Significance of Asymmetric Sites in Choosing Siderophores as Deferration Agents

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The syntheses of the microbial iron chelators L-fluviabactin, its unnatural enantiomer, D-fluviabactin, L-homofluviabactin, and L-agrobactin, are described. The key steps involve the selective bis-acylation of the terminal nitrogens of norspermidine, spermidine, or homospermidine with 2,3-bis(benzyloxy)benzoic acid in the presence of 1,1-carbonyldiimidazole, followed by coupling of the *N*-hydroxysuccinimide ester of CBZ-protected L- or D-threonine with the central nitrogen. The effectiveness of each of these ligands in supporting the growth of *Paracoccus denitrificans* in a low-iron environment and the ability of these compounds to promote iron uptake are evaluated. The stereochemical configuration of the oxazoline ring is shown to be the major structural factor controlling both microbial growth stimulation and iron uptake. L-Fluviabactin, L-homofluviabactin, and L-agrobactin all promoted growth and iron uptake; D-fluviabactin was only marginally active. As with the microorganism's native siderophore, L-parabactin, all three ligands in the L-configuration investigated exhibited biphasic, i.e., both high-affinity and low-affinity, kinetics. The high-affinity system (iron concentration < 1 μM) yielded K_m values between 0.11 and 0.23 μM and V_{max} values from 157 to 129 pg-atoms Fe min^{-1} (mg of protein)$^{-1}$, whereas the low-affinity scheme (iron concentration > 1 μM) gave K_m values from 0.53 to 3.5 μM and V_{max} values between 96 and 413 pg-atoms Fe min^{-1} (mg of protein)$^{-1}$. Both L- and D-fluviabactin are very effective at clearing iron from the bile duct-cannulated rodent; when given subcutaneously at a dose of 150 μmol/kg, both ligands had iron clearing efficiencies of >13%, which is much greater than that of desferrioxamine in this model. Thus, by altering the stereochemistry of certain microbial siderophores, it is possible to generate deferration agents that are still effective at clearing iron from animals, yet do not promote microbial growth.

Introduction

Many organisms are auxotrophic for Fe(III), and nature's response to the insolubility of the hydroxide ($K_{sp} = 1 \times 10^{-38}$)[1] formed under physiological conditions has been the development of rather sophisticated iron storage and transport systems. Whereas eukaryotes tend to utilize proteins to transport iron (e.g., transferrin) and store iron (e.g., ferritin), microorganisms utilize low molecular weight ligands, siderophores.[2]

Iron metabolism in primates is characterized by a highly efficient recycling process;[3–6] no specific mechanism exists for eliminating this transition metal. As "excess iron"[7–9] cannot be cleared effectively, its introduction into this closed metabolic loop leads to chronic overload and, ultimately, to peroxidative tissue damage. A number of situations, including high iron diet or abnormally increased absorption of the metal, can result in iron overload. In each of these scenarios, the patient can be treated by phlebotomy.[10] There are, however, iron overload syndromes secondary to chronic transfusion therapy (e.g., aplastic anemia and thalassemia).[11] In patients undergoing this regimen, erythropoiesis is suppressed and iron absorption may be near normal, but each unit of transfused erythrocytes contains 200 to 250 mg of iron. Most thalassemia major patients require 200 to 300 mL kg^{-1} year^{-1} of blood, equivalent to 0.25 to 0.40 mg of Fe kg^{-1} day^{-1}.[12] Phlebotomy is not an option, as the origin of the excess iron is the transfused red blood cells. The only currently available alternative is chelation therapy, in which the chelator must be able to remove at least 0.25 to 0.40 mg of Fe/kg daily. Left untreated, these patients generally die in their second decade.

The commercial introduction of the hydroxamate siderophore desferrioxamine B (DFO)[13] over 30 years ago provided a life-saving treatment for iron overload in chronically transfused thalassemia patients. The subcutaneous (sc) infusion of DFO is regarded as the method of choice for handling transfusional iron overload.[14–16] The drug's efficacy and long-term tolerability are well documented, yet it suffers from a number of shortcomings associated with its marginal oral activity, its high cost of production, and its poor to moderate efficiency. Efficiency is defined as the amount of iron excreted by an animal or human upon administration of a given chelator after subtracting the baseline iron excretion, divided by the theoretical amount of iron excretion that should be stimulated on the basis of the dose and the compound's coordination chemistry; this quotient is expressed as a percentage. DFO can be immunogenic.[17–19] The ligand also has a very short half-life in the body and must, therefore, be administered by continuous sc infusion over long periods of time. At the clinical level, the result is poor patient compliance.[20]

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To circumvent these difficulties, the development of an orally effective iron chelator has been a therapeutic strategy for many years.
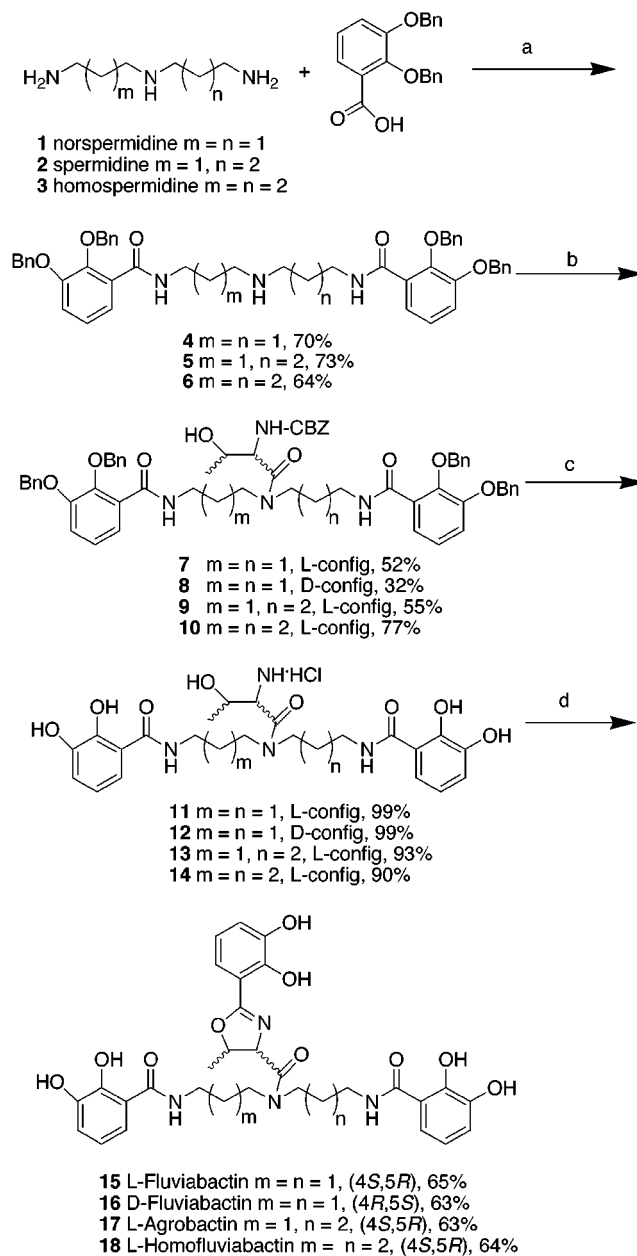
Although many synthetic iron chelators have been studied in recent years as potential orally active therapeutics [e.g., hydroxypyridones[21,22] and bis(*o*-hydroxybenzyl) ethylenediaminediacetic acid analogues[23]], none has yet proven to be completely satisfactory. The siderophores, microbial iron chelators, have remained relatively unexplored in this search. The rate of their isolation and structural elucidation has far surpassed the progress in their evaluation as iron clearing agents.

Siderophores fall primarily into two structural classes, hydroxamates or catecholamides.[24−26] There are several compounds that do not belong to either family, such as pyochelin,[27,28] rhizobactin,[29] and 2-(3′-hydroxypyrid-2′-yl)-4-methylthiazoline-4(*S*)-carboxylic acid (desferrithiocin).[30−35] Of concern regarding the utilization of siderophores as therapeutics for the treatment of iron overload disease is that these ligands could serve to stimulate the growth of pathogenic microorganisms in humans. When the hexacoordinate catecholamide chelator enterobactin was tested as a possible deferrating agent in rodents, a fulminant *Escherichia coli* infection developed in these animals which ultimately resulted in death due to septicemia.[36] Although not all microorganisms cross-utilize siderophores, the phenomenon can occur.[20,37−40] We have demonstrated that L-parabactin both stimulated the growth of *Paracoccus denitrificans* and promoted iron uptake, whereas D-parabactin did neither, as the latter enantiomer was not recognized by the bacterial iron transport apparatus.[41] We also showed that L-parabactin was very efficient at removing iron from rodents and primates.[42] A structurally similar catecholamide, L-fluviabactin, was recently isolated from *Vibrio fluvialis*.[43] In the current study, we assembled L- and D-fluviabactin, L-agrobactin, and L-homofluviabactin, evaluated the cross-utilization of these ligands by *P. denitrificans*, and compared the iron clearing efficacy of D- and L-fluviabactin in a bile duct-cannulated rodent model. The results further underscore the idea that siderophores can serve as a platform in the design of therapeutic deferration agents. Stereochemical modification of the natural product iron chelators can profoundly diminish their capacity to stimulate microbial growth without reducing their iron clearing properties in vivo.

Results

Synthesis. The siderophores agrobactin (**17**)[44] and L-parabactin[45] were first synthesized in this laboratory by cyclocondensation of the appropriate imidate ester with the hydrobromide salt of *gem*-amino alcohol **13** (Scheme 1). N^4-Benzylspermidine was acylated with 2,3-dimethoxybenzoyl chloride (2 equivalents); the central nitrogen was deprotected[46] and coupled with *N*-carbobenzoxy-L-threonine. Sequential removal of the blocking groups afforded synthon **13**. In a subsequent route to L-parabactin,[47] N^1,N^8-bis[2,3-bis(benzyloxy)benzoyl]-spermidine (**5**) was obtained directly from spermidine, and the secondary nitrogen was acylated with *N*-*tert*-butoxycarbonyl-L-threonine. Unmasking of the chiral amino group, cyclization with ethyl 2-hydroxybenzimidate, and deprotection of the catecholamides completed

Scheme 1. Synthesis of L- and D-Fluviabactin (**15** and **16**), L-Agrobactin (**17**), and L-Homofluviabactin (**18**)[a]

1 norspermidine m = n = 1
2 spermidine m = 1, n = 2
3 homospermidine m = n = 2

4 m = n = 1, 70%
5 m = 1, n = 2, 73%
6 m = n = 2, 64%

7 m = n = 1, L-config, 52%
8 m = n = 1, D-config, 32%
9 m = 1, n = 2, L-config, 55%
10 m = n = 2, L-config, 77%

11 m = n = 1, L-config, 99%
12 m = n = 1, D-config, 99%
13 m = 1, n = 2, L-config, 93%
14 m = n = 2, L-config, 90%

15 L-Fluviabactin m = n = 1, (4*S*,5*R*), 65%
16 D-Fluviabactin m = n = 1, (4*R*,5*S*), 63%
17 L-Agrobactin m = 1, n = 2, (4*S*,5*R*), 63%
18 L-Homofluviabactin m = n = 2, (4*S*,5*R*), 64%

[a] Reagents: (a) 1,1-carbonyldiimidazole, CH_2Cl_2; (b) N-CBZ-(D-or L-Thr)-OH, *N*-hydroxysuccinimide, 1,3-dicyclohexyldiimide (for **7−9**) or N-CBZ-L-Thr-OSu (for **10**); NEt_3, CH_2Cl_2; (c) H_2, 10% Pd−C, CH_3OH, HCl; (d) ethyl 2,3-dihydroxybenzimidate,[44] CH_3OH.

the natural product synthesis. The present route to L-(**15**) and D-fluviabactin (**16**), L-agrobactin (**17**), and L-homofluviabactin (**18**) exploits the preferential acylation of linear triamines, the simultaneous removal of amine and phenol protecting groups, and cyclocondensation with ethyl 2,3-dihydroxybenzimidate.[44]

Accordingly, the syntheses of the hexacoordinate chelators **15−18** began with the selective bis-acylation of the primary nitrogens of norspermidine (**1**), spermidine (**2**), or homospermidine (**3**)[48] with 2,3-bis(benzyloxy)benzoic acid[49] in the presence of 1,1-carbonyldiimidazole[50] (Scheme 1), resulting in the corresponding bis-amides, N^1,N^7-bis[2,3-bis(benzyloxy)benzoyl]-norspermidine (**4**, 70%),[51] N^1,N^8-bis[2,3-bis(benzyloxy)benzoyl]spermidine (**5**, 73%),[50−52] and N^1,N^9-bis[2,3-bis-
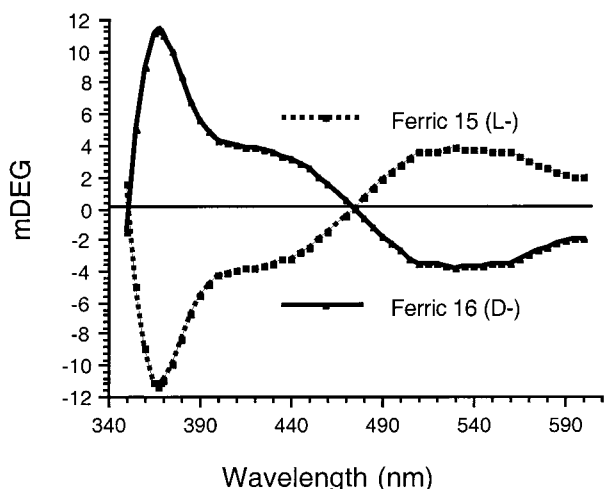
Figure 1. Circular dichroism spectra of ferric L- (**15**, solid line) and D-fluviabactin (**16**, dashed line). The chelates were dissolved to a concentration of 400 μM in Tris-HCl buffer (100 mM Tris, pH 7.4); the cell path length was 1.00 cm.

(benzyloxy)benzoyl]homospermidine (**6**, 64%). Each of these bis-amides (**4**−**6**) was reacted with an activated ester of *N*-carbobenzyloxythreonine. The norspermidine bis-amide (**4**) was coupled with *N*-carbobenzyloxy-L-threonine or with *N*-carbobenzyloxy-D-threonine in the presence of 1,3-dicyclohexyldiimide and *N*-hydroxysuccinimide in triethylamine[45] to generate N^4-(*N*-carbobenzyloxy-L-threonyl)-N^1,N^7-bis[2,3-bis(benzyloxy)benzoyl]-norspermidine (**7**, 52% yield) or its D-threonyl enantiomer (**8**, 32%), respectively. The spermidine and homospermidine bis-amides (**5** and **6**) were also coupled with the activated succinimidyl ester of *N*-carbobenzyloxy-L-threonine in triethylamine to produce the corresponding homologues (**9** and **10**) in 55 and 77% yield, respectively. The carbobenzyloxy (CBZ) and benzyl (Bn) protecting groups of **7**−**10** were simultaneously removed by hydrogenolysis (H₂/Pd−C in methanolic HCl) to afford N^4-L-threonyl-N^1,N^7-bis(2,3-dihydroxybenzoyl)norspermidine (**11**, 99% yield), N^4-D-threonyl-N^1,N^7-bis(2,3-dihydroxybenzoyl)norspermidine (**12**, 99%), and the corresponding L-spermidine and L-homospermidine homologues (**13** and **14**) in 93 and 90% yield, respectively, as their hydrochloride salts. Each of these compounds was then condensed with ethyl 2,3-dihydroxybenzimidate[44] to generate the final products L- and D-fluviabactin (**15** and **16**, 65 and 63% yields, respectively), L-agrobactin (**17**, 63%), and L-homofluviabactin (**18**, 64%). Finally, as expected from the enantiomeric relationship of the fluviabactins **15** and **16**, the circular dichroism spectra of their ferric complexes were antisymmetric (Figure 1).

Biological Evaluations. (a) Microbiology. The impact of the asymmetric siderophores L- and D-fluviabactin, L-parabactin, L-agrobactin, and L-homofluviabactin on the growth of *P. denitrificans* was evaluated at a ligand concentration of 2 μM in minimal salts medium containing the nonutilizable iron chelator, EDDA (Figure 2). Bacterial growth was followed over 48 h by measuring changes in the optical density of the culture at 660 nm (OD$_{660}$). All of the ligands in the L-configuration (**15**, **17**, and **18**) stimulated the growth of *P. denitrificans*, whereas D-fluviabactin did not. We observed the same phenomenon with the enantiomers of parabactin,[41] i.e., the D-parabactin was inactive.
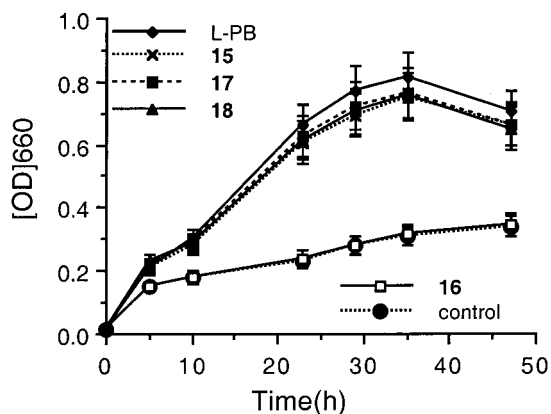

Figure 2. Growth rate of *P. denitrificans* in the presence of L-parabactin ("L-PB") and ligands **15**−**18** (2.0 μM) in low-Fe medium as measured by turbidity of the culture (OD$_{660}$, *y*-axis) vs time (*x*-axis).
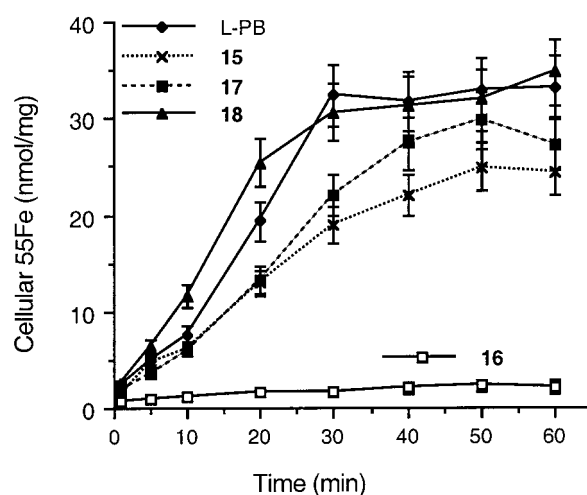

Figure 3. Uptake of [^{55}Fe]ferric ligands by *P. denitrificans*. Stock solutions of [^{55}Fe]ferric chelates of L-parabactin ("L-PB") and compounds **15**−**18** (1.0 μM) were added to late log-phase cultures of *P. denitrificans*. Samples were withdrawn from the cell suspension at the times indicated, chilled, filtered, rinsed, and subjected to scintillation counting.

The observed growth stimulation paralleled the ability to accumulate radiolabeled iron in the presence of the corresponding [^{55}Fe]ferric catecholamide complexes (Figure 3). *P. denitrificans* is able to accumulate iron from the ferric complexes of L-parabactin, L-fluviabactin (**15**), L-agrobactin (**17**), and L-homofluviabactin (**18**), but not from ferric D-fluviabactin (the complex of **16**).

The dramatic differences in the transport kinetics of ferric D-fluviabactin as compared to ferric L-fluviabactin are illustrated in the Lineweaver−Burk plot (Figure 4). These differences in velocity of transport are especially pronounced at lower chelate concentrations (e.g., < 1 μM). In addition to illustrating marked differences in the velocity of transport, Figure 4 suggests other features of mechanistic interest. The kinetic data for ferric D-fluviabactin fit a straight line, and thus obey a simple Michaelis−Menten model (K_m = 3.5 ± 0.8 μM, V_{max} = 96 ± 63 pg-atoms of Fe/min-mg of protein). For ferric L-fluviabactin, it is apparent that the data do not show a linear relationship. The expanded view in Figure 5 emphasizes this nonlinearity. When analyzed separately, the data from 1.0 to 5 μM ferric L-fluviabactin fit one line (K_m = 2.2 ± 1.6 μM) implying a low-affinity
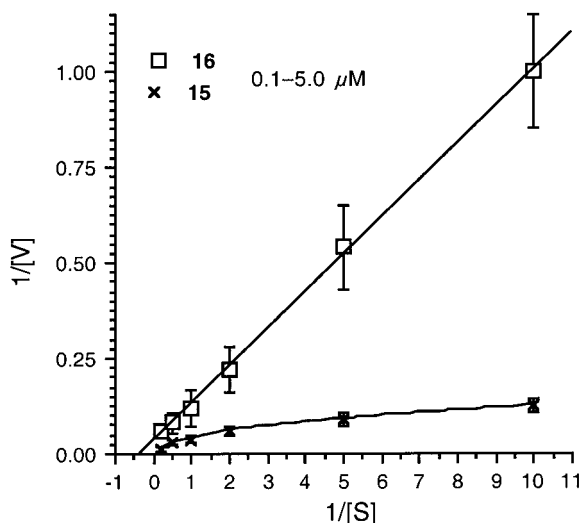
Figure 4. Lineweaver−Burk plot showing the kinetics of iron transport of [^{55}Fe]ferric L-(**15**, x−x) and D-fluviabactin (**16**, squares) at concentrations ranging from 0.1 to 5.0 μM by *P. denitrificans*. The data represent the mean of three experiments at each concentration.
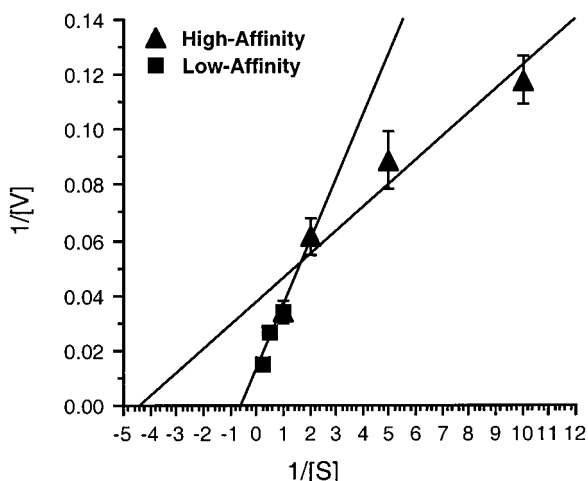

Figure 5. Expanded Lineweaver−Burk plot showing the kinetics of iron transport of [^{55}Fe]ferric L-fluviabactin (**15**) by *P. denitrificans*. The triangles show the high-affinity component of the transport (0.1 μM < [S] < 1.0 μM); the low-affinity system (1.0 μM < [S] < 5.0 μM) is depicted by the squares.

system, whereas the data from 0.1 to 1.0 μM fit another line ($K_m = 0.23 \pm 0.03$ μM), suggesting a high-affinity system. The ferric complexes of other catecholamides, L-agrobactin (**17**) and L-homofluviabactin (**18**), which also contain a chiral oxazoline derived from L-threonine, exhibited biphasic kinetics with a high affinity component ($K_m = 0.1-0.2$ μM; Table 1) as well. *P. denitrificans* thus appears to have a stereospecific high-affinity system to obtain iron from ferric catecholamides under low-iron conditions (<1 μM) in which the stereochemistry of the chiral oxazoline ring plays a critical role.

(b) Clearance of Iron from Bile Duct-Cannulated Rodents. When L- or D-fluviabactin was administered sc to bile duct-cannulated rodents at a dose of 150 μmol/kg, the iron clearing efficacy was very similar to that of L-parabactin given at the same dose. The efficiency of L-parabactin was $14.2 \pm 2.0\%;^{42}$ the efficiencies of **15** and **16** were 13.8 ± 0.6 and $16.0 \pm 5.3\%$, respectively (Table 2). The proportion of the iron that was excreted in the bile was comparable in all three cases, between

Table 1. Kinetics of Accumulation of [^{55}Fe]-Labeled Ferric Complexes of Asymmetric Siderophores by *P. denitrificans*[a]

[^{55}Fe]-chelate[b]	high-affinity component		low-affinity component	
	K_m (μM)[c]	V_{max}[c,d]	K_m (μM)[c]	V_{max}[c,d]
L-fluviabactin (**3**)[e]	0.23 ± 0.03	129 ± 2	2.2 ± 1.6	413 ± 149
D-fluviabactin (**3**)[e]			3.5 ± 0.8	96 ± 63
L-agrobactin (**2**)[f]	0.11, 0.11	157, 153	0.55, 0.53	245, 247
L-homo-fluviabactin (**3**)[e]	0.17 ± 0.04	138 ± 19	1.0 ± 0.1	229 ± 29

[a] Stock solutions of [^{55}Fe]ferric chelates of the siderophores listed were added to late log-phase cultures of *P. denitrificans* grown in low-iron liquid medium. Samples were withdrawn from the cell suspension every 20 s for 2 min, quickly chilled, filtered, rinsed, and subjected to scintillation counting. [b] The number in parentheses is the number of assays at each chelate concentration (0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 μM). [c] The kinetic data were utilized in the computer generation of plots, which were then used to estimate K_m and V_{max}. [d] Expressed as pg-atoms Fe min^{-1} (mg of protein^{-1}). [e] Mean $\pm$ standard error. [f] The data from the two individual experiments are reported.

Table 2. Iron Clearance Stimulated by L-Fluviabactin, D-Fluviabactin, and L-Parabactin[a]

compound	efficiency (%)[b]	distribution of excreted Fe (%)
L-fluviabactin (**3**)	13.8 ± 0.6	84.6 bile, 15.4 urine
D-fluviabactin (**3**)	16.0 ± 5.3	90.8 bile, 9.2 urine
L-parabactin[c] (**8**)	14.2 ± 2.0	90.3 bile, 9.7 urine

[a] The ligands were administered to non-iron-overloaded, bile duct-cannulated rats (n − value in parentheses) as a sc injection at a dose of 150 μmol/kg. Bile samples were collected at 3-h intervals for 48 h; urine samples were taken every 24 h over the experimental period. [b] The efficiency of each chelator was calculated by subtracting the iron excretion of control animals from the iron excretion of treated animals. This number was then divided by the theoretical output on the basis of a 1:1 ligand-iron complex; the result is expressed as a percentage. [c] These results are from ref 42.
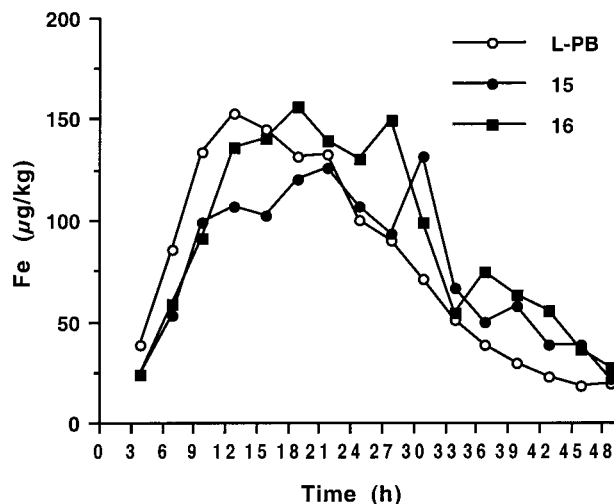

Figure 6. Time course of mean biliary iron excretion in normal rats after administration of L-parabactin (L-PB, $n = 8$, open circles),[42] L-fluviabactin (**15**, $n = 3$, filled circles), or D-fluviabactin (**16**, $n = 3$, filled squares). All chelators were given sc at a dose of 150 μmol/kg.

84 and 91% (Table 2). Finally, the deferration kinetics were also similar for the three ligands (Figure 6), i.e., a protracted biliary excretion phase which returned to baseline 48 h after dosing.

Discussion

The currently accepted drug for the treatment of transfusional iron overload is DFO, a natural product

iron chelator (siderophore) isolated from *Streptomyces pilosus*.[13] As a consequence of poor patient compliance, investigators have continued to search for alternative ligands, focusing on synthetic chelators. It is interesting that, although numerous siderophores from various microorganisms have been isolated and characterized, very few have been pursued as therapeutics.

There is one potential problem implicit in utilizing a siderophore to treat patients with transfusional iron overload: the ligand can promote the growth of indigenous microorganisms. This phenomenon has been observed in rodents which were given enterobactin and subsequently died from septicemia[36] as well as in patients using DFO.[20,37] Cross-utilization of other siderophores by heterologous bacterial species is well established.[38,39] This is certainly the case in the present work; the ferric complexes of L-agrobactin, isolated from *Agrobacterium tumefaciens*,[53] and of L-fluviabactin, produced by *V. fluvialis*,[43] were both utilized by *P. denitrificans*.

In this study, modification of the polyamine backbone of the siderophore does not substantially affect either the ability of the ligands to support bacterial growth or the kinetics of iron uptake by *P. denitrificans*. Both the growth curves and the K_m values were similar for L-fluviabactin (**15**), which possesses a norspermidine backbone, L-agrobactin (**17**), which contains a spermidine moiety, and L-homofluviabactin (**18**), which has a homospermidine backbone. However, the relatively small structural alteration of inverting the configuration of two carbon atoms (4 and 5) within the oxazoline ring is sufficient to render the siderophores unable to promote microbial growth. This inversion changes the stereochemistry of the ferric complex from Λ to Δ, as inferred from the CD spectra, a configuration that cannot be utilized by the microorganism. D-Fluviabactin (**16**) does not promote the growth of *P. denitrificans*. This further supports our previous findings with L- and D-parabactin.[41]

Likewise, the kinetics of ferric L- and D-fluviabactin transport mirrored the results from our earlier publication.[54] The Λ complex of fluviabactin apparently is recognized by a stereospecific, high-affinity mechanism which is regulated by iron concentration. We have isolated such a receptor from iron-starved *P. denitrificans* outer membranes.[55] Such stereospecificity of iron transport was reported earlier by Neilands et al. for enterobactin; the Fe(III) complex of synthetic enantioenterobactin (derived from D-serine) failed to promote the growth of enterobactin-deficient *E. coli* mutants.[56] High-affinity iron uptake systems are widespread among prokaryotic and eukaryotic microorganisms.[57–60]

On the other hand, utilization of iron from the Δ complex by *P. denitrificans* likely is via a nonstereospecific, low-affinity system, as postulated by Wee et al.[61] These uptake systems, which do not appear to be subject to regulation by iron concentration, have been characterized in many microorganisms, both prokaryotic and eukaryotic, in the past decade.[58–60,62] Many of these systems operate via a reductase; systems that utilize ferrisiderophore reductases have been characterized in microbial species as diverse as *Mycobacterium smegmatis*[63] and *Saccharomyces cerevisiae*.[64] A nonstereospecific alternative to a reductase is a ligand exchange mechanism similar to those proposed for mycobactin-mediated iron transport in *Mycobacterium* species[65] and more recently for amonabactin transport in *Aeromonas hydrophila* 495A2.[66] We have noted previously that an attractive model involved a stereospecific high-affinity binding step requiring the L-oxazoline ring, followed by a nonstereospecific postreceptor processing involving hydrolysis of the oxazoline ring of ferric L-parabactin ($E_0' = -0.673$ mV[67]) to the open-chain threonyl structure of ferric parabactin A ($E_0' = -0.400$ mV[67]) from which iron might be more readily removed, perhaps by a nonstereospecific reductase. In this earlier work, we assessed the contribution of parabactin A to iron transport via a ligand exchange mechanism. Under the conditions of the study, such exchange seemed insufficient to account for the V_{max} observed for iron accumulation from ferric parabactin A.[54] It is certainly true that iron exchange can take place between heterologous and native siderophores, promoting microbial growth. Nevertheless, on the basis of the work of Neilands et al. with enantiomeric enterobactin in *E. coli*,[56] our earlier studies with L- and D-parabactin in *P. denitrificans*,[41,54] and the results presented here, the proof of concept is provided that changing the stereochemistry of a siderophore compromises that siderophore's ability to transport iron and thereby promote the growth of the organism which synthesized the siderophore.

The iron clearing efficiencies of L- and D-fluviabactin are quite high and similar to each other, 13.8 ± 0.6 and $16.0 \pm 5.3\%$, respectively, and are also within error of the efficiency of L-parabactin.[42] These efficiencies are also between five and six times that of sc-administered DFO in the rodent model.[42] Furthermore, a comparably protracted biliary iron excretion was observed with all three of the catecholamides. The current observations suggest that, given the many siderophores that could serve as platforms from which to contruct therapeutic iron chelators, those with asymmetric centers are the best choice. In these systems, changing the asymmetric center(s) is likely to result in ligands that will not support microbial growth, yet could still function as deferration agents.

Experimental Section

General. *N*-Carbobenzyloxy-L-threonine and *N*-carbobenzyloxy-D-threonine were purchased from Sigma Chemical Co. (St. Louis, MO), *N*-carbobenzyloxy-L-threonine-1-(*N*-succinimidyl) ester was purchased from Bachem Bioscience Inc. (King of Prussia, PA), and all other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Fisher Optima grade solvents (Fisher Scientific, Pittsburgh, PA) were used. DMF was distilled under N_2 and stored over molecular sieves. Distilled solvents were employed for reactions involving chelators. Organic extracts were dried with sodium sulfate unless otherwise indicated. Glassware for chelator reactions and purification steps were soaked in 3 N HCl for 15 min, rinsed with distilled water then distilled ethanol, and dried prior to use. Silica gel 60 (70–230 mesh) obtained from EM Science (Darmstadt, Germany) or Lipophilic Sephadex LH-20 from Sigma was used as indicated for column chromatography. Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Proton NMR spectra were obtained on a Varian Unity 300 at 300 MHz in CD_3OD at ambient temperature unless otherwise indicated; chemical shifts are given in parts per million downfield from an internal tetramethylsilane standard with coupling constants (J) in Hz.

High-resolution mass spectra were obtained utilizing FAB ionization from a glycerol matrix on a Fennigan 4516. Optical rotations were determined at 589 nm (sodium D line) with a Perkin-Elmer 341 polarimeter using a 10-cm cell path length in the indicated solvent; *c* is expressed as g of compound/100 mL. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA). Circular dichroism (CD) spectra were obtained utilizing a Jasco J500C spectropolarimeter operating in analog mode with a cell path length of 1.00 cm.

All chemical components of the liquid culture media were purchased from Fisher Scientific (Pittsburgh, PA). Trypticase soy agar and trypticase soy broth were procured from Becton-Dickinson (Cockeysville, MD). The optical density of bacterial cultures was measured at 660 nm (OD_{660}) on a Perkin-Elmer Lambda 3B spectrophotometer (Norwalk, CT). Ethylenedi-amine-di(*o*-hydroxyphenyl acetic acid) (EDDA) and trisodium nitriloacetate (NTA) were obtained from Sigma. [^{55}Fe]Ferric chloride (specific activity, 41.8 Ci g^{-1}) in 0.5 M HCl and Biofluor Scintillation Cocktail were purchased from DuPont/New England Nuclear (Boston, MA). Chelex 100 resin was purchased from Bio-Rad (Hercules, CA). The C_{18} silanol reversed-phase chromatographic support was procured from J. T. Baker (Phillipsburg, NJ), and the polyethyleneimine cellulose thin-layer ion exchange chromatographic support was obtained from Merck (Cherry Hill, NJ). The glass fiber filters used in the accumulation and kinetic assays (Whatman GF/F Cat. No. 1825-025) were obtained from Fisher. L-Parabactin, the reference compound in the bacterial growth and iron accumulation studies (Figures 2 and 3), was synthesized by a known method.[45]

Cremophor RH-40 was obtained from BASF (Parsippany, NJ). Sprague−Dawley rats were purchased from Charles River (Wilmington, MA). Nalgene metabolic cages, rat jackets, and fluid swivels were obtained from Harvard Bioscience (South Natick, MA). Intramedic polyethylene tubing PE-50 was obtained from Fisher.

N^1,N^7-Bis[2,3-bis(benzyloxy)benzoyl]norspermidine (4).[51] The reagents 2,3-bis(benzyloxy)benzoic acid[49] (3.34 g, 10 mmol) and 1,1-carbonyldiimidazole (1.62 g, 10 mmol) were dissolved in CH_2Cl_2 (100 mL) and stirred for 1 h at room temperature under a nitrogen atmosphere.[50] A solution of **1** (0.67 g, 5.1 mmol) in CH_2Cl_2 (10 mL) was added, and the mixture was stirred overnight. The resulting solution was washed with 2% NaOH (100 mL), H_2O (100 mL), and brine (100 mL) and then dried over $MgSO_4$ and filtered. Solvent removal in vacuo followed by flash chromatography of the residue on silica (5% EtOH in EtOAc) gave **4** (3.57 g, 70%) as a colorless oil: ^{1}H NMR (CDCl$_3$) δ 1.52−1.63 (m, 4 H), 2.45−2.50 (m, 4 H), 3.28−3.36 (m, 4 H), 5.07 (s, 4 H), 5.15 (s, 4 H), 7.10−7.49 (m, 26 H), 7.64−7.68 (m, 2 H), 8.04−8.08 (br, 1 H).

N^4-(*N*-Carbobenzyloxy-L-threonyl)-N^1,N^7-bis[2,3-bis-(benzyloxy)benzoyl]norspermidine (7). A solution of 1,3-dicyclohexylcarbodiimide (0.34 g, 1.30 mmol) in CH_2Cl_2 (20 mL) was added to a solution of *N*-carbobenzyloxythreonine (0.33 g, 1.50 mmol) and *N*-hydroxysuccinimide (0.19 g, 1.65 mmol) in CH_2Cl_2 (20 mL) and stirred at room temperature for 18 h. The mixture was filtered; **4** (1.0 g, 1.3 mmol) and triethylamine (0.20 g, 2.0 mmol) were added. The resulting mixture was stirred 24 h, concentrated in vacuo, then dissolved in EtOAc (100 mL). The solution was washed with H_2O (2 × 50 mL), 10% citric acid (50 mL), and H_2O (100 mL), dried, and filtered. Solvent removal in vacuo followed by flash chromatography on silica (1:1 EtOAc CHCl$_3$) gave **7** (0.68 g, 52%) as a viscous oil: ^{1}H NMR δ 1.08−1.11 (m, 3 H), 1.51−1.72 (m, 4 H), 2.92−3.31 (m, 8 H), 3.83−3.92 (m, 1 H), 4.38−4.42 (m, 1 H), 5.02−5.21 (m, 10 H), 7.11−7.51 (m, 31 H). Anal. ($C_{60}H_{62}N_4O_{10}$) C, H, N.

N^4-L-Threonyl-N^1,N^7-bis(2,3-dihydroxybenzoyl)norsper-midine Hydrochloride (11). A mixture of **7** (0.75 g, 0.75 mmol) and 10% Pd−C (0.10 g) in HCl-saturated CH_3OH (50 mL) was stirred at room temperature for 1 h under a H_2 atmosphere at ambient pressure. The mixture was filtered through acid-washed Celite and concentrated in vacuo to give 0.40 g (99%) of **11** and used without further purification. An

analytical sample was obtained by column chromatography on LH-20 (10−15% EtOH in toluene) and gave **11** as a white solid: ^{1}H NMR δ 1.25−1.28 (m, 3 H), 1.84−2.02 (m, 4 H), 3.38−3.78 (m, 8 H), 4.02−4.10 (m, 1 H), 4.18−4.21 (m, 1 H), 6.68−6.75 (m, 2 H), 6.91−6.95 (m, 2 H), 7.18−7.21 (m, 2 H). Anal. ($C_{24}H_{33}ClN_4O_8$) C, H, N.

N^4-[2-(2,3-Dihydroxyphenyl)-(4*S*,5*R*)-*trans*-5-methyl-2-oxazoline-4-carboxamido]-N^1,N^7-bis(2,3-dihydroxyben-zoyl)norspermidine (L-Fluviabactin; 15). Ethyl 2,3-dihy-droxybenzimidate[44] (0.22 g, 1.21 mmol) was added to a solution of **11** (0.16 g, 0.30 mmol) in CH_3OH (20 mL). The reaction mixture was heated at reflux under a N_2 atmosphere for 30 h and then concentrated in vacuo. Column chromatography on LH-20 (10% EtOH in toluene) gave **15** (0.12 g, 65%) as a white glass: ^{1}H NMR (CD$_3$OD, 50 °C) δ 1.41 (d, 3 H, *J* = 6.4), 1.85−1.96 (m, 2 H), 2.03−2.14 (m, 2 H), 3.40−3.92 (m, 8 H), 4.80 (d, 1 H, *J* = 6.5), 5.21−5.30 (m, 1 H), 6.61−6.76 (m, 3 H), 6.86−6.97 (m, 3 H), 7.12−7.22 (m, 3 H); HRMS *m/z* calcd for $C_{31}H_{35}N_4O_{10}$ 623.2353 (M + H), found 623.2339; $[\alpha]_D^{24}$ = +89.18 (*c* = 1.00). Anal. ($C_{31}H_{34}N_4O_{10}\cdot0.5$ H_2O) C, H, N.

N^4-(*N*-Carbobenzyloxy-D-threonyl)-N^1,N^7-bis[2,3-bis-(benzyloxy)benzoyl]norspermidine (8). A solution of *N*-carbobenzyloxy-D-threonine (0.76 g, 3.00 mmol) and 1,3-dicyclohexylcarbodiimide (0.63 mg, 3.04 mmol) in CH_2Cl_2 (40 mL) and a solution of **4** (1.50 g, 1.96 mmol) and *N*-hydroxy-succinimide (0.35 g, 3.04 mmol) in CH_2Cl_2 (40 mL) were combined; triethylamine (0.31 g, 3.06 mmol) was added. Reaction conditions, workup, and purification were as de-scribed for **7** and afforded **8** (0.62 g, 32%) as a viscous oil: ^{1}H NMR δ 1.08−1.11 (d, 3 H, *J* = 7), 1.51−1.72 (m, 4 H), 2.92−3.31 (m, 8 H), 3.83−3.92 (m, 1 H), 4.38−4.42 (m, 1 H), 5.02−5.21 (m, 10 H), 7.11−7.51 (m, 31 H). Anal. ($C_{60}H_{62}N_4O_{10}$) C, H, N.

N^4-D-Threonyl-N^1,N^7-bis(2,3-dihydroxybenzoyl)norsper-midine Hydrochloride (12). A mixture of **8** (0.30 g, 0.30 mmol) and 10% Pd−C (0.15 g) in HCl saturated CH_3OH (50 mL) was reacted under a H_2 atmosphere as described for **11**. The resulting white solid (**12**, 0.16 g, 99%) was used without further purification. An analytical sample was obtained by column chromatography on LH-20 (15% EtOH in toluene) and gave **12** as a white solid: ^{1}H NMR δ 1.25−1.28 (d, 3 H, *J* = 7), 1.84−2.02 (m, 4 H), 3.38−3.78 (m, 8 H), 4.02−4.10 (m, 1 H), 4.18−4.21 (m, 1 H), 6.68−6.75 (m, 2 H), 6.91−6.95 (m, 2 H), 7.18−7.21 (m, 2 H). Anal. ($C_{24}H_{33}ClN_4O_8$) C, H, N.

N^4-[2-(2,3-Dihydroxyphenyl)-(4*R*,5*S*)-*trans*-5-methyl-2-oxazoline-4-carboxamido]-N^1,N^7-bis(2,3-dihydroxyben-zoyl)norspermidine (D-Fluviabactin; 16). Utilizing the conditions, procedures, and workup described for **15**, ethyl 2,3-dihydroxybenzimidate[44] (0.12 g, 0.66 mmol) was reacted with **12** (0.15 g, 0.28 mmol). Column chromatography of the resulting residue on LH-20 (15% EtOH in toluene) gave **16** (0.11 mg, 63%) as a white glass: ^{1}H NMR (CD$_3$OD, 50 °C) δ 1.41 (d, 3 H, *J* = 6.4), 1.85−1.96 (m, 2 H), 2.03−2.14 (m, 2 H), 3.40−3.92 (m, 8 H), 4.80 (d, 1 H, *J* = 6.5), 5.21−5.30 (m, 1 H), 6.61−6.76 (m, 3 H), 6.86−6.97 (m, 3 H), 7.12−7.22 (m, 3 H); HRMS *m/z* calcd for $C_{31}H_{35}N_4O_{10}$ 623.2353 (M + H), found 623.2334; $[\alpha]_D^{24}$ = −85.86 (*c* = 1.00). Anal. ($C_{31}H_{34}N_4O_{10}\cdot0.5$ H_2O) C, H, N.

N^1,N^8-Bis[2,3-bis(benzyloxy)benzoyl]spermidine (5).[50] A mixture of 2,3-bis(benzyloxy)benzoic acid (2.60 g, 7.78 mmol), 1,1-carbonyldiimidazole (1.26 g, 7.77 mmol), and **2** (0.56 g, 3.86 mmol) in CH_2Cl_2 (10 mL) was reacted and worked up as described for **4**. Flash chromatography of the resulting residue on silica (10% CH_3OH in CHCl$_3$) gave **5** (2.2 g, 73%) as a pale yellow oil: ^{1}H NMR δ 1.37−1.44 (m, 4 H), 1.56−1.66 (m, 2 H), 2.38−2.44 (m, 2 H), 2.44−2.55 (m, 2 H), 3.20−3.26 (m, 4 H), 5.08 (s, 4 H), 5.16−5.17 (m, 4 H), 7.10−7.50 (m, 26 H).

N^4-(*N*-Carbobenzyloxy-L-threonyl)-N^1,N^8-bis[2,3-bis-(benzyloxy)benzoyl]spermidine (9). Utilizing the proce-dures described for **7**, *N*-carbobenzyloxy-L-threonine (0.76 g, 3.00 mmol), 1,3-dicyclohexylcarbodiimide (0.63 g, 3.04 mmol) in dry CH_2Cl_2 (40 mL), **5** (1.5 g, 1.93 mmol), and *N*-hydroxy-succinimide (0.35 g, 3.04 mmol) in dry CH_2Cl_2 (40 mL) and triethylamine (0.31 g, 3.06 mmol) were combined and reacted.

After workup, flash chromatography of the resulting residue on silica (1:1 EtOAc CHCl$_3$) gave **9** (1.08 g, 55%) as a viscous oil: ^{1}H NMR δ 1.13–1.16 (m, 3 H), 1.56–1.76 (m, 6 H), 2.94–3.35 (m, 8 H), 3.72–3.86 (m, 1 H), 4.32–4.41 (m, 1 H), 5.06–5.25 (m, 10 H), 7.10–7.50 (m, 31 H). Anal. (C$_{61}$H$_{64}$N$_4$O$_{10}$) C, H, N.

N^4-L-Threonyl-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine Hydrochloride (13). A mixture of **9** (0.31 g, 0.31 mmol) and 10% Pd–C (0.15 g) in HCl-saturated CH$_3$OH (30 mL) was reacted under a H$_2$ atmosphere as described for **11**. The resulting white solid (**13**, 0.16 g, 93%) was used without further purification. An analytical sample was obtained by column chromatography on LH-20 (15% EtOH in toluene) and afforded **13** as a white solid: ^{1}H NMR δ 1.07–1.10 (m, 3 H), 1.52–1.73 (m, 6 H), 2.91–3.32 (m, 8 H), 3.82–3.91 (m, 1 H), 4.36–4.44 (m, 1 H), 6.67–6.73 (m, 2 H), 6.90–6.93 (m, 2 H), 7.19–7.23 (m, 2 H). Anal. (C$_{25}$H$_{35}$ClN$_4$O$_8$) C, H, N.

N^4-[2-(2,3-Dihydroxyphenyl)-(4S,5R)-$trans$-5-methyl-2-oxazoline-4-carboxamido]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine (L-Agrobactin; 17). Ethyl 2,3-dihydroxybenzimidate[44] (0.12 mg, 0.66 mmol) was added to a solution of **13** (0.14 g, 0.25 mmol) in CH$_3$OH (30 mL); reaction conditions and workup were as described for **15**. Column chromatography of the resulting residue on LH-20 (15% EtOH in toluene) gave **17** (0.10 g, 63%) as a white glass: ^{1}H NMR (CD$_3$OD, 50 °C) δ 1.40 (d, 1.26 H, $J = 6.4$) 1.45 (d, 1.74 H, $J = 6.4$), 1.56–1.96 (m, 5 H), 2.00–2.13 (m, 1 H), 3.34–3.90 (m, 8 H), 4.78 (d, 0.46 H, $J = 6.2$) 4.85 (d, 0.54 H, $J = 6.5$), 5.20–5.29 (m, 1 H), 6.61–6.76 (m, 3 H), 6.86–6.97 (m, 3 H), 7.11–7.24 (m, 3 H); HRMS m/z calcd for C$_{32}$H$_{37}$N$_4$O$_{10}$ 637.2510 (M + H), found 637.2510. Anal. (C$_{32}$H$_{36}$N$_4$O$_{10}$·0.5 H$_2$O) C, H, N.

N^1,N^9-Bis[2,3-bis(benzyloxy)benzoyl]homospermidine (6). A mixture of 2,3-bis(benzyloxy)benzoic acid (2.10 g, 6.28 mmol), 1,1-carbonyldiimidazole (1.02 g, 6.29 mmol), and **3**[48] (0.5 g, 3.14 mmol) in CH$_2$Cl$_2$ (10 mL) was reacted and worked up as described for **4**. Flash chromatography of the resulting crude material on silica (10% CH$_3$OH in CHCl$_3$) gave **6** (1.6 g, 64%) as a pale yellow oil: ^{1}H NMR δ 1.41–1.48 (m, 8 H), 2.51–2.58 (m, 4 H), 3.22–3.28 (m, 4 H), 4.85 (s, 4 H), 5.09 (s, 2 H), 5.18 (s, 2 H), 7.01–7.50 (m, 26 H).

N^5-(N-Carbobenzyloxy-L-threonyl)-N^1,N^9-bis[2,3-bis(benzyloxy)benzoyl]homospermidine (10). A mixture of **6** (1.50 g, 1.89 mmol), N-carbobenzyloxy-L-threonine-1-(N-succinimidyl) ester (1.00 g, 2.85 mmol), and triethylamine (0.20 g, 1.98 mmol) in CH$_2$Cl$_2$ (100 mL) was stirred overnight. The resulting solution was washed with H$_2$O (100 mL), 10% citric acid (100 mL), and brine (100 mL) and then dried and filtered. Solvent removal in vacuo followed by flash chromatography of the residue on silica (1:1 EtOAc/CHCl$_3$) gave **10** (1.49 g, 77%) as a viscous oil: ^{1}H NMR δ 1.09–1.11 (d, 3 H, $J = 7$), 1.31–1.54 (m, 8 H), 3.01–3.28 (m, 8 H), 3.84–3.93 (m, 1 H), 4.42–4.45 (m, 1 H), 5.07–5.16 (m, 10 H), 7.09–7.48 (m, 31 H). Anal. (C$_{62}$H$_{66}$N$_4$O$_{10}$) C, H, N.

N^5-L-Threonyl-N^1,N^9-bis(2,3-dihydroxybenzoyl)homospermidine Hydrochloride (14). A mixture of **10** (0.60 g, 0.59 mmol) and 10% Pd–C (0.20 g) in HCl-saturated CH$_3$OH (50 mL) was reacted under a H$_2$ atmosphere as described for **11**. The resulting white solid (**14**, 0.30 g, 90%) was used without further purification. An analytical sample was obtained by column chromatography on LH-20 (15% EtOH in toluene) to give **14** as a white solid: ^{1}H NMR δ 1.19–1.21 (d, 3 H, $J = 7$), 1.41–1.54 (m, 8 H), 3.11–3.36 (m, 8 H), 4.44–4.53 (m, 1 H), 4.92–5.12 (m, 1 H), 6.84–7.28 (m, 6 H). Anal. (C$_{26}$H$_{37}$ClN$_4$O$_8$) C, H, N.

N^5-[2-(2,3-Dihydroxyphenyl)-(4S,5R)-$trans$-5-methyl-2-oxazoline-4-carboxamido]-N^1,N^9-bis(2,3-dihydroxybenzoyl)homospermidine (L-Homofluviabactin; 18) Ethyl 2,3-dihydroxybenzimidate[44] (0.20 g, 1.1 mmol) and **14** (0.30 g, 0.53 mmol) in CH$_3$OH (50 mL) were reacted and worked up as described for **15**. Column chromatography of the resulting residue on LH-20 (15% EtOH in toluene) gave **18** (0.22 g, 64%) as a white glass: ^{1}H NMR (CD$_3$OD, 50 °C) δ 1.44 (d, 3 H, $J = 6.4$), 1.55–1.88 (m, 8 H), 3.36–3.79 (m, 8 H), 4.83 (d, 1 H, $J = 6.5$), 5.19–5.29 (m, 1 H), 6.63–6.76 (m, 3 H), 6.85–6.98 (m, 3

H), 7.12–7.23 (m, 3 H); HRMS m/z calcd for C$_{33}$H$_{39}$N$_4$O$_{10}$ 651.2666 (M + H), found 651.2664. Anal. (C$_{33}$H$_{38}$N$_4$O$_{10}$·0.5 H$_2$O) C, H, N.

Low-Fe Defined Minimal Salts Medium. Glassware was presoaked in 3 N HCl for 15 min and rinsed well with water; all water used was distilled in a Mega-Pure still (Corning Costar, Corning, NY) and passed through a deionizing cartridge (Sybron-Barnstead, Milwaukee, WI) before use.

For a 12-L batch of culture medium, succinic acid (70.8 g), KH$_2$PO$_4$ (48.0 g), Na$_2$HPO$_4$ (60.6 g), and NH$_4$Cl (19.2 g) were dissolved in H$_2$O (10 L); the solution was autoclaved and was then stored at 4 °C for 3–4 days to allow Fe salts to coagulate.[68] After filtration through a 0.2-μm membrane and adjustment of the pH to 7.0, the solution was passed through a column of Chelex 100 resin (1500 g). The pH was again adjusted to 7.0, Chelex-treated Tween 80 (300 mL) was added, and the final volume was adjusted to 12 L (final Tween concentration, 0.5%). The following were then added to the medium to provide divalent cations and trace metals which were removed during Chelex treatment (final concentration in parentheses): MgSO$_4$ (1.7 mM), Ca^{2+} (182 μM), Mn^{2+} (10 μM), Zn^{2+} (1 μM), Cu^{2+} (0.1 μM), and Co^{2+} (0.01 μM).[54] Atomic absorption analysis revealed that the liquid culture medium had an Fe concentration of < 0.1 μM; thus, the degree of low-iron stress could then be controlled by addition of a known quantity of ferric(NTA)$_2$ to the medium. "Transport buffer" is the culture medium without additional Fe.

Preparation of Labeled Chelates. Stock solutions of labeled chelates of compounds **15–18** were prepared according to the ligand exchange method described previously for [^{55}Fe]-ferric parabactin with ferric (NTA)$_2$.[54] Briefly, [^{55}Fe]ferric-(NTA)$_2$ was prepared from ^{55}FeCl$_3$ in 0.5 M HCl and trisodium NTA in 10% NTA excess in 0.1 N HCl; the pH was adjusted to 7.0 with a Tris buffer solution (500 mM). Ligand exchange was accomplished employing a 10% molar excess of ligand in EtOH and ferric (NTA)$_2$; Tris hydrochloride (500 mM, pH 7.4) was used to dilute the labeled chelate solution to 300 μM. Purification on a C$_{18}$ silanol reversed-phase column (2:5 CH$_3$-OH/H$_2$O) yielded the labeled chelate solution, which was then adjusted with Tris hydrochloride (100 mM, pH 7.4) to a concentration of 100–300 μM as determined spectrophotometrically at the visible λ_{max} of the chelates: ferric fluviabactin ($\epsilon_{508} = 3.8 \times 10^3$ M^{-1} cm^{-1}); ferric homofluviabactin ($\epsilon_{516} = 3.1 \times 10^3$ M^{-1} cm^{-1}); ferric agrobactin ($\epsilon_{507} = 3.6 \times 10^3$ M^{-1} cm^{-1}). The purified chelates were homogeneous by thin-layer ion exchange chromatography on polyethyleneimine cellulose (2:2:1 THF/CH$_3$OH/10 mM tetrabutylammonium phosphate, pH 7.0); their respective R_f values were 0.59–0.63, 0.60–0.64, and 0.60–0.64. When the ferric fluviabactin complex was dissociated by addition of pH 3.0 phosphate buffer and extracted with EtOAc, the organic layer contained only the intact oxazoline, fluviabactin, as determined by thin-layer chromatography on silica gel (94:6 CHCl$_3$/CH$_3$OH), $R_f = 0.40–0.44$.

Preparation of Unlabeled Chelates for CD Spectra. Unlabeled ferric L-fluviabactin and ferric D-fluviabactin solutions utilized for the CD spectral studies employed unlabeled Fe(NTA)$_2$ and were prepared as described above for the labeled chelates.

Bacterial Strains. *P. denitrificans* (ATCC strain 17741) was maintained on trypticase soy agar plates. Unless otherwise indicated, all liquid cultures were incubated at 30 °C with rotary shaking (120 rpm). Individual bacterial colonies were inoculated into trypticase soy broth (20 mL) in 250-mL culture flasks and incubated for 24 h. Inoculations were then made into the defined medium containing 1 μM Fe(III) (50 mL) to give a starting OD of 0.015 and incubated for 16 h. This culture, in turn, was used to inoculate a 250-mL culture flask containing defined medium with 0.5 μM Fe(III) (50 mL) to give a starting OD of 0.040. After a 12-h incubation, the cells were in late logarithmic growth (3.0 < OD$_{660}$ < 3.5), and the medium was positive for catechols.[54] Cells were harvested by centrifugation (15 min at 1100g) at 15–20 °C, washed twice with transport buffer, and resuspended in fresh transport buffer to an OD$_{660}$ reading of 1.000.

Protein Determination. The protein concentration in the cell suspensions was estimated by the method of Lowry et al.[69] with the modifications for membrane protein samples as given by Markwell et al.[70] using bovine serum albumin as the standard. The cell density of the suspensions was estimated spectrophotometrically as described previously;[54] 1 OD_{660} unit refers to the amount of cells yielding an OD_{660} of 1.000 when suspended in transport buffer at room temperature. Under the growth conditions described, 1 OD_{660} unit corresponded to ~0.25 mg of protein.

Growth Studies. The effects of D- and L-fluviabactin, L-homofluviabactin, and L-agrobactin on the growth of *P. denitrificans* in iron-deficient media were assessed. Iron-free ligands (2 μM in CH_3OH) were added to empty, sterile flasks, evaporated to dryness under nitrogen [which was passed through a 0.22-μm pore size filter (Millipore)], and redissolved in sterile medium. For these studies, low-iron defined minimal salts medium was formulated as described above except that EDDA was added (1.1 mM final concentration) to the medium.[41] Bacterial suspension was added to the medium such that the starting OD_{660} was 0.017. Growth rates were determined by monitoring OD_{660} readings vs time (t = 5, 10, 20, 24, 36, and 48 h; Figure 1). The results of the growth studies are reported as OD_{660} vs time.

Accumulation of Radiolabeled Ligands. A cell suspension with an initial OD_{660} = 1.00 (50 mL) was added to each 250-mL flask and incubated for 15 min before addition of stock solutions of labeled chelates (1 mL; final concentration 1 μM; specific activity, 0.033 μCi mL^{-1}). Samples of cell suspensions (5 mL) were aseptically removed at t = ca. 0, 5, 10 min, and at 10-min intervals through 60 min and immediately diluted with cold transport medium (5 mL) to stop the reaction. Whatman GF/F fiber glass filters, which had been presoaked for 24 h in 500 μM unlabeled chelate and rinsed with chilled transport buffer (5 mL), were used to separate cells from medium by rapid filtration. After additional rinsing with transport buffer (2 × 5 mL), the filters were dried at 70 °C and placed in a scintillation vial containing Biofluor (10 mL). Liquid scintillation counting was performed in a Beckman LS6000IC counter (Beckman, Palo Alto, CA). Control values of labeled chelates adsorbed to the filters in the absence of cells were measured and subtracted from the values obtained in the presence of cells. Uptake of labeled chelates by *P. denitrificans* is reported as the net amount of radiolabeled Fe per mg of cell suspension.

Kinetic Transport Assay. Polystyrene conical-bottom tubes (50-mL) were used as incubation vessels. The cell suspensions (OD_{660} = 1.00, 3.45 mL/tube) were incubated with shaking in a water bath (30 °C) for 15 min. After the addition of radiolabeled chelate (50 μL), samples (500 μL) were withdrawn every 20 s for 2 min; the reaction was stopped by adding the sample to 5 mL of chilled (0 °C) transport buffer. Triplicate 100-μL portions of each incubation mixture were counted to verify the amount of label added. Preparation and counting of the samples were as described for the accumulation assay above.

The data were manipulated as described in an earlier publication.[54] Briefly, the six datum points taken during the first 2 min for each assay tube were fitted to a regression line with a slope equal to the initial reaction velocity (v_0). These initial rates, expressed as pg-atoms ^{55}Fe min^{-1} (mg of protein)$^{-1}$, were then used to generate Lineweaver−Burk plots by using Cleland's Fortran program[71] to estimate K_m, V_{max}, and their standard errors. Rates varied linearly with the number of cells present (OD_{660} from 0.5 to 2.000). Data were corrected for cell growth that had occurred during the experiment.

Cannulation of Bile Duct in Rats. The cannulation has been described in detail in earlier publications.[31,72] Briefly, male Sprague−Dawley rats averaging 400 g were housed in Nalgene plastic metabolic cages during the experimental period and were given free access to water. The animals were anesthetized using sodium pentobarbital (55 mg/kg) given ip. The bile duct was cannulated using 22-gauge polyethylene PE-50 tubing. The cannula was inserted into the duct about 1 cm from the duodenum and tied in place. After threading through the shoulder, the cannula was passed from the rat to the swivel inside a metal torque-transmitting tether, which was attached to a rodent jacket around the animal's chest. The cannula was directed from the rat to a Gilson microfraction collector (Middleton, WI) by a fluid swivel mounted above the metabolic cage. Bile samples were collected at 3-h intervals for 48 h. Urine samples were taken every 24 h. Sample collection and handling were as previously described.[31] The efficiency of each chelator was calculated by subtracting the iron excretion of control animals from the iron excretion of treated animals. This number was then divided by the theoretical output on the basis of a 1:1 ligand-iron complex; the result is expressed as a percentage.

Atomic Absorption Iron Determinations. Bile and urine samples were analyzed on a Perkin-Elmer 5100 PC atomic absorption spectrophotometer fitted with a model AS-51 autosampler using a quartz sampling probe as previously described.[30]

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