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Microbial growth promotion studies of exochelin MN and analogues thereof

Li Dong[1], Marvin J. Miller[1] & Ute Möllmann[2]
[1]*Department of Chemistry and Biochemistry, 251 Nieuwland Science Hall, University of Notre Dame, Notre Dame, IN 46556, USA*
[2]*Hans-Knöll Institut für Naturstoff-Forschung, Jena, Germany*
[*]*Author for correspondence (Tel: 574-631-7571; E-mail : mmiller1@nd.edu)*

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Abstract

The ability of exochelin MN and three synthetic analogues to promote the growth of various strains of mycobacteria and Gram-negative bacteria was investigated. The results indicated that growth promotion ability of these compounds depends either on ligand exchange with mycobactin or on the exochelin permease. Despite stronger iron complexing capacity, the structural analogues showed weaker growth promotion ability than exochelin MN, which further supported our hypothesis of pH-dependent iron(III)-release of exochelin MN.

Introduction

One of the strategies employed by microorganisms to assimilate physiologically essential iron is the expression of siderophores and utilization of the complementary uptake and transport systems (Neilands 1995). The ability to acquire iron from an iron deficient environment and transport it inside the cell has a direct impact on the viability and virulence of pathogenic microbes (Neilands 1993). Although more and more information on iron transport mechanisms has been revealed (Braun & Braun 2002, Winkelmann 1991, Winkelmann & Carrano 1997, Wooldridge & Williams 1993), there are many details yet to be elucidated. This is especially important in the context of increasing recognition of the potential of iron transport systems as targets and drug delivery vehicles in the treatment of microbial infections (Miller & Malouin 1993, Roosenberg *et al.* 2000, Vergne *et al.* 2000).

Exochelin MN (**1a**, Figure 1) is an extracellular siderophore isolated by Ratledge and co-workers from the culture broth of *Mycobacterium neoaurum* in 1995 (Sharman *et al.* 1995). The molecule possesses impressive biological properties. It can transport iron not only into nonpathogenic *M. neoaurum* but also into *M. leprae* cells, which are causative agents of leprosy (Sharman *et al.* 1996). Structurally, exochelin MN contains an unusual ligand, *threo*-β-hydroxy-L-histidine, which has only been reported as a key iron binding component of pseudobactin PF244 (Hancock *et al.* 1993).

Recently, the first total synthesis of exochelin MN and analogues thereof has been achieved in this group (Dong & Miller 2002). At the same time, we have proposed a novel mechanism for the reversible coordination of iron(III) by exochelin MN. Intrigued by the unusual iron chelating ligand of the imidazole group (Sugiura 1980), we suggested that the iron binding ability of exochelin MN was strongly influenced by subtle changes of pH within the physiological range. Under neutral or basic conditions, exochelin MN, a hexadentate ligand, should be able to acquire iron effectively from the growth media. While at slightly acidic pH, protonation of the imidazole nitrogen of its constituent β-hydroxyhistidine would drastically reduce its affinity for iron (tetradentate ligand) and subsequently trigger the release of iron. This hypothesis was supported by the results from a study of the iron(III) coordination properties of exochelin MN. The first protonation constant for Fe(III)-exochelin MN

complex was determined to be 6.58 ± 0.05 (Dhungana *et al.* 2002). These data strongly indicated that the dissociation of iron(III) from imidazole ligand could be readily induced by a slight increase of environmental acidity.

As part of these studies, several exochelin MN analogues were designed (**1b–d**, Figure 1). In these compounds, the imidazole of β-hydroxyhistidine was replaced by phenol and catechol which are common iron-binding ligands found in siderophores. However, these ligands, unlike imidazole, are not readily protonated in their Fe(III)-complex forms under physiological conditions. We expect that the iron-binding and growth promotion studies of exochelin MN and analogues could provide further information to evaluate our theory. The following is an account of these investigations with other known siderophores, exochelin MS, mycobactin J, desferal and ferricrocin for comparison.

Methods and materials

Synthesis

Exochelin MN and analogues **1b–d** were synthesized according to the reported procedures (Dong & Miller 2002).

Biological assays

Bacterial strains. The Gram-negative bacteria are wild type strains from culture collections (*Pseudomonas aeruginosa* ATCC27853, ATCC 9027, NCTC 10662; *Escherichia coli* ATCC 25922), from the stock of the Hans-Knöll Institute (HKI), Jena, Germany (*P. aeruginosa* SG137), and a penetration mutant (K799/WT, Zimmermann 1979) or siderophore biosynthesis and tonB mutants.

Strain	Iron related marker	Origin/Reference
P. aeruginosa PAO 6609	pvd-	J.-M. Meyer, University of Strasbourg, France
P. aeruginosa K648	pch-, pvd-	K. Poole, University of Kingston, Canada
E. coli H 1443	aroB-	(Hantke, 1990)
E. coli AB2847	aroB-	V. Braun, University of Tübingen, Germany
E. coli BR 158	tonB-, aroB-	V. Braun, University of Tübingen, Germany

The mycobacteria are a wild type strain from the stock of HKI (*M. smegmatis* SG987), a transformation mutant (*M. smegmatis* mc^2155, Snapper *et al.* 1990) and mutants thereof in siderophore biosynthesis and transport (Schumann *et al.* 1998; Schumann & Möllmann 2001) as well as an exochelin and mycobactin negative mutant of the HKI stock (*M. phlei* 239-M77).

M. smegmatis strain	Biosynthesis of		Exochelin uptake
	Exochelin	Mycobactin	
SG 987	+	+	+
SG 987-M10	−	+	+
mc^2 155	+	+	+
mc^2 155-M24	+	−	+
mc^2 155-B1	−	+	+
mc^2 155-M24-B3	−	−	+
mc^2 155-M24-U3	+	−	−

Siderophores

Exochelin MS(Rb) (**2**, Figure 1) was cultivated and isolated by Dr R. Reissbrodt Wernigerode, Germany from *M. smegmatis* SG 987. It was used as a crude extract and consequently in higher concentrations. Mycobactin J (**3**, Figure 1) was from Rhone Merieux, Laupheim, Germany. Desferal (**4**, Figure 1) mesylate was from Sigma, Germany. Ferricrocin (**5**, Figure 1) was kindly provided by Prof. H.P. Fiedler, Tübingen, Germany.

Siderophore assays

Utilization of siderophores was determined by a growth promotion assay as described (Schumann *et al.* 1998; Schumann & Möllmann 2001). Alternatively, the following assay medium was used for the *E. coli* mutants and for *P. aeruginosa* PAO 6609: NaCl (0.5%), tryptone (0.8%), agar (1%), dipyridyl (150 μmol) and EDDHA (150 μmol). Strains were suspended in the iron depleted agar media (pH 6.8) hindering normal bacterial growth. The inoculated media was poured into petri dishes. Siderophore solutions were applied on paper discs of 6 mm in diameter on the surface of the agar plates. Growth zones surrounding the discs were read after 1 day for *P. aeruginosa* and *E. coli* strains and after 2 days for *M. smegmatis* strains. Determination of the relative iron complexing capacity of the desferri- or ferri-siderophores was performed by the chrome azurol S (CAS) assay (Schwynn & Neilands 1987) and demonstrated by the diameter of orange halo in mm for a 5 μl sample on a CAS agar plate. Antimicrobial activity was determ-

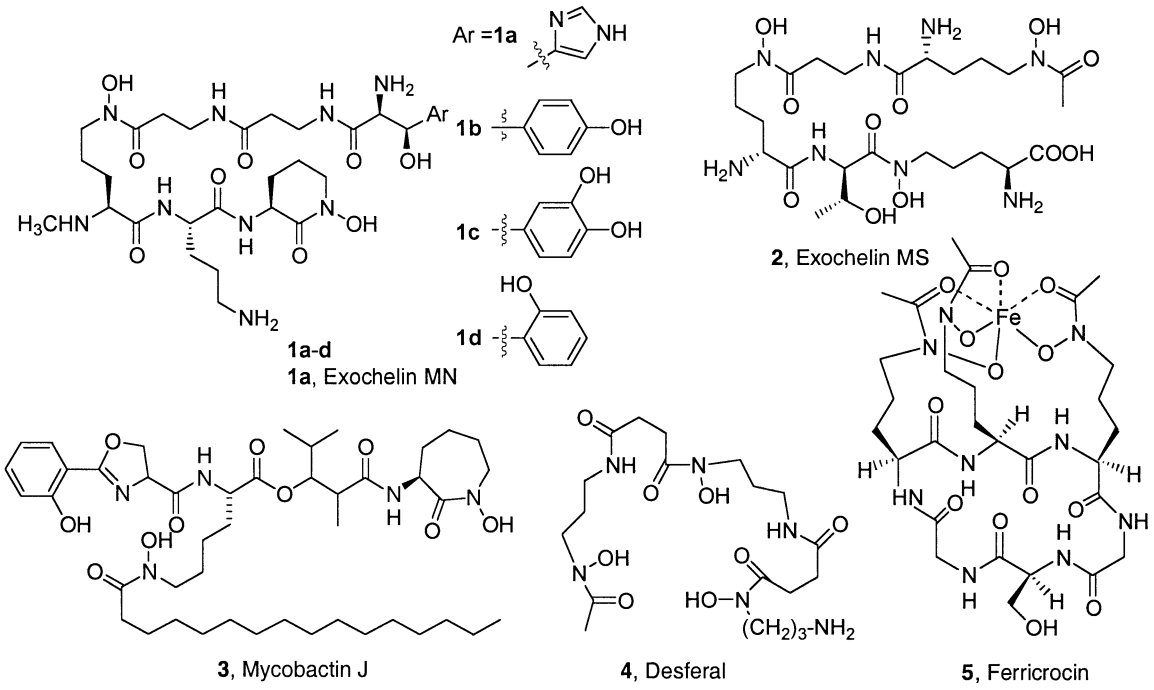

Fig. 1. Exochelin MN (**1a**), analogues **1b-d**, exochelin MS (**2**), mycobactin J (**3**), desferal (**4**) and ferricrocin (**5**).

Table 1. Growth promotion in mm (growth zones) of selected mycobacterial strains by exochelin MN analogues **1b–d** relative to the natural siderophores exochelin MN (**1a**), exochelin MS(Rb) (**2**) and mycobactin J (**3**).

Strain	Exochelin MN[a]	Exochelin MS[a]	1**1b**[a]	**1c**[a]	**1d**[a]	Mycobactin J[a]
Concentration [mg/mL]	1	10	1	1	1	0.4
CAS-Reaction	5	0	10	9	9	0
M. smegmatis SG 987	23	26	15	8	19	18
M. smegmatis SG 987-M10	18	21	11	0	12	16
M. smegmatis mc^2 155	25	27	16	10	17	17
M. smegmatis mc^2 155-M24	25	26	11	10z	13	15
M. smegmatis mc^2 155-B1	26	25	14	11z	15	16
M. smegmatis mc^2 155-M24-B3	0	9z	0	0	0	16
M. smegmatis mc^2 155-M24-U3	0	10z	0	0	0	15
M. phlei 239-M77	22	21	A	0	15A	15

[a]Diameter of zones of growth promotion in mm for a 5 μg sample in agar diffusion on a petri dish. A: Indication of growth. z: weak growth zone

ined by an agar diffusion assay according to Deutsches Arzneibuch (1986). All compounds were tested in a concentration of 5 μg/well (50 μl of a 100 mg/l solution).

Results and discussion

Biological assays

The CAS reaction showed that we had desferri- (exochelin MN, analogues **1b–d** and desferal) and ferri-siderophores (exochelin MS(Rb), mycobactin J, and ferricrocin). Since the diameters of the orange halos in the CAS reaction are directly related to the

Table 2. Growth promotion in mm (growth zones) of select Gram-negative bacteria by exochelin MN analogues **1b–d** relative to the natural siderophores exochelin MN (**1a**), exochelin MS(Rb) (**2**) and desferal (**4**).

Strain	Exochelin MN[a]	Exochelin MS[a]	**1b**[a]	**1c**[a]	**1d**[a]	Desferal[a]
Concentration [mg/mL]	1	10	1	1	1	1
CAS-Reaction	5	0	10	9	9	14
P. aeruginosa SG 137	24	22	17	17	18	35
P. aeruginosa ATCC 27853	24	26	20	22	23	40
P. aeruginosa ATCC 9027	28	24	23	22	22	32
P. aeruginosa NCTC 10662	25	27	13	18	19	43
P. aeruginosa K799/WT	25	26	17	18	18	39
E. coli ATCC 25922	32	35	24	18	26	25

[a]Diameter of zones of growth promotion in mm for a 5 μg sample in agar diffusion on a petri dish.

Table 3. Growth promotion in mm (growth zones) of select Gram-negative bacteria mutants by exochelin MN analogues **1b–d** relative to the natural siderophores exochelin MN (**1a**), exochelin MS(Rb) (**2**), desferal (**4**) and ferricrocin (**5**).

Strain-iron related marker	Exochelin MN[a]	Exochelin MS[a]	**1b**[a]	**1c**[a]	**1d**[a]	Desferal[a] /Ferricrocin
Concentration [mg/mL]	1	10	1	1	1	1
CAS-Reaction	5	0	10	9	9	D 14/F 0
P. aeruginosa ATCC 27853/WT	22	25	13	20	17	D 40
27853/WT initial growth zones	15	19	9	14	11	D 35
P. aeruginosa PAO 6609-pvd	15	25	11	10	11	F 32
P. aeruginosa K648-pch, pvd	18	30	10	0	12	D 29
E. coli H 1443-aroB	0	0	0	0	0	F 28
E. coli AB 2847-aroB	0	0	0	0	0	F 27
E. coli BR 158-ton B, aroB	0	0	0	0	0	F 0

[a]Diameter of zones of growth promotion in mm for a 5 μg sample in agar diffusion on a petri dish.

relative iron binding abilities of siderophores, the assay indicated that analogues **1b–d** were able to bind iron more strongly than exochelin MN as oxygen ligands were known to have higher affinity for iron than nitrogen ligands.

The results of the microbial assays of exochelin MN and analogues for strains of *M. smegmatis* showed that exochelin MN had growth stimulation ability comparable to exochelin MS(Rb), and better than that of mycobactin J (Table 1), which was expected considering the role exochelins play in the iron transport process (Matzanke 1997). As exochelin MS(Rb) was a crude extract, there may be components different from exochelin which had a minor growth promoting activity in the mutants B3 (exochelin (-), mycobactin (-)) and U3 (mycobactin (-), exochelin permease (-)). Normally no growth promotion was expected in both of these mutants as the uptake of exochelin-bound iron depends either on ligand exchange with mycobactin or on the exochelin permease. This was apparently true for exochelin MN and analogues.

Despite their stronger iron-binding ability, analogues **1b–d** were less effective than exochelin MN in growth promotion assays for strains of *M. smegmatis* (Table 1). One plausible explanation was that although these analogues were able to acquire iron from the media, the iron could not be efficiently transferred to mycobactin and further utilized by the cell. At the outset of designing the analogues, we intended to take advantage of the significantly different protonation constants between imidazole and phenol (catechol) Fe(III)-complexes to test our hypothesis of pH-dependent iron release of exochelin MN (*vide supra*). Gratifyingly, these results fit nicely into our

theory and provided additional evidence for this novel iron discharge pathway. The exceptionally low protonation constant of the catechol ligand presumably further blocked the transfer of iron to mycobactin and consequently decreased the growth promotion activity of compound **1c**, which was especially obvious for strains *M. smegmatis* SG987-M10 and mc^2 155-B1.

Unlike mycobacteria, Gram-negative bacteria do not utilize exochelin-mycobactin iron exchange processes to assimilate iron. As a consequence, we did not expect significant differences in the growth promotion capability among exochelin MS, exochelin MN and analogues **1b–d** since they should all function as common siderophores. Microbial assays of these compounds for selected Gram-negative bacteria supported our prediction (Table 2). Desferal, (K_{assoc} = 30.6) (Hider 1984; Harris *et al.* 1979) which is known for its strong iron-complexing ability, proved to be the best promoter in these assays.

In order to prove that the above growth promotion resulted from the uptake of ferri-exochelin MN and analogues instead of being a consequence of ligand exchange, we conducted additional assays with the siderophore biosynthesis and tonB mutant strains (Table 3). The pyoverdin and pyochelin single or double mutants of *P. aeruginosa* were shown to be able to assimilate exochelin without ligand exchange as demonstrated by the observed growth stimulation of these strains. The stronger growth promotion of the wild type strains can be an overlap/addition effect from exochelin and the indigeneous siderophores produced by the organisms. The initial growth zones detected on the plates with the wild type are similar in size to those of the mutants (Table 3). However, in contrast to the mutant strains, the growth zones of the wild type further increased due to the beginning of siderophore biosynthesis.

The *E. coli* siderophore mutants do not utilize exochelins, which might be explained by the much higher affinity of enterobactin to iron than that of the *Pseudomonas* siderophores as well as exochelins and analogues. Therefore, we assume that the growth promotion of *E. coli* strains is due to a ligand exchange process between the exochelins and enterobactin.

Finally, exochelin MN and analogues were tested for antimicrobial activity on a variety of representative Gram-positive and Gram-negative bacteria as well as yeast and fungi (list not shown). Not surprisingly, no inhibition of growth of any of the test strains was observed as these compounds were active in growth promotion.

Conclusions

The growth promoting ability of exochelin MN and several structural analogues was assessed for various strains of mycobacteria and Gram-negative bacteria. Analogues **1b–d** showed lower growth promotion activity for mycobacteria although they were stronger iron binders than exochelin MN, which provided further support for our hypothesis of pH-dependent iron release of exochelin MN. As for Gram-negative bacteria, no significant difference in activity was found apparently because they utilize different iron transport mechanisms. We believe studies in this area could further advance our understanding of the iron acquisition mechanisms of mycobacteria and facilitate the development of novel antimycobacterial agents in the future.

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