MDA	Pro	AHMOD	Ala	Aib	Ile	Val	Aib	Aib	AMAE
A_2	MDA	Pro	AHMOD	Ala	Aib	Val	Ile	Aib	Aib	AMAE
A_3	MDA	Pro	AHMOD	Ala	Aib	Ile	Ile	Aib	Aib	AAE
B_1	MDA	Pro	AHMOD	Ala	Aib	Val	Val	Aib	Aib	AMAE
B_2	MDA	Pro	AHMOD	Ala	Aib	Ile	Val	Aib	Aib	AAE
B_3	MDA	Pro	AHMOD	Ala	Aib	Val	Ile	Aib	Aib	AAE
D_1	MDA	Pro	AHMOD	Ala	Aib	Val	Val	Aib	Aib	AAE
D_2	MOA	Pro	AHMOD	Ala	Aib	Ile	Val	Aib	Aib	AMAE
D_3	MOA	Pro	AHMOD	Ala	Aib	Val	Ile	Aib	Aib	AMAE
E_1	MOA	Pro	AHMOD	Ala	Aib	Val	Ile	Aib	Aib	AAE
E_2	MOA	Pro	AHMOD	Ala	Aib	Ile	Val	Aib	Aib	AAE
E_3	MOA	Pro	AHMOD	Ala	Aib	Val	Val	Aib	Aib	AMAE
F	MOA	Pro	AHMOD	Ala	Aib	Val	Val	Aib	Aib	AAE
Heliof. A	MOA	Pro	AHMOD	Ala	Aib	Ile	Ile	Aib	Aib	AAE
Heliof. B	MOA	Pro	AHMOD	Ala	Aib	Ile	Ile	Aib	Aib	AMAE

b)

Roseoferin	1*	B_1*	B_2	B_3	B_4	B_5	B_6	B_7	B_8	$(M+H)^+$
G	169	266	479	550	649	762	875	960	1045	1178
C_1	169	266	479	550	635	748	861	946	1031	1164
C_2	169	266	479	550	649	748	861	946	1031	1164
A_1	169	266	479	550	635	748	847	932	1017	1150
A_2	169	266	479	550	635	734	847	932	1017	1150
A_3	169	266	479	550	635	748	860	946	1031	1150
B_1	169	266	479	550	635	734	833	918	1003	1136
B_2	169	266	479	550	635	748	847	932	1017	1136
B_3	169	266	479	550	635	734	847	932	1017	1136
D_1	169	266	479	550	635	734	833	918	1003	1122
D_2		238	451	522	607	720	819	904	989	1122
D_3		238	451	522	607	706	819	904	989	1122
E_1		238	451	522	607	706	819	904	989	1108
E_2		238	451	522	607	720	819	904	989	1108
E_3		238	451	522	607	706	805	890	975	1108
F		238	451	522	607	706	805	890	975	1094
Helioferin A		238	451	522	607	720	833	918	1003	1122
Helioferin B		238	451	522	607	720	833	918	1003	1136

Abbreviation: MDA; 2-methyldecanoic acid, MOA; 2-methyloctanoic acid, AHMOD; 2-amino-4-methyl-6-hydroxy-8-oxo-decanoic acid, Pro; L-proline, Ala; L-alanine, Aib; α-aminoisobutyric acid, Ile; L-isoleucine, Val; L-valine, AMAE; 2-[(2′-aminopropyl)-methylamino]-ethanol, AAE; 2-(2′-aminopropyl)-aminoethanol.

type fragments (B_1 to B_4), *i.e.* cleavage of the amide bond with the charge remaining at the *N*-terminus[5]. Fragments B_2 to B_8 were also visible in the electrospray ionization MS^n spectra (see below). The 2-amino-4-methyl-6-hydroxy-8-oxo-decanoic acid (AHMOD) moiety was characterizable with both MS methods.

CID-MS^n investigations using an ion trap mass spectrometer (Finnigan LCQ; Finnigan, Bremen, Germany; helium as collision gas) of the homologous pseudo-molecular ions of *m/z* 1178, 1164, 1150, 1136, 1122, 1108, and 1094, respectively, indicated the presence of a mixture of isomers with different amino acid sequences. A typical

Fig. 3a. CID-MS^2 (ion-trap) of $[M+H]^+$ of roseoferins with *m/z* 1136.

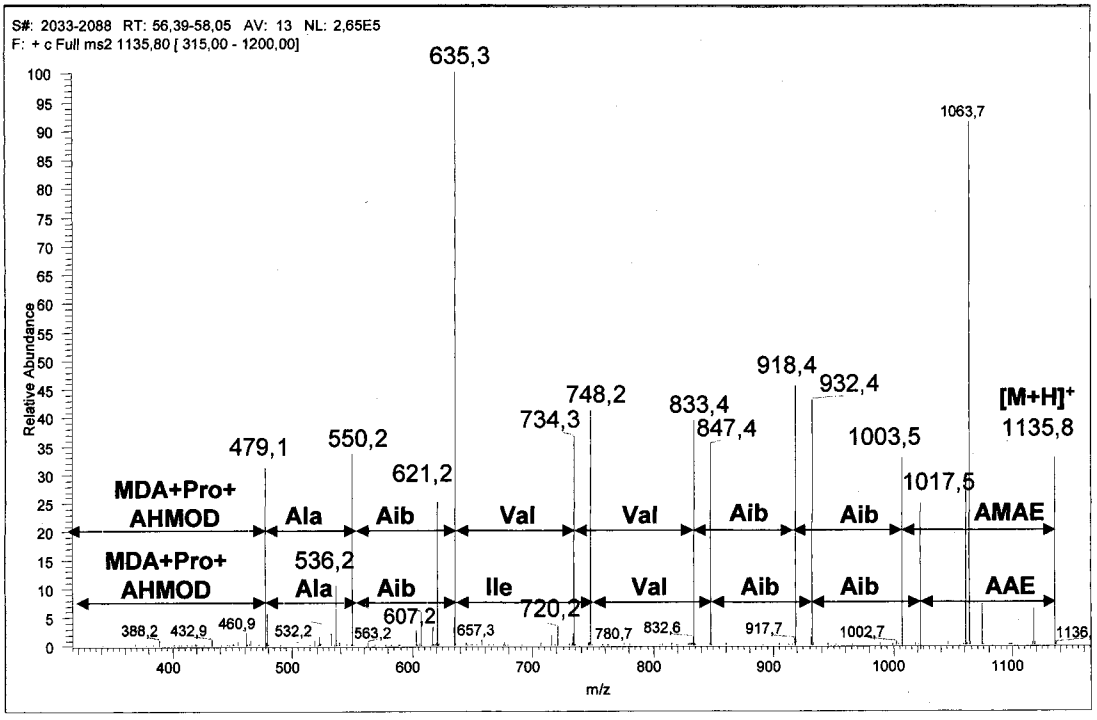


Fig. 3b. ESI-CID-MS^3 (ion-trap, helium as collision gas) of *m/z* 932.4 furnished by the MS^2 experiment with $[M+H]^+$ *m/z* 1136.

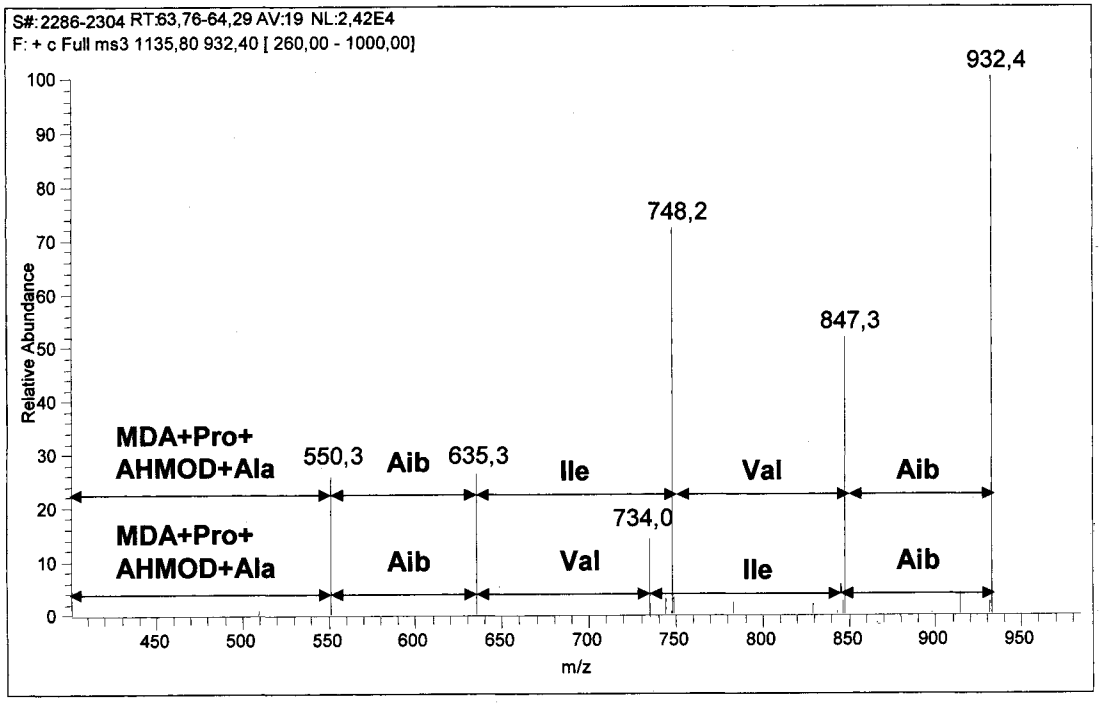
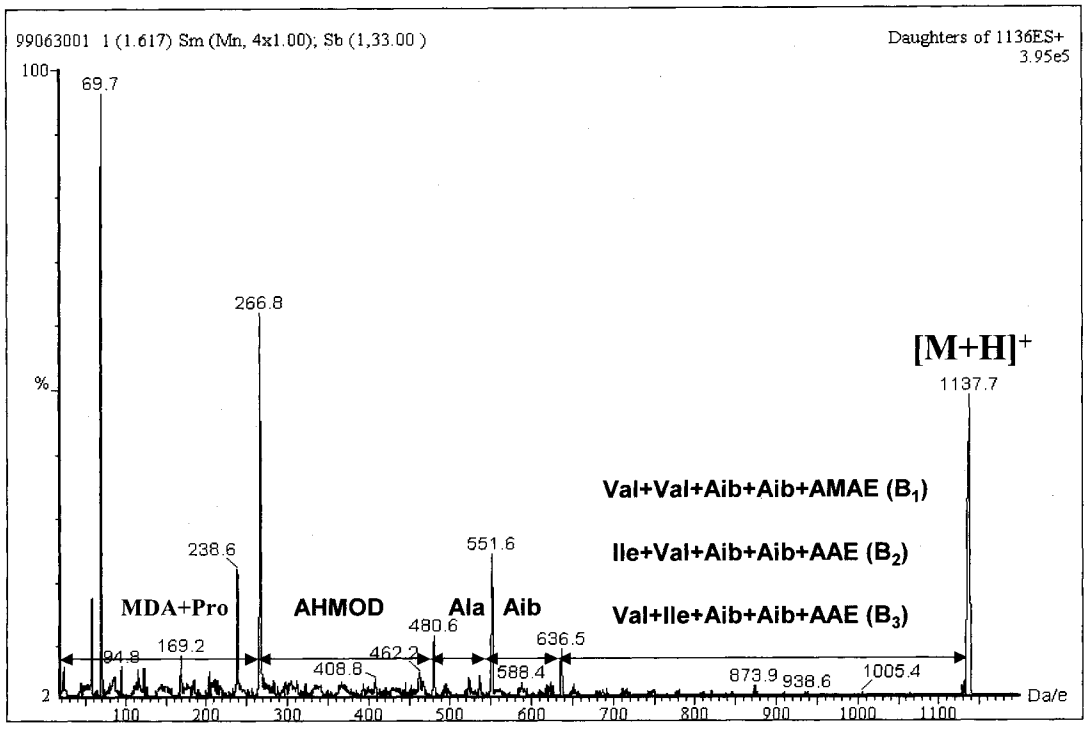


Fig. 4. Triple-quadrupole-ESI-CID-MS/MS of $[M+H]^+$ ion of roseoferins with *m/z* 1136 (argon as collision gas).



example of the fragmentation pattern of the roseoferins is depicted in Fig 3a, showing the ESI-CID-MS^2 of the $(M+H)^+$-ion of *m/z* 1136. The *N*-terminus of all members of the roseoferin family consists either of a 2-[2′-(aminopropyl)methylamino]ethanol (AMAE) or 2-[2′-(aminopropyl)amino]ethanol (AAE) moiety according to the presence of fragments of *m/z* 1003.5 ($[M+H]^+-133$ Da) or *m/z* 1017.5 ($[M+H]^+-119$ Da), respectively.

Any of these fragment ions served as the origin of further B-type fragmentations. However, elucidation of the amino acid sequence of the isomeric compounds could be achieved by CID-MS^3 impact experiments of different CID-MS^2 daughter ions. Sequencing of roseoferin B_2 grand-daughter ion with *m/z* 932.4 by ESI-CID-MS^3 is shown in Fig. 3b. This experiment revealed the additional presence of roseoferin B_3 due to the appearance of the diagnostic fragment with *m/z* 734.

The occurrence of additional diagnostic MS^n fragments (see Fig. 3a; *m/z* 536, 607, 621, and 720, respectively) provided evidence for a series of further minor compounds, but a complete structure assignment by MS was not possible due to their comparatively low concentration in the mixture.

The triple quadrupole ESI-CID-MS/MS of *m/z* 1136 ($[M+H]^+$ ion) is depicted in Fig 4. Thus, the structures of the roseoferins B_1, B_2, and B_3 could be confirmed by the occurrence of the peptide fragments B_1~B_4.

The structures of the roseoferins D_1, D_2, D_3, E_1, E_2, E_3, and F resemble those of the previously described helioferins A and B[2] but are distinguishable by the replacement of amino acids 6 and 7, respectively. According to *m/z* 238 (MOA-Pro; Fig. 4) the roseoferins D_1, D_2, D_3, E_1, E_2, E_3, and F contain the same terminal fatty acid (2-methyloctanoic acid, MOA) as helioferins A and B. However, in the roseoferins A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, and D_1 the fragment of the *C*-terminal fatty acid (methyldecanoic acid; *m/z* 169; (MDA)) and MDA-Pro (*m/z* 266) displayed an additional 28 mass unit suggesting the presence of a dimethyl or dimethylene homologue of the methyloctadecanoic acid moiety (MOA).

Additional proof for the structure of the roseoferin complex (**I**) was furnished by ESI-MS of roseoferin hydrolysate showing the $[M+H]^+$ ions of Pro (*m/z* 116), Aib (*m/z* 104), Ala (*m/z* 90), Val (*m/z* 118), Ile (*m/z* 132), and the *N*-terminal AMAE (*m/z* 133) and AAE (*m/z* 119)

Table 3. Antimicrobial activity of the roseoferin complex (**I**).

Organism	MIC [μg/ml]
Micrococcus luteus ATCC 10240	3.12 *
Enterococcus feacalis 1528	12.5*
Mycobacterium smegmatis SG 987	6.25*
Mycobacterium aurum SB 66	6.25*
Mycobacterium vaccae IMET 10670	3.12*
Mycobacterium fortuitum B	25*
Mycobacterium cheloni B	50*
Candida albicans BMSY 212	1.56**
Sporobolomyces salmonicolor SBUG 549	0.39 ***
Penicillium notatum JP 36	0.39 ***

* MIC obtained from microtiter test[12]

** MIC obtained from microtiter test[13]

*** MIC obtained from agar well diffusion assay[11]

moieties. The *m/z* 214 corresponded to the diagnostic $[M+H]^+$ ion of 4-methyl-6-(2-oxobutyl)-2-piperidine carboxylic acid (MOBPA) which arises under acidic conditions from the 2-amino-4-methyl-6-hydroxy-8-oxo-decanoic acid (AHMOD) moiety of helioferins[2], leucinostatins[8~9], roseoferins and trichopolyns[7].

Special investigations were aimed at the structure elucidation of the MDA moiety of the roseoferins A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, D_1 and G . CID-MS/MS of *m/z* 266 (B_1 in Table 1) confirmed that this fragment was composed of a proline and a MDA unit due to the appearance of *m/z* 169 (MDA) as a daughter ion. HRESI-MS investigations (Finnigan 95XL) disclosed $C_{16}H_{28}NO_2$ for 266.2112 (calcd. 266.2120). This molecular composition suggested that the MDA-Pro moiety exceeds the comparable MOA-Pro unit in the helioferins A and B[2] by a C_2H_4 moiety. Supporting evidence was inferred from the formation of mono-2,4-dinitrophenylhydrazones by members of the roseoferin complex (*e.g.* *m/z* 1314.2 as the $[M+H-OH]^+$ ion of roseoferin $A_1/A_2/A_3$ mono-2,4-dinitrophenyl-hydrazones).

Gradient HPLC-coupled MS of the fatty acids (MDA, MOA) extracted from the roseoferin hydrolysate ([solvent A: 2 mM ammonium formiate, solvent B: AcCN, gradient: 1 minute at 0.5% B, 16 minutes at 99.5% B, 17 minutes at 99.5% B, 21 minutes at 0.5% B, 25 minutes stop] column: YMC-Pack ODS-AQ, 120 Å (150×3) mm, YMC Europe GmbH, flow rate: 0.5 ml/minute, coupled to Finnigan LCQ revealed *m/z* 185 $[M-H]^-$ for a peak appearing at 14.56 minutes as well in the positive ion mode the corresponding $[M+H]^+$ and $[M+Na]^+$ ions. HREI-MS of *m/z* 185.1529 ($[M-H]^-$; calcd. 185.1542) afforded the chemical formula $C_{11}H_{21}O_2$.

Finally, the α-position was suggested for the methyl group of the methyldecanoic acid moiety (MDA) by HREI-MS (high-resolution sector field instrument AMD 402, AMD Intectra, Harpstedt, Germany, direct inlet; 70 eV) due to the two prominent fragments with *m/z* 74.0364 ($C_3H_6O_2$, calcd. 74.0368) and *m/z* 87.0441 ($C_4H_7O_2$, calcd. 87.0446), respectively, appearing in addition to EI-MS of *m/z* 186 (M^+). (*R*)-(−)-2-Methyldecanoic acid (MDA) was previously discovered as a constituent of the trichopolyns[6] and of the uropygial gland wax of various birds such as the oystercatcher[7].

Similar to the helioferins A and B[2] the roseoferin complex (**I**) displays strong antibiotic activity versus Gram-positive bacteria and *Mycobacterium* sp., but no inhibitory effect was observed against Gram-negative bacteria such as *Escherichia coli* SG 458 and *Pseudomonas aeruginosa* K 799/61. However, a number of filamentous fungi and yeasts was highly sensitive to the roseoferins (Table 3).

Usually, the strain produces roseoferin A_1 as the major compound as measured by the intensity of relative

Table 4. Antiproliferative and cytotoxic effects of roseoferins (**I**)[14].

Effect	Cell line	IC_{50} [μg/ml]
Antiproliferative effect	L 929 (mouse fibroblast cells)	0.4
	K 562 (human leukemia)	0.08
Cytotoxic effect	HeLa (human cervical carcinom)	3.3 (IC_5 0.2)

pseudomolecular ions during ESI-MS, followed by the roseoferins B_1, C and D, respectively. Roseoferins A_2, A_3, B_2, B_3, E, F and G are minor compounds that were assigned structurally solely by mass spectrometrical methods.

Table 3 displays antimicrobial activities of the roseoferin complex against filamentous fungi yeasts and Gram-positive bacteria. An interesting fact was the high sensitivity of *Candida albicans* and some species of Mycobacteria.

Roseoferin exhibits highly antiproliferative effects against several mouse fibroblast and cancer cell lines, which render it promising as an antitumor drug. The antibiotic thus appears as a new representative of the small group of aminolipopeptide antibiotics which was represented thus far by the trichopolyns, leucinostatins, and helioferins[2,6,8,9].

It appears as a remarkable fact that all these compounds and roseoferin (**I**) are produced by deuteromycetes such as *Paecilomyces lilacinus*[8], *Trichoderma polysporum*[6] and *Mycogone rosea*[2] which either grow saphrophytically or parasitically on fruiting bodies of basidiomycetes[10].

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