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Oxachelin, a Novel Iron Chelator and Antifungal Agent from *Streptomyces* sp. GW9/1258

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Abstract During a screening campaign for new antimicrobial and antifungal secondary metabolites from several thousand actinomycetes, a novel compound, isolated by activity guided fractionation, was oxachelin (**1**) from the new *Streptomyces* sp. GW9/1258. Oxachelin shows strong antibiotic activities against several fungi and Gram(+) bacteria. Additionally, oxachelin is a strong complexing ligand for Fe^{3+} (siderophore), possibly making it useful *e.g.* for iron excess diseases.

Keywords oxachelin, antifungal agents, iron chelator, *Streptomyces*, microbial products

Introduction

Iron in form of its cations Fe^{2+} and Fe^{3+} plays a pivotal role in many biological systems, and iron deficiency diseases are prevalent especially in developing countries, but also in industrialized regions. On the other hand, an excess of iron causes severe damages and plays a pivotal role in diseases like Friedreich's ataxy [1], cardiomyopathy [2], aplastic anemia [3], thalassemia [4], hepatitis [5], Parkinson's Disease (PD) [6], Alzheimer's Disease (AD) [7] or amyotrophic lateral sclerosis (ALS) [8]. Therefore, it is essential to develop new drugs which are able to modulate the iron resorption, distribution and excretion in humans. With the siderophore oxachelin (**1**) we isolated a new natural product which may be useful as a lead structure for the treatment of above mentioned diseases.

Siderophores are widely known from natural sources, especially from soil bacteria which are using those molecules to assimilate and transport Fe^{3+}. A broadly used iron chelator is deferoxamine (desferrioxamine, desferrin), originally isolated from *Streptomyces pilosus* [9~11] and now used as its mesylate salt (Desferal®) for several iron-related diseases. Structurally closely related are maduraferrin and the madurastatins from *Actinomadura madurae* [12, 13].

Materials and Methods

General

Nuclear magnetic resonance spectra (NMR) were measured on a Bruker Avance DRX 500 MHz spectrometer with the solvent signals as internal standard. Analytical HPLC examinations were run on a Waters Millennium system with two independent pumps (Model 590) and a PDA 996 photodiode array detector, using a acetonitril - water gradient on a Chromolith SpeedROD C-18e column (Merck, Darmstadt). Ultraviolet-visible (UV-VIS) spectra were taken directly from the analytical HPLC-PDA runs and show relative intensities. Size exclusion chromatography was conducted on Kronlab glass columns (2.5×100 cm) with Sephadex® LH-20 (Amersham Biosciences) as column material and methanol as eluent. High resolution mass spectra were run on a Micromass LCT with a TOF detector, combined with a Hewlett-Packard 1100 analytical HPLC.

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Microorganism

The producing strain GW9/1258 was identified as a new *Streptomyces* sp. (personal communication Prof. Dr. Reiner M. Kroppenstedt, DSMZ, Braunschweig, Germany) and deposited in the strain collection of the laboratory of soil microbiology *Labor Gruen-Wollny*, Giessen, Germany. The microorganism can be cultivated on standard cultivation media like YMG medium.

Fermentation

Strain GW9/1258 was grown for 7 days at 27°C in 4×1 liter shaking flasks each containing 250 ml of YMG medium: 4 g glucose, 4 g yeast extract and 10 g malt extract in one liter of deionized water, pH adjusted to 6.5 to 7 before sterilization.

Results and Discussion

Isolation

The whole culture broth (1 liter) was evaporated to dryness under reduced pressure at 40°C and the resulting solid was extracted four times with 150 ml methanol. After removal of the solvent, 500 mg of the resulting thick oily gum (5.5 g in total) were separated into fractions by preparative HPLC (column 4×25 cm) using a water - acetonitrile gradient on reversed-phase C18. The most active fraction (tested against *Staphylococcus aureus*) was further purified on Sephadex LH-20 (column 2.5×100 cm) with methanol as eluent. The activity was tested again and the most active fractions were combined and evaporated to dryness, which resulted in 10 mg oxachelin (**1**) in form of a white fluffy powder. This material was subsequently used for structure elucidation.

Structure Elucidation

Oxachelin (**1**) is a polar substance which is very soluble in DMSO (>50 mg/ml), less soluble in water and methanol and very poorly in chloroform or ethyl acetate. It shows a $[M-H]^-$ peak at m/z=634 in the (−)ESI-MS, a $[M+H]^+$ peak at m/z=636 and a $[M+Na]^+$ peak at m/z=658 in the (+)ESI-MS, corresponding to a molecular mass of 635 Da. Additionally, there is sometimes an accompanying signal observable at m/z=689 with varying intensities, pointing to the formation of a $[M-2H+Fe^{3+}]^+$ peak. This finding was a first hint to the iron complexing properties of **1**. The HR-EI mass spectrum shows a molecular ion peak at m/z=635.2603, indicating a molecular formula of $C_{27}H_{37}N_7O_{11}$, which is in good accordance with the elemental analysis (C 50.77, H 5.85, N 15.50).

An H/D exchange experiment analyzed by (+/−)ESI-MS using the direct injection mode revealed the presence of 8 exchangeable protons.

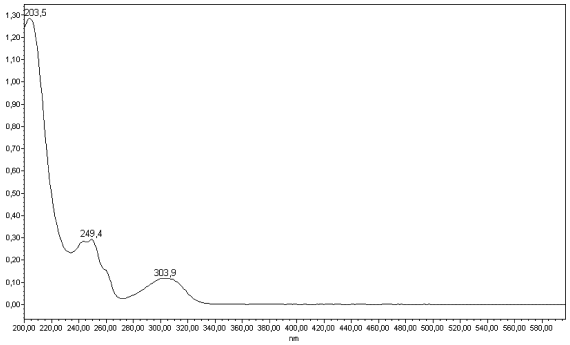

Fig. 1 Qualitative UV-spectrum of oxachelin in acetonitrile/water 2 : 1.

The UV-spectrum of **1** (Fig. 1) is not very characteristic, nevertheless pointing to a substituted aromatic system with λ_{max}=203, 245, 249, 255 (sh) and 304 nm.

In the ^{1}H NMR of **1**, there are visible several characteristic signals. Typical for an oxygen-substituted aromatic ABCD-spin system are the four signals at δ_H 6.93 (t), 6.99 (d), 7.45 (t) and 7.63 (d), with δ_C 119.07, 116.61, 134.00 and 128.07, respectively. In the range of 7.85~8.24 ppm, there are observable the typically broad signals of 4 CO–NH–protons, showing no correlation to a carbon atom in the gHSQC and suggesting a peptide core structure. In correspondence to this, there are 4 α-CH visible at δ_H 4.17 (attached to a carbon atom which is split into two signals at δ_C=52.40+52.60), 4.29 (δ_C 49.42), 4.36 (δ_C 55.32) and 5.06 (δ_C 67.24). Interestingly, there are four more carbon signals at δ_C 22.50+22.52, 28.50+28.55, 46.00+48.77, 157.00+161.74 which are also observed as "doublets", indicating the presence of two rotamers. All those "split" carbon atoms can be assigned to a *N*5-hydroxy-*N*5-formyl-ornithine side-chain, where the splitting of the signals is explained by the presence of two relatively stable conformations generated by the rotation of the formyl-group. At higher temperatures, these signals show coalescence and are observed as broad singlets. This phenomenon is well known from substances with similar structural features like formobactin from *Nocardia* sp. [14], brasilibactin A from *N. brasiliensis* [15] or the nocardimicins from *N. nova* [16, 17].

Taking together the information from 1D and 2D NMR experiments (gCOSY, gTOCSY and gHSQC-TOCSY), the partial structures shown in Fig. 2 can be defined:

The above mentioned partial structures were assembled to the final structure of oxachelin (**1**) using the information received from ^{1}H,^{13}C-gHMBC, ^{1}H,^{15}N-gHMBC and

Table 1 NMR data of oxachelin (**1**) in DMSO-d_6 (500/125 MHz)

Position	δ_C		δ_H (J Hz)	
1	109.33 s		—	
2	159.07 s		—	
3	116.61 d		6.99 (d 8.4)	
4	134.00 d		7.45 (ddd 8.4, 7.4, 1.5)	
5	119.07 d		6.93 (t 7.4)	
6	128.07 d		7.63 (dd 7.4, 1.5)	
7	166.07 s		—	
8	—		—	
9	67.24 d		5.06 (dd 10.4, 7.7)	
10	69.59 t		4.48 (dd 8.7, 7.7)	
			4.63 (dd 10.4, 8.7)	
11	—		—	
12	169.09 s		—	
13	—		8.24 (s br.)	
14	55.32 d		4.36 (m)	
15	169.35 s		—	
16	—		8.10 (d 8.4)	
17	52.4 d	[52.6 d][a]	4.17 (m)	
18	171.12 s		—	
19	—		7.89 (m)	
			7.95 (m)	
20	35.38 t		3.22 (m)	
21	35.22 t		2.23 (t 7.3)	
22	169.98 s		—	
23	—		8.13 (d 8.4)	
24	49.42 d		4.29 (m)	
25	164.90 s		—	
26-OH	—		9.70 (s)	
27	51.20 t		3.44 (m)	
28	20.25 t		1.84 (m)	
29	26.63 t		1.59 (m)	
			1.86 (m)	
30	61.72 t		3.62 (dd 5.4)	
30-OH	—		4.98 (s br.)	
31	28.50 t	[28.55 t][a]	1.44 (m)	[1.64 (m)][a]
32	22.50 t	[22.52 t][a]	1.46 (m)	[1.54 (m)][a]
33	46.00 t	[48.77 t][a]	3.32 (m)	[3.37 (m)][a]
34-OH	—		9.70 (s)	
35	157.00 d	[161.74 d][a]	7.85 (s br.)	[8.21 (s br.)][a]

[a] Duplicate signals due to rotamers.

ROESY experiments. Hence, oxachelin is a new member of the siderophore family isolated from actinomycetes. A possible biosynthesis of **1** starts from a pentapeptide core structure, to which a salicylic acid is subsequently added and then further modified by hydroxylation, formylation and cyclization.

Besides the above mentioned siderophores formobactin, brasilibactin A and the nocardimicins, there are several more natural products which are structurally very closely related to oxachelin. The characteristic chromophore of **1** formed by the phenol-dihydrooxazole moiety are also present in brasilibactin A, the nocardimicins, antibiotic BE 32030 from *Nocardia* sp. [18], antibiotic BMS 199687 from *Actinomadura ferruginea* [19], the mycobactins from *Mycobacterium* species [20], acinetobactin from *Acinetobacter baumannii* [21], the anachelins from the cycnobacterium *Anabaena cylindrica* [22], or antibiotic L654040 from *Streptoverticillium syroense* [23]. The *N*-hydroxy-*N*-formyl side chain is described for example in formobactin, brasilibactin A, the nocardimicins or asterobactin from *N. asteroides* [24]. A cyclic and hydroxylated form of ornithine or lysine is observed in exochelin MN from *M. neoaurum* [25], Coelichelin from *Streptomyces coelicolor* [26] or again formobactin, brasilibactin A, the nocardimicins, the mycobactins, antibiotic BE 32030, antibiotic BMS 199687 or antibiotic L 654040.

Metal Complexing Properties

To investigate qualitatively the metal complexing properties of oxachelin, 200 μl of different metal salt solutions (150 μM) were added to 200 μl of a solution of **1** (300 μM) in deionized water, and the resulting mixture was subsequently analyzed by HPLC. In the case of a complex formation between oxachelin an the metal ion, an approximatly 50% reduction of free **1** is observed compared to non-complexing cations, indicating an equimolar complexation mechanism. Concurrently, complex formation is revealed in the HPLC chromatogram by the formation of a new peak, possessing a higher polarity and a UV spectrum with a bathochromic shift compared to uncomplexed **1**. Using this methodology, a 1 : 1 complex formation of oxachelin with Fe^{3+}, Fe^{2+}, Co^{2+}, Al^{3+}, Cr^{3+}, Ni^{2+} and Cu^{2+} could be shown, whereas Mg^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+} or Zn^{2+} are not complexed by **1**. The strong iron complexing properties of **1** are also expressed by the fact, that Fe^{3+} is removed from $Fe(SCN)_3$ and also from deferoxamine-Fe (Desferal®-bound iron III) by oxachelin, but not from $K_3[Fe(CN)_6]$. On the other hand, Fe^{3+} cannot be removed from the complex with **1** by addition of CN^- or H_2S.

Biological Activities

Oxachelin was screened against different pathogenic fungi in the microdilution assay with concentrations of 125 ng/ml~125 μg/ml:

In the agar diffusion assay, oxachelin shows considerable

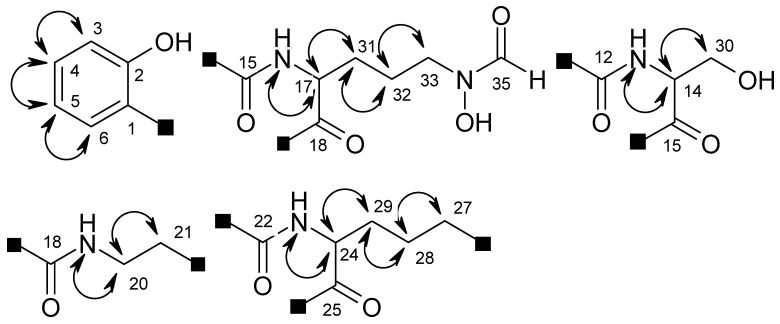

Fig. 2 Partial structures of **1** derived from gCOSY, gTOCSY and gHSQC-TOCSY experiments (DMSO-d_6, 500/125 MHz). Numbering according to Table 1 and Fig. 3.

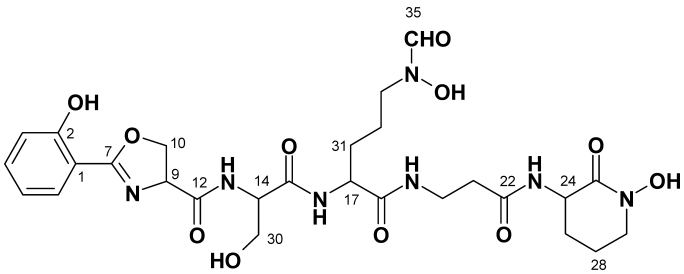

Fig. 3 Structure of oxachelin (**1**).

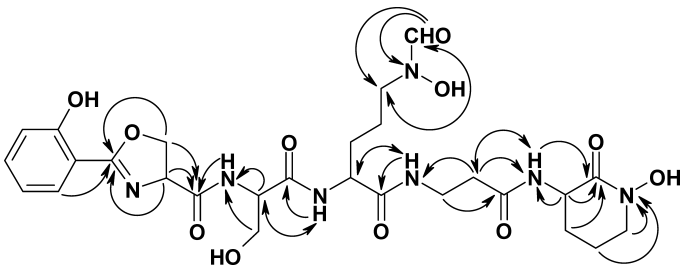

Fig. 4 Indicative $^{1}H,^{13}C$-gHMBC (→), $^{1}H,^{15}N$-gHMBC (→) and ROESY (↔) correlations in **1** (DMSO-d_6, 500/125/50 MHz).

activity against *Staphylococcus aureus* ATCC 6538. Here, an inhibition zone of 12 mm in diameter is observed (filter disc i.d. 6 mm, c = 1 mg/ml, 20 μl/disc).

Oxachelin shows no cytotoxicity in the tumor cell lines MCF-7 (ATCC HTB-22), L-929 (DSMZ ACC 2) or HEP-G2 (ATCC HB-8065) in concentrations up to 80 μM.

Discussion

Oxachelin (**1**) represents a novel natural product siderophore isolated from *Streptomyces* sp. GW9/1258, possessing selective metal complexing properties. Especially iron III is strongly bound, making **1** potentially valuable for the therapy of iron overload diseases like thalassemia, hepatitis or Parkinson's disease. Additionally, strong and selective antifungal and antibacterial effects

Table 2 Antimicrobial spectrum of **1**

Fungal species	MIC_{50} (μM)	MIC_{90} (μM)
Candida albicans ATCC 90028	12	>200
Candida glabrata DSM 6425	6	50
Cryptococcus neoformans ATCC 90112	1.6	3
Rhizomucor pusillus ATCC 36606	12	24
Fusarium solani CBS 181.29	>200	>200
Aspergillus fumigatus HD 3482/01*	50	200
A. flavus HD 2026/01*	100	>200
Trichophyton rubrum EF-S-714*	3	6
Microsporum canis EF-S-713*	3	6

* Clinical isolates Prof. Dr. med. Reinhard Kappe, Labor Diagnostika GmbH, Erfurt, Germany.

could be shown, whilst no cytotoxicity was observed.

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