Biol Metals (1991) 4:186–191

BIOLOGY OF METALS



# Chiral linear hydroxamates as biomimetic analogues of ferrioxamine and coprogen and their use in probing siderophore-receptor specificity in bacteria and fungi

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Received April 25, 1991

**Summary.** Linear hydroxamate derivatives, possessing chiral $\alpha$-amino acid moieties, were synthesized and their iron transport activities were studied in bacteria and fungi. No growth-promoting activity could be detected in the Gram-positive hydroxamate-auxotroph *Aureobacterium flavescens* JG9. However, Gram-negative enterobacteria, such as *Escherichia coli*, *Pantoea agglomerans* and *Hafnia alvei* were able to utilize iron from these analogues. Uptake of $^{55}$Fe-labeled analogues was inhibited by sodium azide, suggesting an active transport process. The receptors involved during uptake in enterobacteria were identified by using appropriate indicator organisms which are defective in the transport of either ferrioxamines (*P. agglomerans* FM13), coprogens (*H. alvei*), or both of these siderophore classes (*E. coli fhuE*). Our data suggest that the chiral hydroxamates are recognized by the ferrioxamine receptor (FoxA) and the coprogen receptor (FhuE) at a ratio which depends on the optical $\Lambda/\Delta$ isomer fraction and the nature of side chains. Transport was also observed in the fungus *Neurospora crassa*, known to take up coprogen rather than ferrioxamines, suggesting that in this fungus the synthetic analogues behave like coprogen.

**Key words:** Iron transport – Siderophore receptors – Siderophore analogues – Ferrioxamine – Coprogen – Biomimetics

## Introduction

Bacteria and fungi are known to take up iron via siderophores. Hydroxamate-type siderophores may be of fungal (ferrichromes, coprogens) or bacterial (ferrioxamines) origin. The Gram-positive siderophore-auxotroph *Aureobacterium flavescens* JG9 has been shown to utilize a variety of hydroxamate-type siderophores, whereas catecholate-type siderophores are not recognized. Gram-negative enterobacteria have been shown to utilize, besides catecholate-type siderophores, various hydroxamate-type siderophores (ferrichromes, coprogens, ferrioxamines, aerobactin), irrespective of whether or not they orginate from bacteria or fungi. The ability of *Escherichia coli* to take fungal hydroxamate siderophores is generally dependent on the presence of FhuA and FhuE outer-membrane receptor proteins and of further periplasmic and cytoplasmic membrane proteins (Hantke 1983; Braun et al. 1991; Köster 1991). We have recently shown that species of *Enterobacter agglomerans* (*Erwinia herbicola*), now named *Pantoea agglomerans* (Gavini et al. 1989), and *Hafnia alvei* produce ferrioxamines (Berner et al. 1988; Reissbrodt et al. 1990). Ferrioxamines have been described earlier as typical siderophores of *Streptomyces* species (Bickel et al. 1960) and *Pseudomonas stutzeri* (Meyer and Abdallah 1980). Moreover, a novel outer-membrane receptor protein (FoxA) involved in ferrioxamine transport in *P. agglomerans* (*E. herbicola*) has recently been detected (Berner and Winkelmann 1990). The FoxA protein showed a molecular mass of 76 kDa, which is the same as that found for the coprogen receptor in *E. coli* (Sauer et al. 1987). Earlier investigations with synthetic *retro*hydroxamate ferrichromes revealed good growth-promoting activity in *A. flavescens* JG9 (Emery et al. 1984; Shanzer et al. 1988; Shanzer and Libman 1989). In the present investigation, the biological activity of ferrioxamine analogues possessing chiral amino acid residues was studied in microorganisms utilizing ferrioxamine and/or coprogen.

## Materials and methods

*Strains and growth conditions. Arthrobacter flavescens* JG9 (ATCC 29091), now reclassified as *Aureobacterium flavescens* JG9, was kindly provided by P. Szaniszlo (University of Texas, Austin). *Escherichia coli* H1443 (*aroB*) and its corresponding *fhuE* mutant H1774 were from K. Hantke (Tübingen). *P. agglomerans* (*Erwinia herbicola* K4) was from U. Ullmann (Kiel). The derived ferrioxamine-receptor-deficient (*foxA*) mutants, FM13 and FM30, were

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obtained by nitrosoguanidine mutagenesis and ferrimycin selection as described earlier (Berner and Winkelmann 1990). *Hafnia alvei* 7473 is a wild-type strain (Reissbrodt et al. 1990) which we found to be unable to take up coprogen-bound iron, suggesting that a coprogen receptor is absent or inactive. All strains were maintained on agar slants made of nutrient broth. For transport experiments and growth-promotion tests, cells were grown in M9 minimal salts medium containing 0.4% glucose, autoclaved separately. *Neurospora crassa arg-5 ota aga* (ornithine deficient) was maintained on yeast extract/malt extract/glucose agar. Conidia were harvested, grown in asparagine medium and used for kinetic measurements after 8–12 h of germination.

*Bioassays.* The bioassay medium used for *A. flavescens* JG9 contained per litre: 2.0 g $K_2HPO_4$, 0.5 g $(NH_4)_2HPO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 1.0 g yeast extract, 4.0 g glucose (autoclaved separately), 0.4 g agar and 3 µl/20 ml agar of a preculture of *A. flavescens* JG9. The bioassay medium used for *Pantoea* and *Hafnia* strains contained per litre bidistilled water: 12.1 g Tris (Trizma, Sigma), 0.3 g $KH_2PO_4$, 0.5 g NaCl, 1.0 g $NH_4Cl$, 1 mM $MgSO_4 \cdot 7H_2O$ (autoclaved separately), 0.1 mM $CaCl_2 \cdot 2H_2O$ (autoclaved separately), 4.0 g glucose (autoclaved separately), 1 mM ethylenediamine-*N,N'*-bis(2-hydroxyphenylacetic acid) (EDDHA, Fluka; autoclaved separately), pH 7.0 plus 4.0 g agar. The corresponding medium for the *E. coli* (*aroB*) strains contained nutrient broth medium plus 1 mM EDDHA. The inoculum was dependent on the strain used. Per plate, 20 µl of a preculture grown to an absorbance of 0.04–0.1 at 578 nm was used.

*Transport assays.* Transport of $^{55}$Fe-labeled siderophores in bacteria was measured as described previously (Berner and Winkelmann 1990). Transport in the fungus *N. crassa* was according to earlier protocols (Konetschny-Rapp et al. 1988b; Huschka and Winkelmann 1989).

*Siderophores.* All natural hydroxamate siderophores were from the stock of the Institute in Tübingen and the purity was analyzed by HPLC as described earlier (Konetschny-Rapp et al. 1988a). The linear hydroxamates were synthesized by: (a) preparation of the N-protected amino acid residue **1**, (b) coupling of the amino acid residue with *N*-benzylhydroxy-3-propionic acid ethyl ester to the monomeric ester **2**, (c) hydrolysis of the monomeric ester to the monomeric amino acid **3** and (d) oligomerization of three monomeric acids to the trimeric derivatives **4** by Merrifield solid-phase synthesis. These products were purified by column chromatography on silica gel and fully characterized by their sepctroscopic properties. Finally, removal of the protecting groups from **4** by hydrogenolysis provided the linear hydroxamates. The latter were optionally purified by preparative thin-layer chromatography and characterized by their spectra. The detailed experimental procedures will be described in a separate publication.

## Results and Discussion

### *Design and synthesis of biomimetic siderophores*

The naturally occurring linear siderophores, such as ferrioxamine, possess three hydroxamate groups on a string which are bridged by amide- or ester-containing methylene chains. When binding $Fe^{3+}$, the ligands wrap around the guest ion in a loop to form several configurational isomers including *cis* and *trans* isomers of right or left handedness. In our design we aimed at generating singular $Fe^{3+}$-complexes by (a) eliminating the formation of *trans* isomers via contraction of the loops between the binding sites, and by (b) inducing chiral preference via the additon of asymmetric centers. Both these targets were realized by replacing the extended chains of the natural siderophores by shorter amino acid residues in **5**. This change necessarily inverted the directionality of the hydroxamate groups to a *retro* arrangement relative to that of ferrioxamine. This was, however, anticipated to be of minor biological relevance, since inversion of ferrichrome to its *retro*-isomer did not alter, or even reduce, its biological activity (Emery et al. 1984).

According to these considerations, five chiral *retro*-hydroxamates were synthesized that differ in the side chains of the amino acids used. The synthetic strategy involved the preparation of the monomeric hydroxamate units **2** and **3**, and their subsequent oligomerization by Merrifield-type synthesis on solid support.

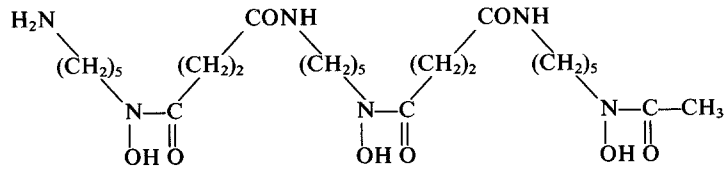
All hydroxamates were fully characterized by their spectroscopic properties. They all were found to bind one equivalent of $Fe^{3+}$, and to form complexes of preferred $\Delta$ configuration when L-amino acids were used, but preferred $\Lambda$ configuration when D-amino acids were used. However, the extent of chiral preference was found to depend on the nature of the amino acids used. While the chiral preference was rather modest with the Ala (P191) and Leu derivatives (P178, MP14, P239), it was significant with the Asp derivative (P238).

### *Biological testing of biomimetic siderophores*

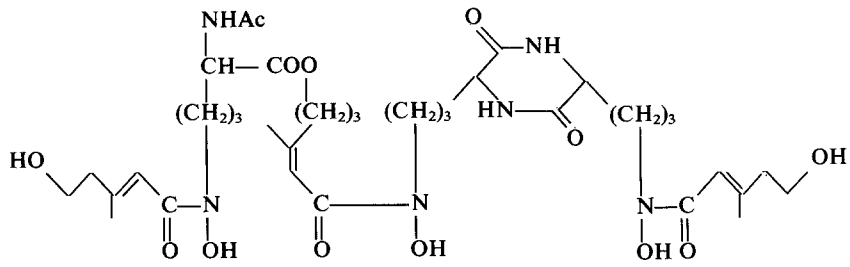
The biological activity of synthetic hydroxamates was studied in bacteria that were known to show growth response in the presence of hydroxamate siderophores (Table 1). Surprisingly, *A. flavescens* JG9 did not respond by growth promotion when the synthetic compounds were added onto filter discs in agar plates seeded with *A. flavescens* JG9, although this indicator strain accepts natural hydroxamate siderophores and *retro*-hydroxamate ferrichromes. Ferrioxamine-utilizing bacteria, such as *P. agglomerans* (*E. herbicola*) and the derived ferrioxamine-receptor-negative mutants FM13 and FM30, were also included in the bioassay. As was shown earlier, ferrioxamines are excluded from transport into these FM-mutant strains. Growth-promotion tests using these two strains and the parent strain revealed that the synthetic compounds are utilized by both parent and mutant strains, suggesting that the synthetic compounds were taken up by additional membrane receptors. As parent and mutant strains still possess an additional coprogen receptor (Berner and Winkelmann 1990), we suggested that the analogues entered the cells via the coprogen receptor.

Coprogens are an important group of linear hydroxamate siderophores in fungi which are known to be utilized by enterobacteria. In the present study two strains were included which were unable to take up coprogen: *H. alvei*, a wild-type strain which we found to be unable to utilize iron from coprogen, and *E. coli* H1774, a coprogen-receptor-deficient *fhuE* mutant derived from the parent *aroB* strain *E. coli* H1443.

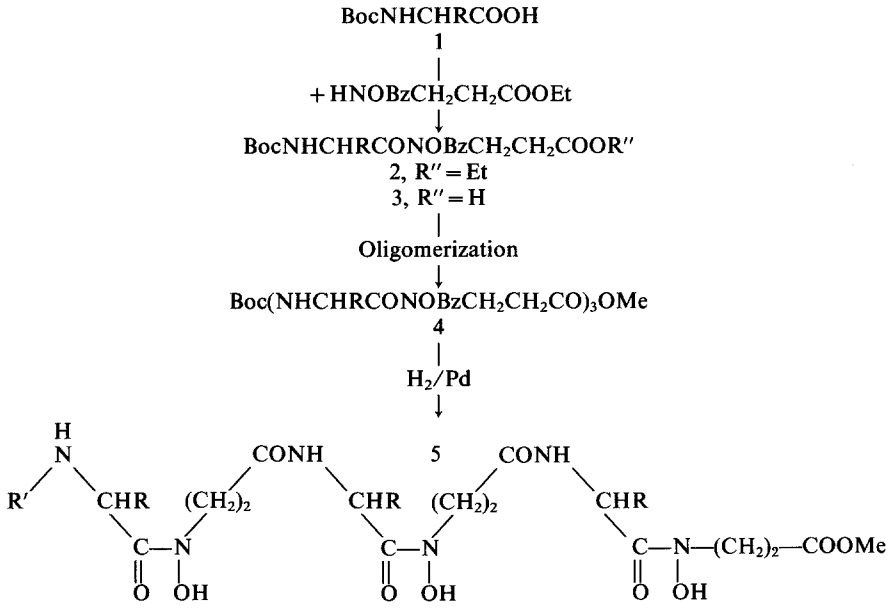
As shown in Table 1, growth promotion was absent in the *fhuE* mutant, suggesting that in *E. coli* the FhuE



Ferrioxamine B



Coprogen



| Code | R | R′ |
|---|---|---|
| P191 | L-Me | Boc |
| P178 | L-iBu | Boc |
| MP14 | L-iBu | H |
| P239 | D-iBu | Boc |
| P238 | L-$CH_2CONEt_2$ | Boc |

**Table 1.** Growth-promotion tests with indicator strains

| Strain | relevant genotype | Coprogen | Ferri-oxamine B | P191 −5.8 | P178 −3.7 | P238 −6.5 | P239 +3.4 |
|---|---|---|---|---|---|---|---|
| *P. agglomerans* K4 | parent | + | + | + | + | + | + |
| *P. agglomerans* FM13 | *foxA* | + | − | + | + | + | + |
| *P. agglomerans* FM30 | *foxA* | + | − | + | + | + | + |
| *E. coli* H1443 | parent *aroB* | + | (+) | + | + | + | + |
| *E. coli* H1774 | *fhuE* | − | (+) | − | − | − | − |
| *H. alvei* 7473 | wild type | − | + | − | − | − | − |
| *A. flavescens* JG9 | sid.-auxotroph | + | + | − | − | − | − |

Growth promotion tests were carried out on agar plates containing 1 mM EDDHA as described in Materials and methods. Symbols: + growth, (+) poor growth, − no growth. The numbers given below the compound numbers correspond to the Cotton effects for the exiton transitions in the $Fe^{3+}$ complexes at 230 nm. The magnitude of the numbers are directly related to the optical purity of the complexes. Negative values indicate preferential $\Delta$-configuration, positive values preferential $\Lambda$-configuration

**Table 2.** Time-dependent uptake of $^{55}$Fe-labeled compounds

| Strain | Time (min) | Uptake (pmol/mg) of | | | | |
|---|---|---|---|---|---|---|
| | | ferriox-amine B | P191 | P178 | MP14 | coprogen |
| *P. agglomerans* K4 (parent) | 0.5 | 63 | 149 | 312 | 268 | 36 |
| | 2 | 116 | 266 | 375 | 336 | 48 |
| | 4 | 180 | 361 | 466 | 422 | 76 |
| | 6 | 237 | 472 | 554 | 510 | 90 |
| | 8 | 295 | 616 | 638 | 573 | 112 |
| | 10 | 361 | 793 | 727 | 627 | 145 |
| *P. agglomerans* FM13 (ferrioxamine-receptor-deficient) | 0.5 | 39 | 93 | 216 | 239 | 57 |
| | 2 | 30 | 140 | 226 | 253 | 119 |
| | 4 | 31 | 187 | 250 | 270 | 184 |
| | 6 | 30 | 232 | 296 | 310 | 251 |
| | 8 | 30 | 301 | 373 | 370 | 318 |
| | 10 | 29 | 348 | 470 | 397 | 372 |
| *H. alvei* (coprogen-receptor-deficient) | 0.5 | 76 | 97 | —nd | — | 28 |
| | 2 | 152 | 200 | — | — | 32 |
| | 4 | 225 | 290 | — | — | 30 |
| | 6 | 292 | 344 | — | — | 34 |
| | 8 | 354 | 384 | — | — | 34 |
| | 10 | 389 | 445 | — | — | 31 |

Cells were grown in M9 minimal salts medium until an $A_{578}$ of 0.5 was reached. Then $^{55}$Fe-labeled iron chelates were added at 0.5 μM concentration. Samples were taken at intervals, filtered through membrane filters, rinsed with water and the radioactivity was determined in a liquid scintillation counter. nd = not determined

receptor is essential for the transport of the synthetic chiral linear hydroxamate compounds. Similarly, *H. alvei* failed to show growth promotion in the presence of the hydroxamate analogues (Table 1). However, significant transport rates with $^{55}$Fe-labeled P191 could be observed in the absence of EDDHA (Table 2), suggesting that EDDHA interferes with iron uptake from the synthetic analogues in this strain. Moreover, ferrioxamine B was taken up at similar rates by *H. alvei*, while coprogen was not taken up at all. As *Hafnia* utilizes ferrioxamines but not coprogen, it may be inferred that the synthetic hydroxamate analogues entered the cells via the ferrioxamine receptor.

Transport data with $^{55}$Fe-labeled synthetic compounds in *P. agglomerans* K4 (parent) and the derived ferrioxamine-receptor-negative mutant FM13 are shown in Table 2. The transport assay was performed in liquid medium in the absence of EDDHA or any other interfering chelating agent. Time-dependent uptake of $^{55}$Fe-labeled synthetic compounds was observed irrespective of whether the strain was defective in the ferrioxamine receptor. For comparison, uptake of ferrioxamine B is shown to be nearly absent in the FM13 mutant, while it is still present in the parent strain K4. Transport of coprogen was measured additionally and was found to be functioning in both parent and mutant strain.

It is obvious from Table 2 that uptake of iron from the synthetic compounds is significantly reduced in the ferrioxamine-receptor-deficient mutant strain compared to that of the parent strain K4. This suggests, that in *P. agglomerans*, the analogues are partly transported via the ferrioxamine receptor (P191 = 57%, P178, MP14 = 37%) and partly (43% and 63% respectively) via

the coprogen receptor. A similar calculation based on the uptake data of *H. alvei* resulted in 56% of P191 via the ferrioxamine receptor. These values may presumably reflect a combination of two parameters: the respective $\Lambda/\Delta$ isomer ratio and stereochemical demands by the projecting methyl and isobutyl groups respectively. Moreover, the isobutyl residues of the leucine moiety in P178 and MP14 resulted in initially higher binding during the first few minutes compared to the methyl residues of the alanine-containing compound (P191) which may be attributed to differences in lipophilicity.

In order to determine whether or not the transport of the synthetic compounds is an active and energy-consuming process, like that of the natural siderophores, transport was measured in the presence and absence of the respiratory inhibitor sodium azide ($NaN_3$) which is a well known inhibitor of siderophore transport in bacteria and fungi. Figure 1 shows the uptake of $^{55}$Fe-labeled compound P191 and, for comparison, uptake of $^{55}$Fe-labeled ferrioxamine B in the absence and presence of 5 mM sodium azide in *P. agglomerans* K4 (wild type). A significant decrease of uptake (70%) is seen in the presence of the inhibitor for both ferrioxamine B and P191 uptake, suggesting that iron uptake from both the synthetic compound and the natural siderophore requires energy. This suggests that, in enterobacteria, the synthetic analogues are taken up by an active transport process.

*N. crassa* is a fungus that is known to utilize iron from coprogen but not from ferrioxamines (Winkelmann and Zähner 1973). Consequently, uptake of the synthetic compounds may indicate coprogen-like activity. As shown in Fig. 2, time-dependent iron uptake from $^{55}$Fe-labeled P191 was observed corresponding to about 1 nmol/mg dry mass after 15 min of transport into young mycelia. This is about 70% of iron upake measured with labeled coprogen (about 1.5 nmol/mg dry mass). These results suggest that, in the fungal system, the synthetic chiral linear hydroxamates are recognized as coprogen analogues.

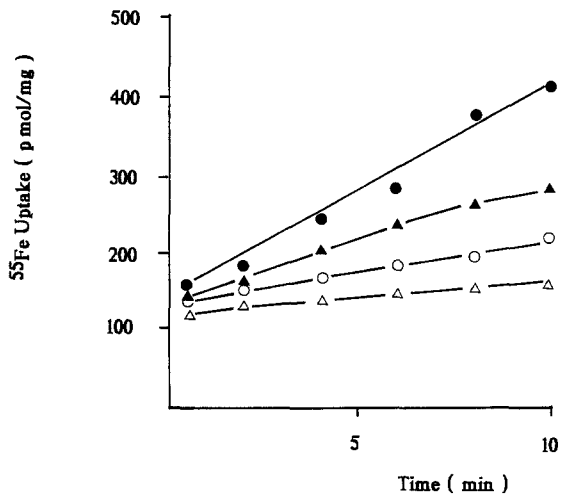



**Fig. 1.** Active transport of ferrioxamine B and compound P191. Transport of $^{55}$Fe-labeled ferrioxamine B and compound P191 was measured in the presence and absence of sodium azide (5 mM). Cells of *P. agglomerans* K4 were grown in M9 minimal salts medium (10 ml). Samples were removed at intervals, filtered through membrane filters, washed and the radioactivity was counted in a liquid sintillation counter. (●) P191; (○) P191 plus sodium azide; (▲) ferrioxamine B; (△) ferrioxamine B plus sodium azide

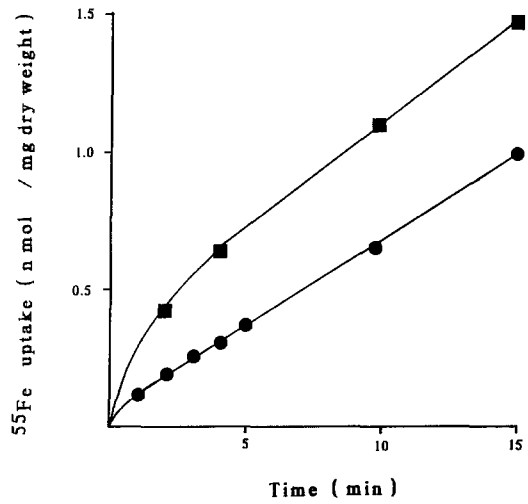



**Fig. 2.** Transport of $^{55}$Fe-labeled coprogen and compound P191 in *N. spora crassa arg-5 ota aga* (siderophore-free mutant). (■) coprogen; (●) compound P191

## Conclusion

Synthetic chiral trishydroxamates of linear topology that form $Fe^{3+}$ complexes of mixed *Δ-cis* and *Λ-cis* configuration were demonstrated to exhibit coprogen- and ferrioxamine-like activity. Specifically, coprogen-like activity was observed in *E. coli*, ferrioxamine-like activity in *Hafnia* and both coprogen- and ferrioxamine-like activity in *P. agglomerans*. The dual activity of these compounds might be rationalized by assuming that the ferrioxamine receptor has a preference for the $\Lambda$-configuration, while the coprogen receptor has a preference for the $\Delta$-configuration. Thus compound P191 and related analogues might work in either system, as they are configurationally not unique. This idea is supported by considering the pronounced similarities between the crystal structures of the two natural siderophores, ferrioxamine and coprogen, when displayed as superposition of their $\Delta$-$Fe^{3+}$ complexes (van der Helm et al. 1987). Experiments are currently in progress to examine this hypothesis by preparing and testing configurationally pure biomimetic analogues on selected indicator organisms.

*Acknowledgements.* This work was supported in part by a grant from the *Deutsche Forschungsgemeinschaft* (Wi 628/4-2). The technical assistance of Maryia Nikoui Bahnamiry and of Rahel Lazar is gratefully acknowledged.

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