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The remarkable hydrophobic effect of a fatty acid side chain on the microbial growth promoting activity of a synthetic siderophore

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Abstract

The ability of synthetic derivatives of the siderophore tripeptide of N^5-hydroxy-N^5-acetyl-L-ornithine to promote the growth of various strains of mycobacteria and Gram negative bacteria was found to depend significantly on the hydrophobic nature of the derivative. Although the tripeptide of N^5-hydroxy-N^5-acetyl-L-ornithine is not normally utilized by mycobacteria, an *N*-terminal palmitoyl derivative mimicked natural mycobactin J in all studies.

Introduction

Siderophores are iron(III) chelators synthesized and secreted by microbes to scavenge and assimilate physiologically essential iron (Neilands 1995). The ability to acquire iron from an iron deficient environment and transport it inside the cell has a direct affect on the viability and virulence of pathogenic microbes (Wooldridge & Williams 1993; Neilands 1993). Siderophore transport systems and siderophore-drug conjugates are being exploited for microbially targeted drug delivery using the 'Trojan Horse' strategy (Miller & Malouin 1993; Roosenberg *et al.* 2000; Miller 1989; Roosenberg & Miller 2000).

Related to our interests in synthesizing siderophore analogs as either microbial growth promoters or growth inhibitors, we decided to incorporate a fatty acid side chain in the tripeptide of N^5-hydroxy-N^5-acetyl-L-ornithine (**1**. Figure 1), the iron chelating ligand found in fungal siderophores, such as ferrichrome and albomycins (Benz *et al.* 1982). A C16 fatty acid residue found in the linear hydroxamic acid in mycobactins (Vergne *et al.* 2000) and the most recently isolated marinobactin E (Martinez *et al.* 2000) was chosen to be incorporated in the *N*-terminus of peptide siderophore component **1** as part of our efforts to prepare siderophore hybrids for structure-activity-relationship (SAR) studies. From the outset, we anticipated that incorporation of this long alkyl side chain in the *N*-terminus of tripeptide siderophore component **1** should not interfere with its iron(III) binding ability, but should change its hydrophobicity. Iron free ligand **1** is very hydrophilic whereas its iron complex **2** is amphiphilic. Addition of a hydrophobic side chain to **1** was anticipated to give **3**, which should have increased hydrophobicity. Exposure of **3** to ferric iron was expected to provide the very hydrophobic iron complex **4** (Figure 1). The hydrophobic interaction between the long alkyl chain of **3** or **4** and microbial cell walls could lead to alteration of microbe selective siderophore utilization and/or unusual biological activity through increased membrane solubility. For example, although natural mycobacterial siderophores are not derived from the tripeptide of N^5-hydroxy-N^5-acetyl-L-ornithine, it was anticipated that these hydrophobically modified forms might behave like mycobactins and affect the growth of mycobacteria.

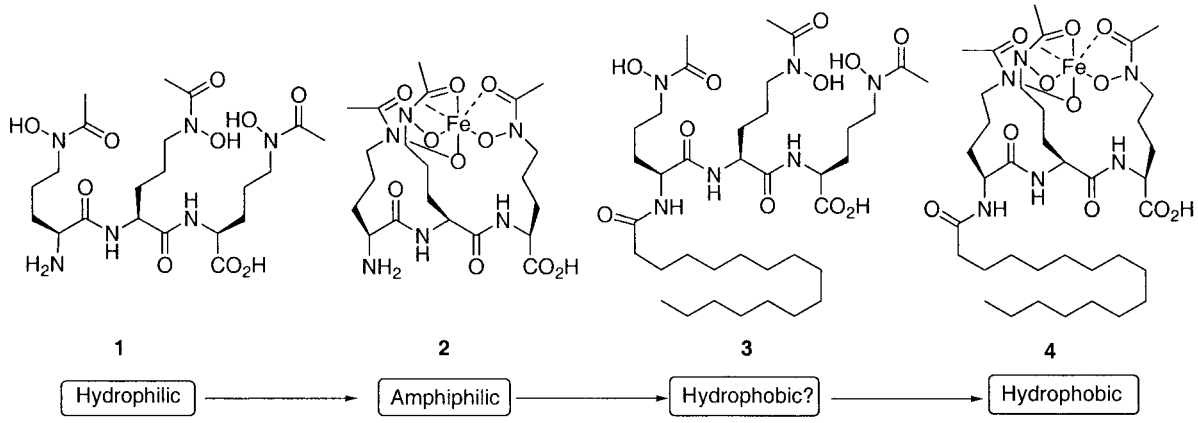

Fig. 1. Structures of tripeptides of N^5-hydroxy-N^5-acetyl-L-ornithine.

Methods and materials

Synthesis

$N^2 - Palmitoyl - N^5 - acetyl - N^5 - hydroxy - l - ornithyl - N^5 - acetyl - N^5 - hydroxy - l - ornithyl - N^5 - acetyl - N^5 - hydroxy - l - ornithine$ (3). To a mixture of N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithine · DIPEA (**1**, (Lin & Miller 1999) 60 mg, 0.0905 mmol) and sodium bicarbonate (15 mg, 0.178 mmol) in DMF (2 ml) at rt, was added the NHS active ester of palmitoic acid (38 mg, 0.107 mmol). The reaction mixture was stirred at *rt* for 48 h. Water and ethyl acetate were added and the pH of the mixture was adjusted to acidic with 0.1 N HCl. Layers were separated and the aqueous layer was extracted with ethyl acetate. The combined extracts were washed with water, dried (Na_2SO_4), filtered, concentrated and separated by reverse phase flash column chromatography (C_{18}, 4:1 to 8:1 CH_3OH/H_2O) to give 11 mg (16%) of analog **3** as an oil. ^{1}H NMR (500 MHz, CD_3OD) δ 4.40 (m, 3H), 3.62 (m, 6H), 2.32 (t, $J = 7.5$ Hz, 2H), 2.10 (m, 9H), 1.69 (m, 12H), 1.31–1.23 (m, 26H), 0.89 (t, $J = 7.5$ Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 176.7, 176.6, 175.4, 174.6, 174.2, 174.0, 173.8, 54.4, 54.3, 54.2, 53.7, 53.6, 48.7, 48.5, 48.4, 48.3, 36.9, 33.2, 31.0, 30.8, 30.6, 30.5, 30.4, 30.3, 30.2, 30.1, 30.0, 29.9, 27.1, 27.0, 24.5, 24.4, 24.3, 24.2, 23.9, 20.4, 14.6; HRFABMS cacld for $C_{37}H_{68}N_6O_{11}Na$ ($M+Na^+$): 795.4844, found: 795.4832.

Biological assays

Bacterial strains.

The Gram-negative bacteria were wild type strains from culture collections (*Pseudomonas aeruginosa* ATCC 27853, ATCC 9027, NCTC 10662; *Escherichia coli* ATCC 25922), from the stock of the Hans-Knöll-Institute (HKI) (SG 137). The mycobacteria represent wild type strains (*Mycobacterium smegmatis* SG 987, from the stock of the Hans-Knöll-Institute, Jena, Germany, *M. smegmatis* mc^{2}155, (Snapper *et al.* 1990) and mutants thereof in siderophore biosynthesis and transport (Schumann *et al.* 1998; Schumann & Möllmann 2000):

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(35)	−	−	+
mc^2 155-M24-U3(47)	+	−	−

Siderophores

Ferrichrome (**6**) and Ferricrocin (**7**) were kindly provided by Prof. H.P. Fiedler, Tübingen, Germany. Mycobactin J (**5**) was from Rhone Merieux, Laupheim, Germany.

Table 1. Growth promotion in mm (growth zones) of selected mycobacterial strains by synthetic peptides **1**–**4** relative to the natural siderophores mycobactin J (**5**), ferrichrome (**6**) and ferricrocin (**7**).

Strain	1[a]	2[a] (1+Fe)	3[a]	4[a] (3+Fe)	5	6	7
CAS-Reaction	+++	−	+++	−	−	−	−
M. smegmatis SG 987	13	30	18	20	16	28	18
M. smegmatis SG 987-M110	12	30	18	19	16	25	15
M. smegmatis mc^2 155	A	24	20	22	17	25	17
M. smegmatis mc^2 155-M24	0	28	13	17	18	25	12
M. smegmatis mc^2 155-B1	A	34	18	22	16	28	13
M. smegmatis mc^2 155-M24-B3(35)	0	25	16	21	15	20	10
M. smegmatis mc^2 155-M24-U3(47)	0	24	13	17	16	18	9

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

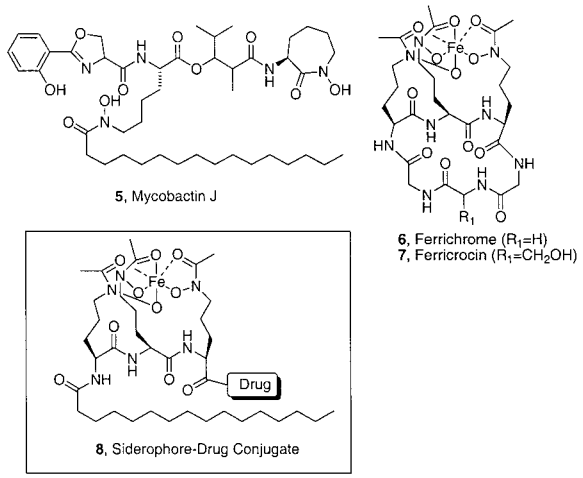

Fig. 2. Mycobactin J (**5**), ferrichrome (**6**), ferricrocin (**7**), and a generic siderophore-drug conjugate (**8**) structure.

Siderophore Assays

Utilization of siderophores was determined by a growth promotion assay as described (Schumann *et al.* 1988; Schumann & Möllmann 2000). Strains were suspended in iron depleted agar media hindering normal bacterial growth. The inoculated media were 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 a positive or negative CAS reaction as indicated in Tables 1 and 2.

Results and discussion

Synthesis

The synthesis of the hydrophobic ferrichrome analog **3** was very straightforward (equation 1). Treatment of readily available siderophore component N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-hydroxy-L-ornithine (**1**) (Lin and Miller 1999) with the *N*-hydroxysuccinimide (NHS) active ester of palmitoic acid afforded the desired analog **3**, albeit in low yield, but without the need to protect any of the several functional groups on **1**.

Biological assays

Selected microbial assays of palmitoyl derivative **3**, its iron complex **4**, its zwitterionic precursor **1**, and corresponding iron complex **2** were then carried out. Indeed, growth promotion assays of the desferri analog **3** showed its siderophore activity, as measured by stimulation of growth, for strains of *M. smegmatis* (Table 1), to be similar to that of mycobactin J (**5**) and comparable to or better than that of ferricrocin (**7**). On the other hand, desferri siderophore component **1** appeared to be too hydrophilic to promote the growth of most of the tested strains of mycobacteria. As expected, the corresponding iron complexes **2** and **4** diplayed increased mycobacterial growth promotion.

While desferri tripeptide **1** was a relatively ineffective growth promoter of mycobacteria, both it and its ferric complex **2** were remarkably effective at promoting the growth of a test strain of *E. coli* and, somewhat less, of *P. aeruginosa* strains (Table 2). The growth enhancement of *E. coli* with tripeptides **1** and **2** was

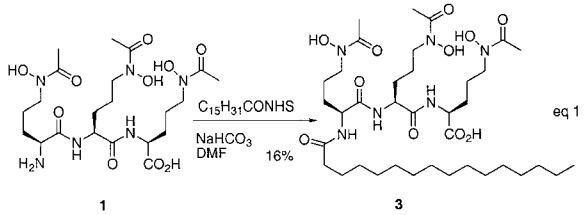

Table 2. Growth promotion in mm (growth zones) of select gram negative bacteria by synthetic peptides **1**–**4** relative to the natural siderophores mycobactin J (**5**, ferrichrome (**6**) and ferricrocin (**7**).

Strain	1[a]	2[b] (1+Fe)	3[a]	4[a] (3+Fe)	5	6	7
CAS-Reaction	+++	−	+++	−	−	−	−
P. aeruginosa SG 137	25	24	14	13	15	31	29
P. aeruginosa ATCC 27853	23	20	14	14	16	35	25
P. aeruginosa ATCC 9027	26	30	15	12	16	35	30
P. aeruginosa NCTC 10662	27	33	17	17	15	40	32
E. coli ATCC 25922	33	34	19	15	10	36	30

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

comparable to that of the natural siderophores ferrichrome (**6**) and ferricrocin (**7**) even though **1** and **2** are structurally simplified relative to the natural cyclic hexapeptides (**6** and **7**) that contain the same iron binding tri-*N*-hydroxy ornithine component. Interestingly, the more hydrophobic analog **3**, its iron complex **4** and mycobactin J (**5**) were significantly less effective growth promoters of the same strain of *E. coli*. Again indicating that the hydrophobic derivatives of the tripeptide behave more like the lipophilic mycobactins. Both **1** and **3**, and their ferric complexes **2** and **4**, stimulated growth of strains of *Pseudomonas* (Table 2), but not to the same extent as did ferrichrome (**6**) or ferricrocin (**7**) (Figure 2). Again, the more hydrophobic palmitoyl-containing analog **3** and its iron complex **4**, were less effective growth promoters for the strains of *Pseudomonas* tested. Thus, for all organisms tested, the hydrophobic derivatives of N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithine behave more like mycobactins than the parent tripeptide or its natural siderophore derivatives, ferrichrome of ferricrocin.

Conclusions

The growth promoting activity of the readily accessible synthetic analog **3** and its ferric complex **4** indicates the important hydrophobic effect of the C16 fatty acid side chain for utilization as a siderophore by mycobacteria and less effective utilization by other strains of bacteria that do not typically rely on membrane bound siderophores. This is especially notable since none of the known natural membrane-bound mycobacterial siderophores are structurally based on the ornithine tripeptide form of **1** (Vergne *et al.* 2000). The growth promoting ability of **3** and **4** also suggests that this synthetic siderophore derivative could serve as a valuable substitute for more complex natural siderophores by relatively simple variation of the hydrophobic or hydrophilic character. Because antimicrobial agents could be readily attached to the pendant free carboxyl group in analog **4**, it might serve as a valuable siderophore component in the design of siderophore-drug conjugates (**8**, Figure 2) for potential treatment of mycobacterial and other pathogenic infections.

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