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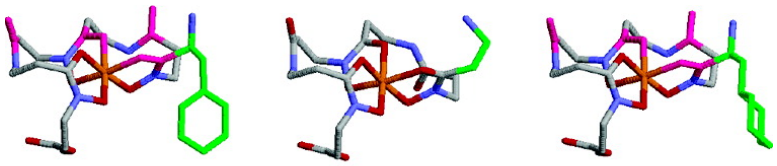

Ferrioxamine B analogs

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Ferrioxamine B Analogues: Targeting the FoxA Uptake System in the Pathogenic *Yersinia enterocolitica*

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Abstract: A series of ferrioxamine B analogues that target the bacterium *Yersinia enterocolitica* were prepared. These iron carriers are composed of three hydroxamate-containing monomeric units. Two identical monomers consist of *N*-hydroxy-3-aminopropionic acid coupled with β-alanine, and a third unit at the amino terminal is composed of *N*-hydroxy-3-aminopropionic acid and one of the following amino acids: β-alanine (**1a**), phenylalanine (**1b**), cyclohexylalanine (**1c**), or glycine (**1d**). Thermodynamic results for representatives of the analogues have shown a strong destabilization (3−4 orders of magnitude) of the ferric complexes with respect to ferrioxamine B, probably due to shorter spacers and a more strained structure around the metal center. No significant effect of the variations at the N-terminal has been observed on the stability of the ferric complexes. By contrast, using in vivo radioactive uptake experiments, we have found that these modifications have a substantial effect on the mechanism of iron(III) uptake in the pathogenic bacteria *Yersinia enterocolitica*. Analogues **1a** and **1d** were utilized by the ferrioxamine B uptake system (FoxA), while **1b** and **1c** either used different uptake systems or were transported to the microbial cell nonspecifically by diffusion via the cell membrane. Transport via the FoxA system was also confirmed by uptake experiments with the FoxA deficient strain of *Yersinia enterocolitica*. A fluorescent marker, attached to **1a** in a way that did not interfere with its biological activity, provided additional means to monitor the uptake mechanism by fluorescence techniques. Of particular interest is the observation that **1a** was utilized by the uptake system of ferrioxamine B in *Yersinia enterocolitica* (FoxA) but failed to use the ferrioxamine uptake route in *Pseudomonas putida*. Here, we present a case in which biomimetic siderophore analogues deliberately designed for a particular bacterium can distinguish between related uptake systems of different microorganisms.

Introduction

Iron is an essential micronutrient for all living organisms and is involved in fundamental enzymatic reactions, such as oxygen metabolism, electron-transfer processes, and synthesis of DNA and RNA. To facilitate adequate iron(III) uptake, microorganisms have developed low molecular weight molecules, termed siderophores or iron carriers. Excreted into the environment, the siderophores bind ferric ions and deliver them to the microorganism via specific membrane receptors and transport proteins. The receptor-regulated process guarantees meticulous control of the intracellular iron concentration and operates against unfavorable concentration gradients. The properties and biological activity of the siderophores are dictated by their structure, chirality, and the extent by which their shape fits the binding sites of specific receptor proteins inside the membrane.[1] The importance of iron-acquisition processes has prompted us[2−4] and others[5−7] to consider artificial siderophores as structural probes for the study of microbial iron uptake processes. Investigation of analogues of the four natural siderophores, enterobactin,[8] ferrichrome,[9,10] coprogen,[11] and ferrioxamine,[11] has provided the methodology in which one can reproduce the

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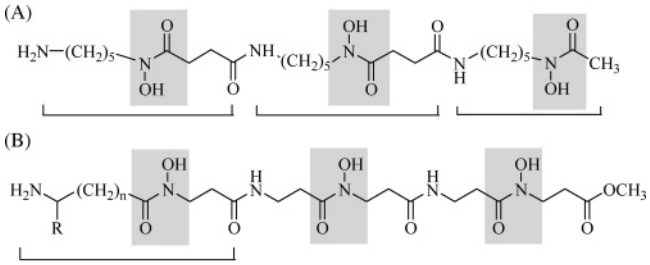
performance of the natural carriers, both in vitro and in vivo. Moreover, the attachment of various fluorescent markers was shown not to alter the microbial activity of the compounds.[12−14]

In this study, we have targeted the Gram-negative bacteria *Yersinia enterocolitica* that have both extremely pathogenic and nonpathogenic strains; *Yersinia pestis* was the cause of the Great Plague (also called Black Death), while *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are mainly associated with gastroenteritis due to contaminated food or water.[15] It has been suggested that supply of iron ions and production of siderophores may be crucial factors for infections in *Yersinia pestis* and in *Yersinia enterocolitica*.[16] *Yersinia enterocolitica* produces the siderophore Yersinobactin, its major source of iron, but is capable of utilizing iron also from exogenic siderophores, such as ferrioxamine B (FOB). This siderophore, isolated from *Streptomyces pilosus*, is utilized by *Yersinia enterocolitica* via the FoxA receptor, which was cloned and sequenced. The FoxA receptor shares a high degree of sequence conservation with *Escherichia coli* ferrichrome outer-membrane receptors (FhuA) and other TonB dependent receptors.[17] The TonB protein is part of a complex known as the Ton system. This complex is believed to undergo conformational changes that are driven by proton motive force (PMF), which in turn enable it to interact with the outer-membrane receptors, thereby activating transport.[18]

Past experiments from our laboratory have shown that while a number of chiral ferrioxamine analogues promoted growth in different microbial systems by using both the ferrioxamine B and the coprogen receptors, thus acting as broad-range carriers,[19] others, such as glutamic acid derivatives, were found to differentiate between analogous receptors of different microorganisms, thus acting as narrow-range carriers.[19,20] These findings together with results concerning species-specific ferrichrome analogues[10,21] have challenged us to design narrow-range analogues of ferrioxamine B.

The major goal of this work was to prepare synthetic siderophores that would be taken up by the bacterium *Yersinia enterocolitica* mainly via the ferrioxamine B uptake system. We have synthesized a retro-hydroxamate family of compounds that mimic the essential structural features of ferrioxamine B. They are composed of three monomeric units, based on variable amino acids and *N*-hydroxy-3-aminopropionic acid, linked to form a linear structure containing three hydroxamic acids. Two of the units are common for all of the derivatives and are built of β-alanine coupled with *N*-hydroxy-3-aminopropionic acid, while the third monomeric unit, next to the N-terminal was altered by using different amino acids, namely, β-alanine (**1a**), phenylalanine (**1b**) cyclohexylalanine (**1c**), and glycine (**1d**) (Chart 1). As the N-terminal part of the molecule is considered to play a crucial role in the recognition and uptake process,[22,23] the effect of modifications near this end in the ferrioxamine B analogues was investigated. Using ^{55}Fe uptake experiments, we were able to corroborate this statement and have found that the modifications at this part of the molecule did affect the uptake mechanism by which the compounds are transported into the microbial cell.

Chart 1. Ferrioxamine B (A) and Its Biomimetic Analogues (B)



1a: R = H, n = 1

1b: R = CH_2-C_6H_5, n = 0

1c: R = CH_2-C_6H_{11}, n = 0

1d: R = H, n = 0

Uptake experiments using the fluorescently labeled molecule **2** demonstrated the ability of this siderophore to serve as a carrier of the fluorophore into the cells, with no impairment to its properties as an iron carrier. Furthermore, the efficient fluorescence quenching of **2** by Fe(III) ions has provided an additional tool for following the pathway of the parent compound **1a**. The observation that **1a** did not use the ferrioxamine B uptake system in *Pseudomonas putida* indicated the selectivity of this compound and is of major importance since **1a** can be envisioned as a specific synthetic siderophore to the pathogenic *Yersinia enterocolitica* via the ferrioxamine B uptake system.

Results

To understand the structural requirements needed for a compound to be transported into *Yersinia enterocolitica* via the FoxA receptor, a set of biomimetic analogues of the natural siderophore ferrioxamine B were synthesized. Ferrioxamine B is known to form five enantiomeric pairs of isomers when binding trivalent metal ions, as was established by studying its kinetically inert Cr(III) complexes.[24] To minimize the number of stereoisomers and confer mainly cisoid complexes, the distances between the hydroxamate binding groups in our new compounds were shortened.[11] Interestingly, a racemic mixture of cisoidal isomers was identified in the crystal structures of ferrioxamine D_1[25] and ferrioxamine E[26] and also in the recently reported structure of ferrioxamine B.[27] In our design, the order of the hydroxamate group was inverted relative to that in the natural compound, thus forming retro-hydroxamate derivatives. This inversion was done to facilitate the synthesis by using

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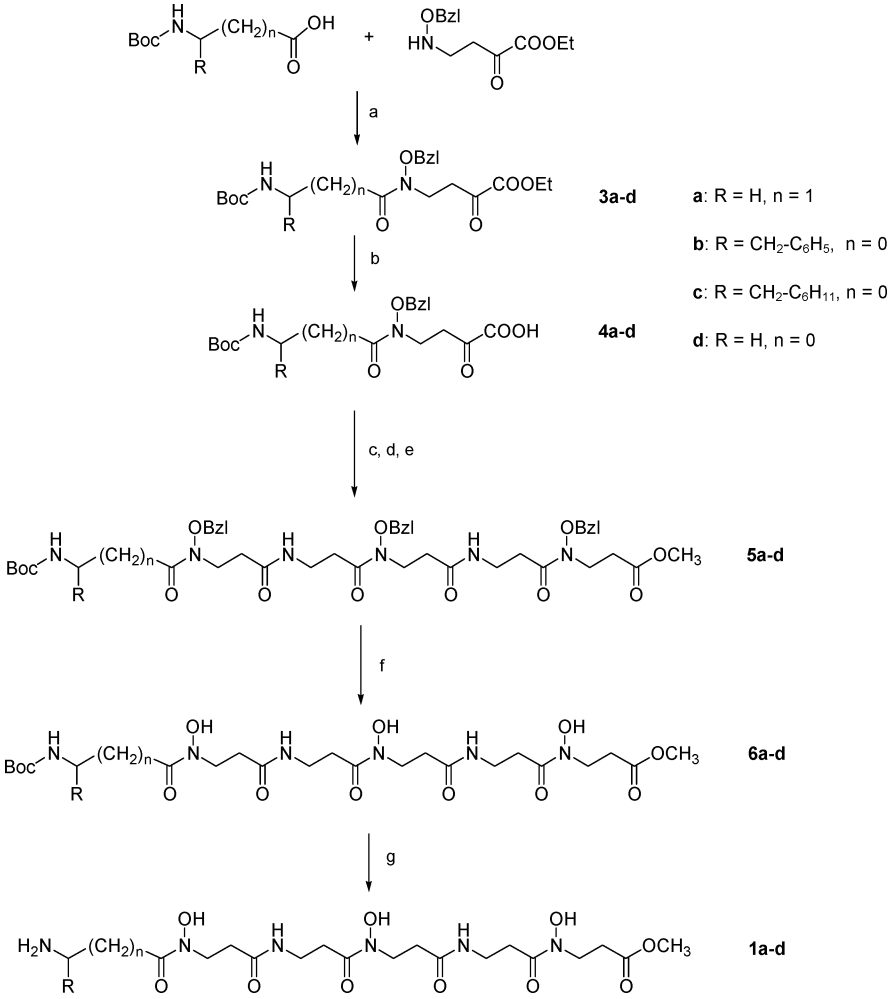
Scheme 1. Synthesis of Ferrioxamine B Analogues[a]



[a] Reagents: (a) DIC, HOBt, ACN; (b) 0.1 N NaOH, MeOH; (c) DIC, HOBt, NMP; (d) 55% TFA in DCM, 5% TEA in DCM; (e) 10% TEA in MeOH, 48 h; (f) H_2, 10% Pd/C, EtOH; (g) 50% TFA, DCM.

natural amino acids as building blocks, while being expected not to affect the iron-binding capabilities nor the biological activities of the retro-compounds.[28] Introduction of a variety of amino acids as part of the N-terminus of the "monomers" enabled insertion of chiral centers, thus directing the complex configuration and modification of the hydrophobicity, bulkiness, and length of this part of the molecule (Scheme 1).

Synthesis and Characterization of the Siderophore Analogues and their Iron(III) Complexes. The monomeric units (**4a**−**d**) were prepared in solution[11] and were assembled to yield the final compounds following the protocols of solid-phase peptide synthesis (Scheme 1).[29] The structures of the products were confirmed by spectroscopic measurements (IR, NMR, UV, and ESI-MS), and the Fe(III) complexes were prepared. This was done by titrations with $FeCl_3$ and was monitored by UV−vis spectroscopy following the increase of the absorption at $\lambda \approx 420$ nm, which is characteristic of iron(III) trishydroxamate.[5] The stoichiometry, which was found to be 1:1, was further confirmed by ESI-MS.

From the CD spectra (Figure 1), it was possible to conclude that the iron complexes of the chiral derivatives **1b** and **1c** adopt a Λ-cis configuration,[30] differing from the achiral compounds, ferrioxamine B, **1a**, and **1d**, which exhibit no Cotton signal. This observation indicates that even a single chiral center in the vicinity of the amino terminus can direct the configuration of the complex.

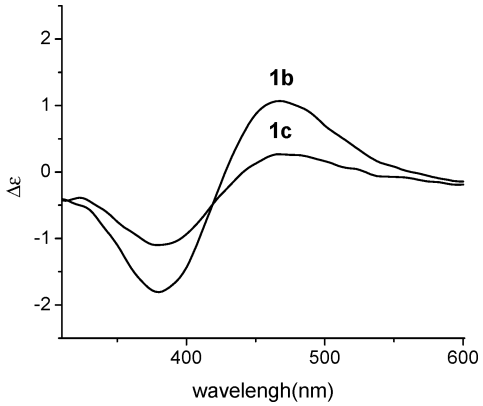


Figure 1. CD spectra of **1b** and **1c**. Solvent: methanol/0.1 N aqueous sodium acetate (4:1). Complex concentration: 0.3 mM.

The EPR signals of the iron complexes of ferrioxamine B and its analogues (**1a**, **1b**, **1c**, **1d**, **6a**, and **2**) were recorded at

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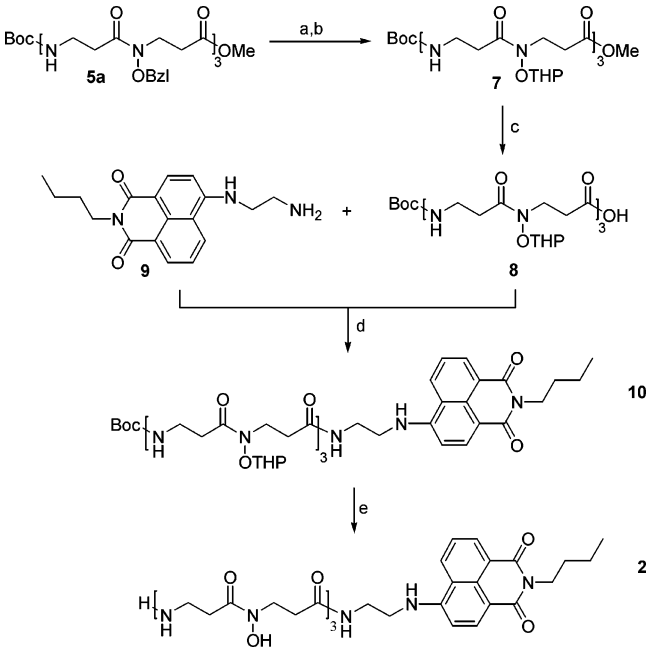
Scheme 2. Protocol for the Synthesis of the Fluorescently Labeled Compound **2**[a]



[a] Reagents: (a) H_2, 10% Pd/C, EtOH; (b) DHP, TsOH; (c) 0.1 N NaOH, MeOH; (d) DIC, HOBt, dry THF; (e) 2.5% TFA in DCM.

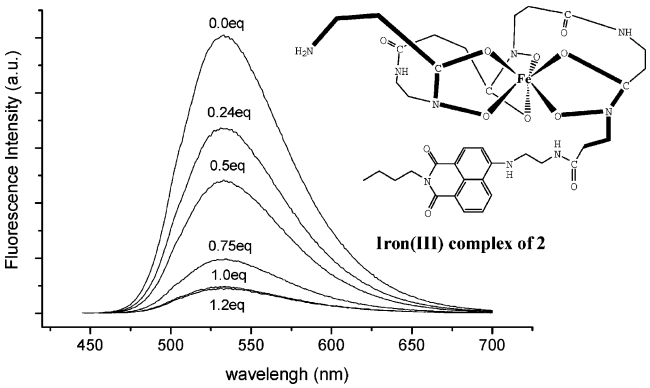


Figure 2. Changes in the fluorescence of **2** on titration with Fe(III) ions.

Table 1. Stability Constants of the Ferric Complexes Formed with Ferrioxamine B, **1a**, **1b**, and **2** (I = 0.1 M, T = 25.0 ± 0.2 °C)

ligand **L**	log $K_{LFe}(3\sigma)$
ferrioxamine B	30.99[a,b]
1a (β-alanine)	26.8(3)[b]
1b (phenylalanine)	26.1(1)[b]
ferrioxamine B	30.0(1)[c]
2	27.2(4)[c]

[a] From refs 35 and 36. [b] With water. [c] With methanol/water (80/20 by weight).

110 °K, and all revealed EPR signals at g = 4.32 and similar line shapes, which is characteristic of Fe(III) in high spin state (S = 5/2) in an environment of low symmetry.[31,32] Since it has been shown that the EPR signal of Fe(III)−ferrioxamine B complexes have g = 4.3,[33] like that obtained in our experiments, we assume that the iron(III) complexes of the analogues, which differ mainly at the terminal domains, have geometry similar to that of the natural siderophore ferrioxamine B. Since we do find differences in the biological behavior among the analogues, we assume that the terminal domains play an important role in the recognition events.

The synthetic protocol for the preparation of the fluorescent derivative **2** is described in Scheme 2. Following hydrogenation of **5a**, THP protection (**7**), and hydrolysis (**8**), the doubly protected **1a** (**8**) was attached via the carboxyl terminal to 6-(ethylamino)-*N*-butyl-1,8-naphthalimides **9** and yielded the fluorescently labeled compound **2** (Scheme 2). Since we have found that by blocking the terminal amine with Boc group, as in **6a**, the inhibition of the compound by ferrioxamine B was decreased, we assume that the free amine at the terminus has a role in the uptake process of ferrioxamine B analogues. Moreover, fluorescent labeling at the carboxyl terminal was expected not to obstruct the recognition of **2** by the ferrioxamine B uptake system since the recognition occurs at the iron(III)-binding domain.[12,21,34] By formation of the iron complex of **2**, the fluorescence of **2** (λ_{em} = 533 nm) is quenched to 10% of its original intensity (Figure 2).

Following the time-dependent fluorescence accumulation in the growth medium, which results from iron release in the cells and secretion of the fluorescent ligand to the medium, and the accumulation of radioactivity in the cells resulting from ^{55}Fe uptake, we were able to track the path of the iron from the environment to the cells.

Binding Properties. The stability constants of three biomimetic analogues of ferrioxamine B (**1a**, **1b**, and **2**) were determined using a fruitful combination of UV−vis absorption spectrophotometry and potentiometry (Table 1). For solubility reasons, a mixed solvent of methanol/water (80/20 by weight) was used for **2**. For the sake of comparison, ferrioxamine B was examined in the same solvent (Table 1).

The comparison of the stability constants (Table 1) shows that analogues **1a**, **1b**, and **2** are about 3−4 orders of magnitude less stable than ferrioxamine B in water and in methanol/water. If the shortening of the spacers between the binding units in the synthetic derivatives does not significantly affect their acido-basic properties,[37] it is of importance in the stability of the corresponding ferric complexes. The decrease in flexibility of the chain is indeed crucial for the ligands to self-organize around the ferric center and to fulfill the octahedral coordination requirements of the metal. Moreover, similar binding constants for **1a**, **1b**, and **2** (Table 1) clearly show that the substitution of the C- or N-terminal by bulky aromatic moieties, namely, benzyl for **1b** and 6-aminonaphthalimide for **2**, does not influence the stability of the ferric complexes.

Microbial Studies. ^{55}Fe uptake studies have shown that the bacteria *Yersinia enterocolitica* sequester iron from all of the current biomimetic analogues of ferrioxamine B at similar efficiencies (Table 2). To establish which of the compounds use the ferrioxamine B uptake system (FoxA), we have used a variety of methods, namely, (i) uptake of ^{55}Fe in the presence

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Table 2. ^{55}Fe Uptake in *Yersinia enterocolitica* WA-C Mediated by the Analogues **1a**−**1d**: Inhibition by the Respiratory Inhibitor, NaN_3, and by Ferrioxamine B (FOB)

	Inhibitor		
siderophore	NaN_3 (%)[a]	FOB (%)[a]	K_{sid}[b] (pmol Fe/min for 5×10^8 bacteria)
ferrioxamine B	70		0.46 ± 0.05
1a (β-alanine)	60	39	0.46 ± 0.05
1b (phenylalanine)	26	10	0.41 ± 0.09
1c (cyclohexylalanine)	56	6	0.45 ± 0.10
1d (glycine)	64	23	0.54 ± 0.15
6a (*N*-Boc β-alanine)	71	14	0.35 ± 0.05
2	81	35	0.43 ± 0.04

[a] For % inhibition = $[1 - (K_{sid + competitor\ or\ inhibitor}/K_{sid})] \times 100$, K = uptake rate. [b] The concentration of Fe(III)−siderophore in the culture medium was 1 μM.

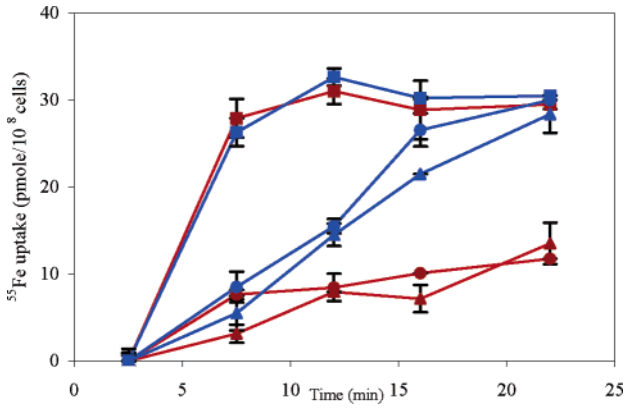

Figure 3. Iron uptake in *Yersinia enterocolitica* WA-C (blue) from ^{55}Fe−FOB (●), ^{55}Fe−**1a** (▲), ^{55}Fe−ferrichrome (■), and in *Yersinia enterocolitica* 12-8 (FoxA$^-$, red) from ^{55}Fe−FOB (●), ^{55}Fe−**1a** (▲), and ^{55}Fe−ferrichrome (■). Fe(III)−siderophore concentration in the culture medium was 2.5 μM.

of NaN_3, which is known for its ability to inhibit energy-dependent processes, (ii) uptake of ^{55}Fe in competition with natural siderophores, (iii) comparison between the uptake rates in the wild-type bacteria *Yersinia enterocolitica* WA-C and the FoxA deficient strain, *Yersinia enterocolitica* 12-8,[38] and (iv) labeling of the bioactive compound with a fluorescent probe and following the time-dependent accumulation of fluorescence in the medium. The results of the inhibition experiments with NaN_3 and ferrioxamine B are summarized in Table 2. As seen from the results, compounds **1a**, **1c**, and **1d** are significantly affected by NaN_3, indicative of the participation of an energy-driven processes in the uptake, while **1b** was less affected. The competition experiments, by incubation of *Yersinia enterocolitica* WA-C with the synthetic analogues loaded with ^{55}Fe-(III) ions, have resulted in iron accumulation inside the bacterial cells, which was reduced by addition of the natural siderophore ferrioxamine B as a competitor (Table 2). Significant inhibition was obtained for **1a** (39 ± 7%), whereas **1d** and **1c** were inhibited to a lesser extent (23 ± 2% and 6 ± 1%, respectively).

To further confirm the assumption that the FoxA uptake system is being utilized, we have carried out another ^{55}Fe uptake experiment using both the wild-type strain and the FoxA deficient strain (Figure 3). It was found that the ^{55}Fe(III) complexes of ferrioxamine B and **1a** have led to accumulation of similar amounts of iron ions in the bacterial cells having the

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Table 3. ^{55}Fe Uptake in *Pseudomonas putida* JM218 Mediated by the Biomimetic Analogues **1a**, **1b**, **1c**, and Ferrioxamine B: Inhibition by NaN_3, Ferrioxamine B, Coprogen, and Ferrichrome

	Inhibitor				
siderophore	NaN_3 (%)	FOB (%)	coprogen (%)	ferrichrome (%)	K_{sid}[b,c]
ferrioxamine B	81		18	35	0.43 ± 0.06
1a (β-alanine)	43	N.I.[a]	N.I.[a]	N.I.[a]	0.32 ± 0.05
1b (phenylalanine)	82	43	33	31	0.73 ± 0.23
1c (cyclohexylalanine)	77	N.I.[a]	18	26	0.68 ± 0.12

[a] N.I. is no inhibition. [b] The concentration of Fe(III)−siderophore in the culture medium was 1 μM. [c] With pmol Fe/min for 5×10^8 bacteria.

FoxA system, while the iron accumulation in the FoxA deficient strain was considerably lower. As a control experiment, it was shown that the uptake of the ferrichrome−^{55}Fe(III) complex by the FoxA deficient mutant was similar to that of the wild type, indicating that this bacterial strain is deficient only in the FoxA uptake system.[39]

When the Fe(III) complex of **2** was added to the culture growth medium of *Yersinia enterocolitica* WA-C, emergence of fluorescence was observed (Figure 4a) as a result of accumulation of the free ligand **2** in the culture media that follows the iron release inside the cells and excretion of the free ligand to the medium. Incubation of the FoxA$^-$ strain *Yersinia enterocolitica* 12-8 with the Fe(III) complex of **2** resulted, as expected, with the appearance of only weak fluorescence in the culture growth medium. It should be noted that the fluorescence of the labeled ferrichrome analogue B9-NBD[13] was not changed, which is further indication that this siderophore utilizes a different receptor and not the FoxA, in contrast to compounds **2** and **1a** that enter the microbial cell via the FoxA receptor, thus mimicking ferrioxamine B. The similar performances of **1a** and **2** as iron carriers (Table 2) indicate that the fluorescent marker at the carboxyl terminus does not modify the ability of the molecule to interact with the receptor, in other words, that the carboxyl terminus may have a lesser role in the uptake process. Additional ^{55}Fe uptake experiments with **2** further support the bioactivity of this analogue and demonstrate the role of the FoxA system (Figure 4b).

^{55}Fe(III) uptake experiments were conducted with the non-siderophore-producing mutant *Pseudomonas putida* JM218 in order to check the selectivity of **1a** (Table 3). This microorganism was used as an indicator strain since it possesses a range of siderophore uptake systems, including those for ferrichrome, ferrioxamine, and coprogen. It was found that while the uptake of ^{55}Fe(III) from **1b** and **1c** was decreased by NaN_3, that from **1a** was significantly less inhibited, suggesting that the transport of **1a** is less dependent on an energy-driven process whereas **1b** and **1c** are dependent on such a process. Corroboration of this finding was achieved by a competition experiment between the ^{55}Fe(III) uptake using **1a** and ferrioxamine B, which has indicated that **1a** could not be transported by the ferrioxamine B uptake system in this bacterium. It is noteworthy that in a competition experiment with ferrichrome or coprogen, known to be utilized by *Pseudomonas putida*, the ^{55}Fe(III) uptake from **1a** was not inhibited, indicating that this process in *Pseudomonas putida* JM218 is not an energy-driven, nonspecific process, but

(39) Ferrichrome is utilized by *Yersinia enterocolitica* via the FcuA receptor: Koebnik, R.; Hantke, K.; Braun, V. *Mol. Microbiol.* **1993**, *7*, 383−393.

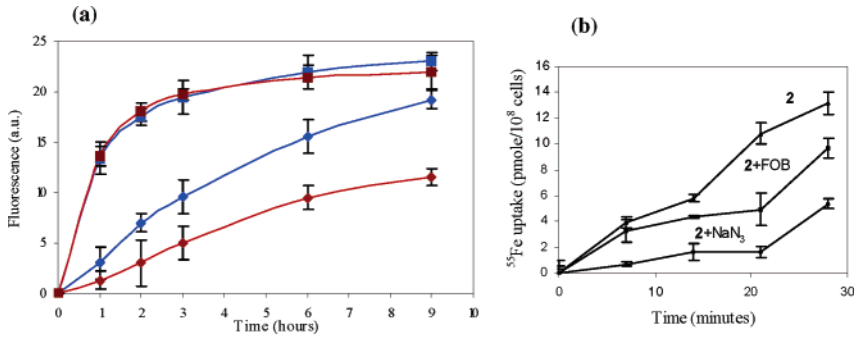

Figure 4. (a) Fluorescence accumulation in cultures of *Yersinia enterocolitica* WA-C (blue) facilitated with Fe(III)−**2** (◆), Fe(III)−B9-NBD (■), and in cultures of *Yersinia enterocolitica* 12-8 (FoxA$^-$, red) facilitated with Fe(III)−**2** (◆) and Fe(III)−B9-NBD (■). (b) Uptake of ^{55}Fe(III)−**2** inhibited by FOB and NaN_3 in *Yersinia enterocolitica* WA-C.

rather based on diffusion. In contrast, **1b** and **1c** enter the cell via an active uptake processes, similar to those of the natural siderophores coprogen, ferrioxamine B, and ferrichrome (Table 3). The same observation was reported by Jurkevitch et al. who have shown that ferrioxamine B and coprogen use different receptors in *Pseudomonas putida* but share common transport elements.[19]

Discussion

A new family of biomimetic analogues that target the ferrioxamine B uptake system in *Yersinia enterocolitica* was prepared. The analogues possess three main features: (i) short bridges between the hydroxamate's binding groups that allow the formation of mainly cisoidal complexes with Fe(III) ions, (ii) inversion of the hydroxamate directionality relative to that in the natural compound, and (iii) modifications at the N-terminus. The structure and the conformation of the chain that holds the terminal amine are believed to play a major role in determining the uptake properties of the various ferrioxamines.[22,23,27,40] Indeed, we have found that altering this part of the molecule affects neither the configuration of the metal center, as shown by EPR, nor the stability of the ferric complexes, as illustrated by similar binding constants (Table 1). By contrast, these modifications drastically affect the uptake mechanisms in *Yersinia enterocolitica*. Even if the ferric species with **1a**, **1b**, and **2** are strongly destabilized with respect to ferrioxamine B, due to the shortening of the bridges between the hydroxamate's binding units of about 30%, the specific chelation remains strong along the receptor binding and uptake process. Moreover, it might facilitate the intracellular release mechanism.

Uptake experiments with NaN_3 have enabled us to determine which of the compounds enter the cells of *Yersinia enterocolitica* by an active transport route. We have found that compound **1b** enters in a passive pathway, whereas compounds **1a**, **1c**, and **1d** make greater use of active routes. Elucidation of this route by means of competition uptake experiments with ferrioxamine B has produced the following bioactivity order: **1a** > **1d** > **1c**. Uptake experiments with the FoxA deficient strain have further confirmed this mechanism. The results may also imply that the structure of the chain between the final hydroxamate group and the terminal amine has an important role in the uptake process. Incorporation of an aromatic side chain, as in **1b**, has led to greater utilization of a nonactive uptake route, while insertion of a bulky side chain, as in the cyclohexyl group in **1c**, has resulted in greater utilization of an active uptake system, different from that of the ferrioxamine B. A shorter chain between the hydroxamate and the terminal amine (**1d** versus **1a**) has decreased the active uptake via the FoxA system. Blocking the terminal amine of **1a** by a Boc group has led to a considerable decrease in the inhibitory effect of ferrioxamine B on the uptake rate (Table 2). However, this effect might be attributed to the bulkiness of the Boc group.

Attaching a fluorescent group at the carboxyl terminus of **1a** did not affect its uptake (**2**, Table 2), indicating that modification at the C-terminus is well tolerated by the receptor with respect to both recognition and uptake. However, inhibition experiments against ferrioxamine B indicated that bulky substituents at the N-terminus decreased susceptibility to competition (namely, **1b**, **1c**, and **6a**), whereas substitution at the C-terminus, as in compound **2**, did not significantly change the susceptibility to competition by ferrioxamine B.

The comparison between mechanisms of uptake in *Yersinia enterocolitica* and in *Pseudomonas putida*, from results of the competition experiments, has indicated that **1a** can target the bacteria *Yersinia enterocolitica* via the ferrioxamine B uptake system (39% inhibition), but it is immune to competition by FOB in *P. putida*. By contrast, **1b** is less susceptible to competition by FOB in *Y. enterocolitica* and more susceptible in *P. putida*. These opposite relative sensitivities to competition show that **1a** and **1b** can differentially target a specific bacterial strain's FOB uptake systems.

We have used Empirical force field calculations[41] for modeling the conformations of the different iron complexes. As can be seen in Figure 5, the differences between the energetically most stable conformations of the iron complexes of the analogues originate mainly from the chain that links the hydroxamate group to the terminal amine. The fact that the cyclic ferrioxamine E efficiently utilizes the FoxA receptor in *Yersinia enterocolitica*[42] may help in elucidating the structural and conformational factors that control the recognition and transport events. The crystal structure of ferrioxamine E, as obtained by van der Helm et al., is essentially a planar structure with two dissimilar faces, one polar and one nonpolar.[26] On the basis of this information, we suggest that forming a "coplanar" conformation similar to that of ferrioxamine E may be the guideline for the analogues' bioactivity. This correlates well with Dhungana and Crumbliss who have suggested that

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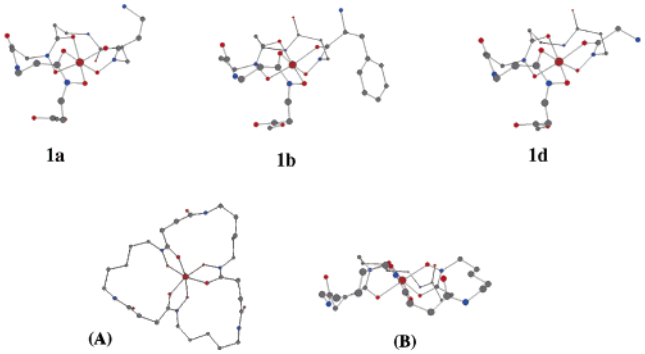

Figure 5. Empirical force field-minimized conformations of **1a**, **1b**, **1d**, and ferrioxamine E: (A) Upper view and (B) side view.

the iron(III) complex octahedron face containing the carbonyl oxygens might be the determining factor for recognition.[27] We assume that the substantial activity of **1a** results from its ability to form an unshielded planar conformation and the appropriate structure of the pendant amine group that contributes an additional favorable interaction with the receptor. Several suggestions as to the nature of this interaction have been postulated, including intramolecular hydrogen bonding, intermolecular second-coordination-sphere for host−guest complexation,[43−45] or a solubilization factor. The decreased inhibition by FOB of **1d** probably results from the chain at the N-terminus, which is too short for such an interaction. Since the length of the methylene chain near the N-terminus is identical in **1b** and **1d**, decreased inhibition by FOB of **1b** might stem from interference of the phenyl at the side chain.

Conclusions

Design and synthesis of "tailor-made" siderophore analogues, which target narrow-range microbial uptake systems, is a very important goal due to their prospective role in therapy and diagnostics. Siderophore−drug conjugates[46] may provide a complementary treatment for systematic infections that complicate the therapy by Desferal,[47] for example, which promotes bacterial growth in *Yersinia enterocolitica* and prevents, therefore, the administration of the drug as long as the infection persists. They may also help in the fight against antibiotic-resistant microorganisms.[7,48]

The ability to prepare siderophore analogues that utilize the ferrioxamine B uptake system in *Yersinia enterocolitica*, which is the major result of this study, is an additional step toward the achievement of these goals.

Experimental Section

General Methods. IR spectra were recorded on a Nicolet 510 FTIR spectrometer. Absorption frequencies are given in cm^{-1}. UV−vis spectra were measured either with a Hewlett-Packard model 8450A diode array or with Varian CARY 50 and CARY 300 spectrophotometers. Molar extinction coefficients are given in M^{-1} cm^{-1} units. Fluorescence spectra were recorded on an SLM-AMINCO MC200 fluorescence spectrophotometer. In biological experiments, fluorescence was measured with an SLM Instrument (Model 4800). ^1H NMR and COSY spectra were measured on a Bruker AMX-400 MHz spectrometer or on a Bruker DPX-250 MHz spectrometer using the solvent deuterium signal as an internal reference. All *J* values are given in hertz. Unless otherwise stated, the spectra were recorded in $CDCl_3$.

EPR spectra were recorded using 1 mM solutions of the iron complexes in methanol, which contained about 10% (v/v) 0.1 N sodium acetate solution. The spectra were recorded at 110 K using an ER 200 D-SRC spectrometer (Bruker).

Mass spectroscopy (ESI-MS) was performed with an LCZ 4000 instrument (Micromass, Manchester, UK), and MALDI-TOF was performed with a 2E instrument (Micromass, Manchester, UK). CD spectra were recorded on an Aviv-202 circular dichroism spectrometer (Lakewood, NJ). Flash chromatography was performed using Merck 230−400 mesh silica gel. The purity of all compounds was controlled by ^1H NMR spectra and by thin-layer chromatography (TLC) using silica gel 60 F-254 on aluminum plates and at least two different solvent systems and was visualized by UV light, ninhydrin, iodine, or $FeCl_3$/EtOH.

Potentiometric titrations were performed using an automatic titrator system, DMS 716 Titrino (Metrohm), with a combined glass electrode (Metrohm 6.0234.100, Long Life) connected to a microcomputer.

Solvents and Materials. Acetonitrile and CH_2Cl_2 were dried by filtration through basic alumina. THF was distilled from Na/benzophenone under Ar. Protected amino acids were purchased from Novabiochem (Laufelfingen, Switzerland). Unless otherwise mentioned, all reagents were purchased from Sigma and were used without further purification. All solvents (HPLC grade) for synthesis and purification were obtained from Labscan (Dublin, Ireland). The following abbreviations were used: DIC = diisopropylcarbodiimide, TsOH = *p*-toluenesulfonic acid, TEA = triethylamine, NMP = *N*-methyl-2-pyrrolidone, DDW = double-distilled water, Cha = 3-cyclohexyl-L-alanine, Gly = glycine, *β*-Ala = *β*-alanine, Phe = L-phenylalanine, HOBt = 1-hydroxybenzotriazole, DCM = dichloromethane, MeOH = methanol, ACN = acetonitrile, TFA = trifluoroacetic acid, Bzl = benzyl, Boc = *tert*-butyloxycarbonyl, Ar = aryl, Ph = phenyl, and Et = ethyl, EtOAc = ethyl acetate.

General Procedures. Compounds **3a**−**d**, **4a**−**d**, and *N*-(benzyloxy)-3-aminopropionic acid ethyl ester were prepared according to published procedures.[11] 6-Ethylamino-*N*-butyl-1,8-naphthalimides (**9**) was prepared according to Chang et al.[49] and de Silva et al.[50]

General Protocol for the Synthesis of the Trimers. The "trimer" was synthesized by solid-phase peptide synthesis procedures using Merrifield resin (chloromethylated polystyrene, 2% divinylbenzene resin). Cesium salts of the first monomeric units were prepared by dissolving the monomers (**4a**−**d**) (1.1 equiv, 1.2 mmol) in MeOH (50 mL) and DDW (5 mL) and were titrated to pH 7 with 20% Cs_2CO_3. The solution was evaporated to dryness in vacuo and dried over P_2O_5 for 5 h. Then, the monomers (1.1 mmol) were shaken with the resin (3.0 g, 0.7 mmol/g) in NMP (25 mL) overnight at 50 °C. The Boc group was removed with 55% TFA in DCM (1 × 2 min, 1 × 30 min), washed with DCM (1 × 2 min), and neutralized with 5% TEA in NMP (2 × 2 min). The second monomer was mixed with HOBt (1.5 equiv, 3.15 mmol), DIC (1.5 equiv, 3.15 mmol), and TEA (3 equiv, 6.3 mmol) in NMP (15 mL) for 10 min and was added to the reaction vessel. The reaction proceeded overnight. After the removal of the Boc group, the coupling of the third monomer was carried out as described for the second one. Each reaction was followed by washes with NMP (5 × 2 min) and DCM (2 × 2min). To ensure completion, the reactions were monitored by ninhydrin.[51] The resin was filtered, dried in vacuo overnight, and suspended in 10% TEA in MeOH. The suspension was

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stirred for 48 h, and the resin was filtered out, washed with $CHCl_3$ and MeOH, and concentrated to dryness. The cleavage reaction was repeated in order to obtain additional product. Chromatography on silica gel afforded the methyl esters of the trimers **5a**, **5b**, **5c**, and **5d**.

Trimer 5a. Purification by chromatography, using $CHCl_3$/MeOH (95:5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.32 (m, 15H, ArH), 6.60 (b, 1H, N*H*CO), 5.31 (b, 1H, N*H*Boc), 4.74 (s, 6H, CH_2Ph), 3.92 (t, 6H, CH_2NOBzl), 3.58 (s, 3H, COOCH_3), 3.41 (m, 4H, NHCH_2), 3.33 (m, 2H, BocNHCH_2), 2.52 (m, 6H, CH_2CONO and CH_2COOMe), 2.48 (m, 2H, CH_2CONO), 2.38 (m, 4H, CH_2CONH), 1.38 (s, 9H, *t*-Bu); ESI-MS: 899.5 $[M + Na]^+$.

Trimer 5b. Purification by chromatography, using $CHCl_3$/MeOH (96:4). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (m, 15H, Ar*H*), 7.19 (m, 3H, C_6H_5), 7.06 (m, 2H, C_6H_5), 6.85 (b, 1H, BocN*H*), 6.48 (b, 1H, N*H*), 5.57 (b, 1H, N*H*), 4.99 (dt, 1H, C_α of Phe), 4.85 (q, 2H, $J = 2.5$, OCH_2Ph), 4.76 (s, 2H, OCH_2Ph), 4.72 (s, 2H, OCH_2Ph), 4.07, 3.70 (m, 1H, CH_2NOBzl), 3.96 (m, 4H, CH_2NOBzl), 3.59 (s, 3H, COOCH_3), 3.47 (m, 3H, CONHCH_2), 3.40 (b, 1H, CONHCH_2), 2.86 (Abq, 2H, $J_1 = 8.75$, $J_2 = 5$, C_β of Phe), 2.56 (t, 6H, CH_2CO), 2.43 (t, 4H, $J =$ 4.25, CH_2CO), 2.27 (m, 2H, CH_2CO of first Boc-monomer), 1.37 (s, 9H, *t*-Bu).

Trimer 5c. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (m, 15H, Ar*H*), 7.01 (b, 1H, N*H*), 6.57 (b, 1H, N*H*), 5.59 (b, 1H, BocN*H*), 4.94 (s, 1H, $C_\alpha H$), 4.80 (m, 5H, CH_2Ph), 4.11 (m, 1H, CH_2NOBzl), 3.99 (m, 4H, CH_2NOBzl), 3.48 (m, 4H, CONHCH_2), 2.60 (m, 10H, CH_2CO), 1.75 (b, 1H, C_6H_{11}), 1.58 (m, 4H, C_6H_{11}), 1.38 (m, 2H, CHC$H_2C_6H_{11}$), 1.14 (m, 4H, C_6H_{11}), 0.9 (m, 1H, C_6H_{11}), 0.7 (m, 1H, C_6H_{11}).

Trimer 5d. ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.36 (m, 15H, Ar*H*), 6.60 (b, 1H, N*H*CO), 5.30 (b, 1H, N*H*CO), 4.78 (m, 6H, CH_2Ph), 4.20 (d, 2H, C_α), 3.94 (m, 6H, CH_2NO), 3.61 (s, 3H, CH_3OCO), 3.45 (m, 2H, CH_2NH), 3.35 (m, 2H, CH_2NH), 2.57 (m, 6H, CH_2CO), 2.40 (m, 4H, CH_2CO), 1.41 (s, 9H, *t*-Bu). ESI-MS: 885.23 $[M + Na]^+$.

Trimer 6a. Hydrogenation of the protected trimer (225 mg, 0.26 mmol) in absolute EtOH (30 mL) with 10% Pd/C (70 mg) proceeded for 4.5 h at room temperature and atmospheric pressure of H_2. Filtration of the catalysts and evaporation of the solvent afforded the free trihydroxamate ligand (151 mg, 0.254 mmol). Yield: 97%. ^{1}H NMR (250 MHz, $CDCl_3$ + CD_3OD): δ 3.59 (m, 6H, $J = 4.5$, CH_2NOH), 3.39 (s, 3H, CH_3OCO), 3.1 (m, 4H, $J = 7.5$, CH_2NH), 3.0 (m, 2H, CH_2NH), 2.37 (m, 4H, CH_2CO), 2.19 (m, 4H, CH_2CO), 1.26 (s, 9H, *t*-Bu). ESI-MS: 629.5 $[M + Na]^+$.

Trimer 6b. The compound was prepared by using the procedure described for compound **6a**. Yield: 86%. ^{1}H NMR (250 MHz, CD_3OD): δ 7.14 (s, 5H, Ar*H*), 4.90 (b, 1H, C_α), 3.75 (m, 6H, CH_2NOH), 3.56 (s, 3H, CH_3OCO), 3.21 (m, 6H, CONHCH_2), 2.56 (m, 8H, CH_2CO), 2.40 (m, 4H, CH_2CO), 1.25 (s, 9H, *t*-Bu). ESI-MS: 977.0 $[M + Na]^+$.

Trimer 6c. The compound was prepared by using the procedure described for compound **19**. Yield: 90%. ^{1}H NMR (250 MHz, CD_3OD): δ 3.75 (m, 6H, $J = 3.75$, CH_2NO), 3.57 (s, 3H, CH_3OOC), 3.33 (t, 4H, CONHCH_2), 2.56 (m, 6H, CH_2CO), 2.41 (m, 4H, CH_2CO), 1.81, 1.61, 1.19, 0.82 (m, 13H, C_6H_{11} and $C_\beta H_2$), 1.3 (9H, *t*-Bu). ESI-MS: 712.9 $[M + Na]^+$.

Trimer 6d. The compound was prepared by using the procedure described for compound **6a**, but **5d** was dissolved in EtOH/EtOAc (1:1). ^{1}H NMR (250 MHz, CD_3OD): δ 4.14 (s, 2H, C_α), 3.80 (m, 6H, CH_2NOH), 3.67 (s, 3H, CH_3OOC), 3.44 (t, 2H, CH_2NH), 3.30 (t, 2H, CH_2NH), 2.60 (m, 10H, CH_2CO), 1.42 (s, 9H, *t*-Bu).

Trimer 1a. 6a (50 mg, 0.08 mmol) was dissolved in dry CH_2Cl_2 (4 mL) and TFA (2 mL), and the reaction was stirred for 30 min at room temperature. The solution was evaporated, and the oily product was redissolved in CH_2Cl_2 and evaporated. The product was then dissolved in MeOH (3 mL), and basic polymer (diethylaminoethylpolystyrene, Fluka, 20 mg) was added to remove the TFA salt. Filtration of the polymer and evaporation of the solvent afforded the free amine trihydroxamate trimer **1a** (40 mg, 0.08 mmol). Yield: 100%. ^{1}H NMR (250 MHz, CD_3OD): δ 5.39 (s, 1H, N*H*CO), 3.78 (m, 6H, CH_2NOH), 3.57 (s, 3H, CH_3OOC), 3.32 (t, 4H, CH_2NH), 3.10 (t, 2H, CH_2NH), 2.77 (t, 2H, NH_2CH2CH_2CO), 2.56 (m, 6H, CH_2CON(OH) and CH_2-CONH), 2.41 (m, 4H, CH_2COOMe and CH_2CONH). ESI-MS: 507.4 $[M + H]^+$. Fe(III) complex, UV−vis: λ_{max} 421 nm, $\epsilon = 2736$. ESI-MS: 560.29 $[M + H]^+$.

Trimer 1b. The compound was prepared by using the procedure described for compound **1a**. Yield: 100%. ^{1}H NMR (250 MHz, CD_3-OD): δ 7.25 (m, 5H, Ar*H*), 4.51 (m, 1H, C*H*CH$_2$Ph), 3.70 (m, 6H, CH_2NOH), 3.54 (s, 3H, CH_3OOC), 3.32 (m, 4H, NHCH_2CH$_2$), 3.87 (m, 2H, CHCH_2Ph), 2.53 (t, 6H, $J = 7.5$, CH_2CO), 2.40 (t, 4H, $J = 5$, CH_2CO). Fe(III) complex, UV−vis: λ_{max} 410 nm, $\epsilon = 2156$. ESI-MS: 636.27 $[M + H]^+$.

Trimer 1c. The compound was prepared by using the procedure described for compound **1a**. ^{1}H NMR (250 MHz, CD_3OD): δ 5.38 (s, 2H, N*H*CO), 4.30 (dd, 1H, C_α *H*), 3.47 (m, 6H, CH_2NOH), 3.56 (s, 3H, CH_2OOC), 3.30 (m, 4H, CONHCH_2), 2.55 (m, 6H, CH_2CO), 1.65 (m, 8H, C_6H_{11}), 1.18 (m, 5H, $C_\beta H_2$ and C_6H_{11}). Fe(III) complex, UV−vis: λ_{max} 415 nm, $\epsilon = 2058$. ESI-MS: 642.37 $[M + H]^+$.

Trimer 1d. The compound was prepared by using the procedure described for compound **1a**. ^{1}H NMR (250 MHz, CD_3OD): δ 4.14 (s, 2H, $C_\alpha H$), 3.90 (m, 6H, CH_2NOH), 3.47 (s, 3H, CH_3OOC), 3.20 (t, 2H, CH_2NH), 2.87 (t, 2H, CH_2NH), 2.58 (m, 10H, CH_2CO). Fe(III) complex, UV−vis: λ_{max} 415 nm, $\epsilon = 2309$.

Boc-[NHCH$_2$CH$_2$CON(OTHP)CH$_2$CH$_2$CO]$_3$−OMe (7). The free trishydroxamate trimer **6b** (140 mg, 0.23 mmol) and 3,4 dihydro-2*H*-pyran (DHP, 0.315 mL, 3.45 mmol) were dissolved in dry CH_2Cl_2 (45 mL), and *p*-toluenesulfonic acid (13.3 mg, 0.069 mmol) was added. The solution was stirred for 5 h at rt. After DHP (0.315 mL, 3.45 mL) was added again, the solution was stirred overnight. The organic mixture was washed with water, dried over Na_2SO_4, and concentrated in vacuo. The crude product was used in the next reaction without further purification. Yield: 61%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.50 (bs, 1H, N*H*CO), 4.88 (bs, 3H, OC*H*OCH$_2$), 4.03 (m, 7H, NC*H*CH$_2$ and OCH_2CH$_2$), 3.86 (m, 1H, OCH_2CH$_2$), 3.75 (m, 1H, OCH_2CH$_2$), 3.68 (s, 3H, CH_3OOC), 3.60 (m, 4H, OCH_2CH$_2$ and NHCH_2), 3.52 (m, 4H, OCH_2CH$_2$ and NHCH_2), 3.40 (m, 1H, OCH_2CH$_2$ and NHCH_2), 1.78 (m, 4H, CH_2CO), 1.59 (m, 2H, CH_2CO), 1.45 (m, 6H, CH_2CO), 1.55 (m, 18H, CH_2 of THP), 1.45 (m, 9H, *t*-Bu).

Boc-[NHCH$_2$CH$_2$CON(OTHP)CH$_2$CH$_2$CO]$_3$−OH (8). The THP-protected trimer **7** (120 mg, 0.14 mmol) was dissolved in MeOH (7 mL), and the mixture was treated with 1 M aqueous sodium hydroxide solution (2.3 mL) at room temperature. The reaction mixture was monitored by TLC every 1 h, and additional portions of aqueous sodium hydroxide solutions were added until all starting material had been consumed. The mixture was washed with EtOAc/hexane (1:1) to remove THP polymer impurities and was cooled in an ice bath and acidified with $KHSO_4$ to pH 2. Methanol was evaporated, and the residue was extracted with ethyl acetate. The organic fraction was washed with water, dried on $MgSO_4$, filtered, and concentrated to afford the trimer acid **8** (50 mg, 0.06 mmol). Yield: 45%. ^{1}H NMR (250 MHz, $CDCl_3$): δ 7.9, 6.75 (b, 1H, N*H*CO), 4.88 (b, 3H, OC*H*OCH$_2$), 3.96 (m, 8H, NCH_2CH$_2$ and OCH_2CH$_2$), 3.50 (m, 10H, NCH_2CH$_2$ and OCH_2-CH$_2$), 2.60 (m, 12H, CH$_2$CO), 1.66 (m, 18H, CH$_2$ of THP), 1.46 (m, 9H, *t*-Bu).

Boc-[NHCH$_2$CH$_2$CON(OTHP)CH$_2$CH$_2$CO]$_3$-6-(ethylamino)-*N*-butyl-1,8-naphthalimide (10). Trimer **8** (50 mg, 0.059 mmol) was dissolved in dry THF (4 mL). The solution was cooled to 0 °C, and HOBt (8 mg, 0.059 mmol), DIC (9μL, 59 mmol), and 6-(ethylamino)-*N*-butyl-1,8-naphthalimide (18.5 mg, 0.059 mmol) were added. The solution was stirred for 1 h at 0 °C and then at room temperature overnight. The THF was evaporated, and the crude product was purified by flash chromatography (2−5% MeOH/$CHCl_3$) to give **10** (18 mg, 0.016 mmol). Yield: 27%. ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.51 (d, 1H, $J = 7.5$, naphthyl), 8.34 (m, 2H, $J = 7$, naphthyl), 7.89 (b, 1H, N*H*CO), 7.58 (t, 1H, $J = 7$, naphthyl), 7.34 (b, 1H, N*H*CO), 6.99 (b,

1H, N*H*CO), 6.56 (d, 1H, $J = 7$, naphthyl), 4.83 (b, 3H, OC*H*OCH$_2$), 4.11 (t, 2H, $J = 7$, CH_2N of imide), 3.74 (m, 9H, CH$_2$CH_2O and CH_2-NO), 3.49 (m, 13H, CH_2NH and CH$_2$CH_2O), 2.69, 2.71 (m, 12H, CH$_2$-CO), 1.65 (m, 22H, CH_2 of *n*-butyl and THP), 1.42 (m, 9H, *t*-Bu).

***H*-[NHCH$_2$CH$_2$CON(OH)CH$_2$CH$_2$CO]$_3$-6-(ethylamino)-*N*-butyl-1,8-naphthalimide (2). 10** (25 mg, 0.022 mmol) was dissolved in dry CH$_2$Cl$_2$ (2 mL) and TFA (20 μL). The reaction was stirred for 4 h at room temperature. The solution was evaporated, and small impurities were removed by MeOH/ether precipitation. The product was then dissolved in MeOH, and basic polymer (*N*,*N*-diethylaminoethylpolystyrene, Fluka, 20 mg) was added to remove the TFA salt. Filtration of the polymer and evaporation of the solvent afforded the free amine trihydroxamate trimer **2** (14 mg, 0.018 mmol). Yield: 82%. ^{1}H NMR (250 MHz, CD$_3$OD): δ 8.47 (m, 2H, naphthyl), 8.35 (d, 1H, $J = 7$, naphthyl), 7.75 (b, 1H, N*H*CO), 7.60 (t, 1H, $J = 7$, naphthyl), 7.35 (b, 1H, N*H*CO), 6.80 (d, 1H, $J = 7$, naphthyl), 4.11 (t, 2H, $J = 5$, CH_2N of imide), 3.83 (m, 6H, CH_2NO), 3.56 (m, 6H, CH_2NH), 3.14 (t, 2H, CH_2NH), 2.84 (t, 2H, $J = 7$, CH_2NH), 2.62, 2.47 (m, 12H, CH_2CO), 1.67 (m, 2H, CH$_3$CH$_2$CH_2CH$_2$), 1.40 (m, 2H, CH$_3$CH_2CH$_2$), 0.97 (m, 3H, CH_3CH$_2$). UV−vis: λ_{max} 425 nm, $\epsilon = 12\,460$. ESI-MS: 808.7 [M + Na]$^+$.

General Protocol for the Determination of Stability Constants. For solubility reasons, solutions of analogues **1a** and **1b** of ferrioxamine B were prepared in distilled water, while that of ligand **2** was prepared in solvent made of 80% spectrophotometric grade methanol (Merck) and 20% water. The ionic strength was fixed at $I = 0.1$ M. As a reference for the iron(III) binding properties by a trihydroxamate ligand, the protonation and stability constants of the methanesulfonate salt of ferrioxamine B (Desferal, Ciba-Geigy), FOB, were also determined under the same experimental conditions.

Water was further purified by passing it through a mixed bed of an ion-exchanger (Bioblock Scientific R3-83002, M3-83006) and activated carbon (Bioblock Scientific ORC-83005). Both methanol and water were deoxygenated by CO_2- and O_2-free argon (Sigma Oxiclear cartridge). All stock solutions were prepared using an AG 245 Mettler Toledo analytical balance (precision 0.01 mg).

The potentiometric and UV−vis titrations were used to determine the stability constants of iron(III) complexes of studied ferrioxamine B analogues.[37]

Circular Dichroism Measurements. Methanolic solutions of ligands **1b** and **1c** were mixed with a methanolic solution of $FeCl_3$ (5mM) in 1:1 stoichiometry and diluted with methanol/0.1 N aqueous NaOAc (4:1) to a final concentration of 0.3 mM. Measurements were preformed after equilibration for 24 h at room temperature. Previously studied compound **4**[11] was used as a reference, demonstrating a relatively lower chiral preference of **1b** and **1c**.

Bacterial Strains. The plasmidless strain of *Yersinia enterocolitica* WA-C and its FoxA$^-$ mutant, WA-C 12-8, which lacks the ferrioxamine receptor FoxA$^-$, were kindly provided by K. Hantke (Tubingen, Germany).[38] The sid$^-$ mutant, *Pseudomonas putida* JM218, was kindly provided by L. C. van Loon (Utrecht, The Netherlands).[52]

Microbial ^{55}Fe Uptake Studies. ^{55}Fe uptake studies were conducted according to Weizman et al.[12] with minor modifications. Bacteria were grown in LMKB medium[53] overnight at 28 °C on a rotary shaker at 180 rpm to a final absorbance of 0.3−0.5 at 620 nm. The bacterial cultures were then centrifuged for 15 min at 5000 rpm, resuspended in fresh half-strength standard succinate medium (SSM)[54] to a final OD_{620} of 0.6, and incubated for 60 min in a water bath at 28 °C. Half-strength SSM medium was unable to support *Yersinia* growth (data not shown), which eliminates possible changes in bacterial cell numbers during the iron uptake studies. The ^{55}Fe−siderophore complex was added to the bacterial culture at a final concentration of 1 μM, unless mentioned otherwise. When appropriate, NaN_3 was added to a final concentration of 5 mM 30 min prior to the addition of the siderophores. For competition tests, the nonlabeled competing Fe−siderophore complex was added to the bacterial culture together with the tested ^{55}Fe−siderophore at the same final concentration. For each time interval, aliquots (0.5 mL) were taken in duplicates, layered onto a mixture of dibutyl/octyl phthalate (1:1 v/v, Sigma), and immediately centrifuged. The supernatant was removed, and the Eppendorf tube tips were cut. Radioactivity in the tips of the tubes containing the bacterial cells was counted by a β-scintillation counter (TR-1900, Packard). Each experiment was performed three times in duplicates.

In Vivo Fluorescence Studies. Bacterial culture was grown as described for the ^{55}Fe uptake studies. The Fe−siderophore complex was added to the bacterial suspension to a final concentration of 5 μM. Aliquots of 0.5 mL were collected and centrifuged in each time interval, and the supernatant was divided into triplicates of 150 μL. The fluorescence of the samples was measured in a 96 wells polystyrene microtiter plate by an ELISA fluorimeter (Fluostar, SLT Salzburg Austria). The samples were excited at 485 nm, and the emission was measured at 538 nm. Results are given in arbitrary fluorescence intensity units. Each experiment was performed three times in triplicate.

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Supporting Information Available: Spectral data for **3a−d** and **4a−d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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