Maltophilin: A New Antifungal Compound Produced by *Stenotrophomonas maltophilia* R3089

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Strains of *Stenotrophomonas maltophilia* R3089, isolated from the rhizosphere of rape plants (*Brassica napus* L.), produced a novel antifungal compound, named maltophilin, which inhibited the growth of various saprophytic, human-pathogenic and phytopathogenic fungi but was inactive against Gram-positive and Gram-negative bacteria. Maltophilin is a novel macrocyclic lactam antibiotic with a molecular mass of 510 mu. The compound was isolated from the culture filtrate by ethyl acetate extraction and gel filtration on Sephadex LH20 and purified by preparative HPLC on reversed phase. The structure of maltophilin was elucidated by electrospray mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy.

Stenotrophomonas maltophilia (formerly *Pseudomonas maltophilia*) is an ubiquitous free-living bacterium which has been isolated from water, soil and the plant rhizosphere[1,2]. It is also known as an opportunistic pathogen of humans isolated from immunocompromised patients[3]. *S. maltophilia*, like other members of the Pseudomonas group is resistant to beta-lactam antibiotics and a wide range of other antibiotics. It is able to excrete various exoenzymes like chitinase, glucanases, proteases and is known to produce biofilms. Although *S. maltophilia* has previously been reported to be involved in antagonistic effects against soil-borne pathogenic fungi in plants[4,5], the antifungal agent has so far not been isolated and characterized. In the present paper we describe the isolation, structure elucidation and biological properties of a novel antifungal compound, named maltophilin (Fig. 1), produced by *S. maltophilia* R3089.

Materials and Methods

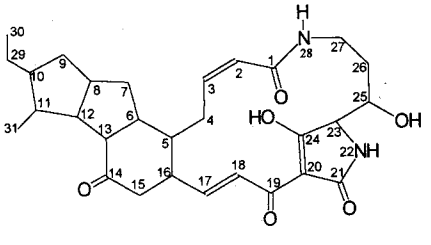
Bacterial Strain

The producing organism *S. maltophilia* R3089 was isolated from the rhizosphere of oilseed rape (*Brassica napus* L. ssp. *oleifera* Metzg. Sinsk) collected at Malchow (Poel, Germany). Identification of the isolate was performed using the Analytical Profile Index (API) strip which consists of a gallery of 20 biochemical tests separated into 7 groups (Bio Meriéux, Nürtingen).

Fermentation Studies

Maltophilin was produced in a 25-liter bioreactor (typ b 25/typ b200, Giovanola Frères S. A., Monthey, Switzerland) under the following conditions: An inoculumn (10%) of a 48 hours old preculture of *S. maltophilia* R3089 grown in the same medium in 500-ml Erlenmeyer flasks (100 ml medium, 120 rpm, 27°C) was added to a 25-liter bioreactor containing 22.5 liters of complex medium (glucose 0.5%, soy peptone 0.5% and $CaCl_2$ 0.1%, pH 7,0). The fermentation was run at 27°C for 96 hours with an agitation rate of

Fig. 1. Structure of maltophilin.



1000 rpm and an air flow rate of 0.5 vvm. To prevent foaming, silicone antifoam (Merck) was added.

Biological Assay

Antifungal and antibacterial activtity was determined by the agar diffusion test on YMG-agar. Paper discs (6 mm diameter) containing 10 μg maltophilin dissolved in methanol were used.

Isolation

The culture filtrate (24 liters) was adjusted to pH 4 with HCl (10 N) and extracted twice with ethyl acetate (6 liters). The extract was evaporated under reduced pressure. The dry residue was dissolved in methanol (10 ml) and purified by gel filtration on Sephadex LH-20 (Pharmacia, Freiburg) using methanol as solvent (flow rate: 2 ml/minute). The fractions containing maltophilin (based on analytical HPLC) were pooled and evaporated under reduced pressure. A further purification was achieved by preparative HPLC on a reversed phase column (Nucleosil 100 C-18, 7 μm, 250 × 16 mm, Grom, Herrenberg) using an isocratic gradient of 60% acetonitrile and 40% water containing TFA (0.08%) and a flow rate of 20 ml/minute over 30 minutes. Fractions containing maltophilin were pooled, dried by evaporation and lyophilized yielding a yellow powder of maltophilin.

Analytical Procedures

Analytical HPLC was performed on Nucleosil RP-18, 5 μm (Grom, Ammerbuch Germany) using a 250 × 4.6 mm column. An isocratic gradient of 60% acetonitril and 40% water containing TFA (0.08%) and a flow rate of 1 ml/minute over 20 minutes was used. UV/Vis spectra were measured in methanol using an HPLC photodiode array detector (HPLC-DAD) with Hewlett Packard LC 1090M.

Mass Spectrometry

Electrospray mass spectra of maltophilin were recorded on a Sciex API III triple-quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray (ionspray) (Sciex, Thornhill, Ontario, Canada). FAB mass spectrometry was recorded on a Finnigan MAT 711A instrument (Bremen, Germany).

NMR Spectroscopy

Two-dimensional NMR spectra were recorded at 300 K on a Bruker AMX2-600 spectrometer (Bruker, Karlsruhe, Germany) at a proton frequency of 600.13 MHz using an inverse triple resonance probe. 4 mg of maltophilin were dissolved in 500 μl of DMSO-d_6. The spectra were referenced to the signal of residual DMSO-d_5 (^{1}H: δ=2.49 ppm; ^{13}C: δ=39.5 ppm). The data set consisted of TOCSY (total correlation spectroscopy), DQF-COSY (double quantum filtered correlation spectroscopy) and P.E. COSY (primitive exclusive correlation spectroscopy) experiments as well as HSQC (heteronuclear single quantum coherence) and HMBC (heteronuclear multiple bond correlation) spectra. In the TOCSY experiment a clean-MLEV spinlock with a duration of 60 ms was used. The HMBC experiment was optimized for a heteronuclear coupling constant of J_{CH}=8 Hz. Pulsed field gradients were applied in the DQF-COSY, HSQC and HMBC experiments for coherence selection. In general, 512 t_1-increments were recorded with a data size of 4096 or 2048 complex points in the time domain. The sweep width was set to 12 ppm in the proton dimension. For the HSQC and the HMBC experiments the sweep widths in the indirect dimensions were 160 and 200 ppm, respectively. Data processing consisted of twofold zerofilling in both dimensions and apodization with a $\pi/2$ shifted squared sine-bell window function.

Results

Fermentation

The amount of maltophilin produced by *S. maltophilia* (strain 3089) in a 25-liter bioreactor reached a maximum after 48 hours of cultivation. Fig. 2 presents the data from a typical fermentation (25 liters) and gives information on maltophilin production and growth of *S. maltophilia* (OD_{578}). Maltophilin production began after 24 hours and the maximum of production was observed after 48 hours of cultivation. The yield of maltophilin was 45 mg/liter.

Isolation

Isolation and purification of maltophilin from the culture broth of *S. maltophilia* R3089 cultivated in a 25-liter bioreactor is summarized in the scheme depicted in Fig. 3.

Structure Elucidation

The molecular mass of maltophilin has been determined by electrospray mass spectrometry to be 510 amu. The molecular formula is in agreement with HRFAB mass spectra to be $C_{29}H_{39}N_2O_6$ (m/z [M+H$^+$] exp.:

Fig. 2. Production of maltophilin in a 25-liter-fermenter.
■ Maltophilin, ▲ OD_{578}.

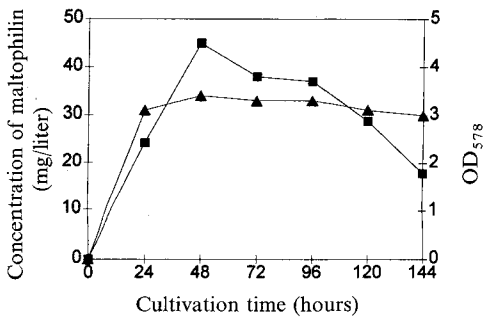


Fig. 3. Isolation and purification scheme for maltophilin.

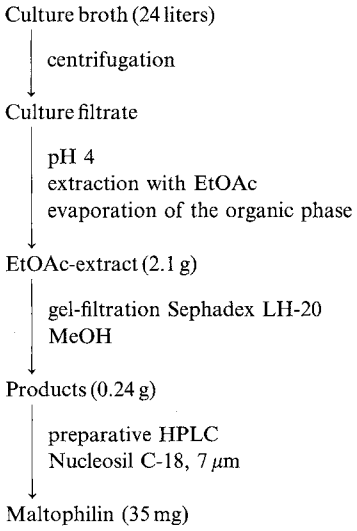


Fig. 4. UV spectrum of maltophilin in methanol.

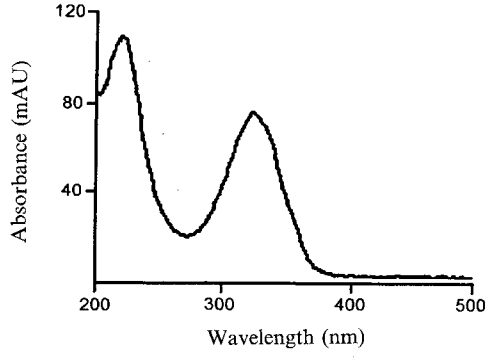


Table 1. ^{1}H and ^{13}C NMR data of maltophilin.

Position	^{1}H [ppm]	^{13}C [ppm]	Observed HMBC signals
1	—	165.1	H2, H3, H27a, H28
2	5.71	123.9	H3, H4a
3	5.92	137.9	H2, H4a, H4b, H5
4a	3.62	27.3	H2
4b	1.98	27.3	
5	1.89	42.8	H3, H4a, H7a, H13, H15a, H15b, H16, H17
6	2.08	50.7	H4a, H5, H13
7a	1.05	38.0	H6, H8, H9a
7b	2.05	38.0	
8	2.33	40.1	H7b, H9a, H12
9a	0.82	39.4	H7a, H29a, H29b
9b	2.03	39.4	
10	1.35	52.9	H9b, H29b, H31
11	1.07	46.4	H9b, H13, H31
12	2.20	50.0	
13	2.35	62.6	H7b, H11, H12, H15a
14	—	207.0	H12, H13, H15a, H15b
15a	2.10	45.2	H17
15b	2.57	45.2	
16	2.37	47.4	H5, H15a, H15b, H17, H18
17	6.63	147.3	H15a, H15b, H16
18	6.86	121.4	H16, H17
19	—	171.5	H17, H18
20	—	100.4	H18, H22
21	—	175.3	H24
22	8.92	—	
23	3.86	68.2	H22
24	—	192.6	H22, H23
25	3.80	69.7	H23, H26, H27a, H27b
26a	1.19	30.6	H23, H25, H27a, H27b
26b	1.39	30.6	
27a	2.56	36.0	H25, H28
27b	3.24	36.0	
28	7.96	—	
29a	1.00	25.1	H30
29b	1.50	25.1	
30	0.83	12.0	H10, H29a, H29b
31	0.95	17.3	H10, H11, H12

511.28195; calc.: 511.28078). The UV absorption maximum at 320 cm^{-1} suggests the presence of a conjugated system (Fig. 4). The IR-spectrum indicated the presence of a carbonyl group (1708 cm^{-1}) as well as a carbonyl group conjugated to a double bond (1648 and 1590 cm^{-1}). Complete resonance assignment of maltophilin (Fig. 1) has been accomplished by means of TOCSY, HSQC and HMBC experiments and is given in Table 1. Resonances in the ^{1}H and ^{13}C NMR spectra indicated the presence of two unsaturated systems (^{1}H: 5.71, 5.92, 6.63, and 6.86 ppm; ^{13}C: 123.9, 137.9, 147.3, and 121.4 ppm). The homonuclear coupling constants of the two proton pairs are typical for a *Z*-configuration in the case of H-2 and H-3 ($^{3}J_{HH}=11.5$ Hz) and an *E*-configuration for H-17 and H-18 ($^{3}J_{HH}=15$ Hz). The signal at 207.0 ppm in the ^{13}C spectrum was assigned to a keto group. Connectivities between structural elements were established with DQF-COSY and HMBC experiments. The amide proton at 7.96 ppm, one of the protons at C-27 and the olefinic protons H-2 and H-3 show a C-H long-range correlation to the same amide carbonyl carbon at 165.1 ppm, thus establishing the connectivity between C-1 and N-28. The other pair of olefinic protons H-17 and H-18 also shows a long range correlation to a carbonyl carbon at 171.5 ppm as well as to a carbon at 100.4 ppm (C-20) which in turn correlates to the amide proton H-22, thus proving the connectivity between C-19 and C-20. Connectivities in the tricyclic part of the molecule could also be assigned with the HMBC spectrum (see Table 1). Due to signal overlap the relative configurations could not be assigned.

Table 2. Antifungal spectrum of maltophilin.

Test organism	Diameter[a] of inhibition zone (mm)
Oomycetes:	
Pythium ultimum KSSF[b]	8
Zygomycetes:	
Entomophtora virulenta TÜ[c] 263	9
Mucor miehei Cooney & Emmerson TÜ 284	11
Ascomycetes und Deuteromycetes:	
Ashbya gossypii	8
Aspergillus terreus Thom TÜ 155	11
Botrytis cinerea Persoon TÜ 157	7
Candida albicans TÜ 164	9
Fusarium solani TÜ 535	10
Neurospora crassa 74 A*	16
*Neurospora crassa slime**	12
Paecilomyces variotii Bainer TÜ 137	10
Penicillium notatum Westling TÜ 136	10
*Penicillium puberulum**	14
*Rhizoctonia solani**	9
*Rhodotorula rubra**	12
Saccharomyces cerevisiae Hansen TÜ 125	—
Trichoderma pseudokoningii Rifai CBS[d] 931.69	10

[a] Paper disks (6 mm diameter) with 10 μg maltophilin.
[b] Kulturensammlung, Shell-Forschung.
[c] Strain collection University of Tübingen/Microbiology.
[d] Centraalbureau voor Schimmelcultures Baarn.
* Strain collection University of Tübingen/Prof. Winkelmann.

Biological Properties

The antimicrobial activities of maltophilin against various bacteria and fungi were tested in the agar diffusion assay. As shown in Table 2, maltophilin exhibited biological activity against a broad spectrum of fungi, whereas the Gram-positive and Gram-negative bacteria tested were not sensitive.

Discussion

Previous work from our laboratory has shown that the production of antifungal agents is common among bacteria occuring in soil, water and plant material. Thus herbicolins A and B, isolated from *Erwinia herbicola* (A111) and iturin AL, isolated from *Bacillus subtilis* (A114) were identified as cyclic peptide antibiotics[6~8] having a strong antifungal activity. The present antibiotic, named maltophilin, was isolated from a strain of *Stenotrophomonas maltophilia* (previously *Pseudomonas maltophilia*) which is known to be associated with roots of several plants, especially rape (*Brassica napus*). Maltophilin is a macrocyclic lactam antibiotic which seems to be biosynthetically linked to the polyketides. The polyketides represent a large family of structurally diverse natural products which are predominantly produced by Streptomycetes and which have recently received increasing attention due to the possibility of engineered bio-synthesis[9]. Maltophilin resembles the previously described alteramide A, isolated from a marine *Alteromonas* spp. strain[10] and is also related to discodermide, isolated from the marine sponge *Discoderma dissoluta*[11]. Moreover, the producing bacterium, *S. maltophilia*, is able to produce both, maltophilin and alteramide A, suggesting a biosynthetic relationship between both compounds.

Acknowledgments

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