Microbial Iron Chelators as Drug Delivery Agents: The Rational Design and Synthesis of Siderophore–Drug Conjugates†

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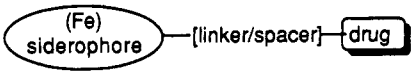
Virulence and even survival of most microbes during infections depends on their ability to compete effectively for key nutrients. Iron assimilation is especially important because of its critical physiological role in cellular redox chemistry. To overcome the extreme insolubility of iron, many microbes synthesize and excrete powerful ferric ion-selective chelating agents, called siderophores.[1] These sequestering agents facilitate active transport of chelated iron into cells where, by modification, reduction, or siderophore decomposition, it is released for use by the cell.[2] Studies of siderophores and their transport processes have revealed considerable information on important aspects of iron metabolism.[3] Related studies also suggest potentially important therapeutic applications of siderophores.[4,5] For example, inhibition of siderophore biosynthesis, siderophore excretion, or iron complex recognition or transport might significantly limit microbial growth. Another approach is to use siderophores as drug delivery agents for the treatment of pathogenic infections. This Account describes efforts to design and synthesize siderophore–drug conjugates and to determine if microbial iron-assimilation systems can be used for drug transport.



The generalized conjugate consists of four components: iron, ligands (the siderophore), linker, and drug. The primary requirements in the design of siderophore conjugates are that the molecules will bind iron yet allow the complex to be recognized and actively transported. The tremendous affinity of siderophores for ferric ion is reflected by association constants ranging from $\leq 10^{30}$ to $\sim 10^{50}$ and assures that appropriately constructed compounds will effectively bind iron. Recognition and transport may or may not depend on peripheral substitution. The nature of the linker will determine whether the drug will remain attached to the complex or be released chemically or enzymatically within the cell.[6] The choice of the drug depends on the biochemical target and its compatibility with the conjugation process. The assembly of conjugates can proceed by derivatization of natural siderophores (semisynthesis) or by total synthesis. Both approaches require consideration of the components of the siderophores.

Siderophore and Siderophore Component Syntheses

Most microbes utilize hydroxamic acids, catechols, and/or α-hydroxy carboxylic acids as the metal-binding components of siderophores. The evolutionary choice of these groups reflects the high affinity of oxygen ligands for "hard" ferric ions.[7] The most effective siderophores contain three bidentate ligands for complete octahedral coordination of ferric ion and to minimize the entropic effects of sequestering a single ferric ion with separate ligands. Representative structures of the more than 200 known siderophores are shown in Figures 1–3. First inspection suggests that siderophores are rather complex molecules which may

Marvin Miller was born in 1949 and grew up in Dickinson, ND. He was inspired to be an organic chemist by the enthusiasm of Professor S. P. Pappas, his undergraduate mentor, at North Dakota State. Professor Marc Loudon introduced him to hydroxamic acid chemistry during Ph.D. studies at Cornell. Subsequent to an NIH postdoctoral fellowship with Professor Henry Rapoport at Berkeley, Dr. Miller joined the faculty of Notre Dame in 1977. Current research interests include synthesis and studies of biologically important molecules. He is sustained by the love of his family: Patty, Chris, Katie, Joey, and Carl.

François Malouin was born in Montreal in 1961. He did his undergraduate studies in biological sciences at the Université de Sherbrooke and undertook afterward a specialty (M.S.) in medical microbiology and immunology with Dr. François Lamothe at the Université de Montréal in 1982. He then further specialized in the molecular basis of antimicrobial drug resistance in the laboratory of Dr. Lawrence E. Bryan at the University of Calgary, where he completed his Ph.D. degree in 1988. After postdoctoral training with Dr. Thomas R. Parr, Jr., at Lilly Research Laboratories in Indianapolis, he is now assistant professor at Université Laval, where he continues his efforts in elucidating the mode of action of new antibacterial agents and defining the mechanisms of resistance in bacteria. He has been a Scholar of the Medical Research Council of Canada since 1990.

† Dedicated to M.J.M.'s mother, E. Miller, on the occasion of her 70th birthday and to Professor J. B. Neilands on the occasion of his retirement from the Department of Biochemistry, U. C. Berkeley.

(1) Winkelman, G., Ed. *Handbook of Microbial Iron Chelates*; CRC: Boca Raton, FL, 1991.
(2) Hider, R. C. *Structure Bonding (Berlin)* **1984**, *58*, 25.
(3) (a) Bullen, J. J., Griffiths, E., Eds. *Iron and Infection*; Wiley: New York, 1987. (b) Winkelman, G., van der Helm, D.; Neilands, J. B. *Iron Transport in Microbes, Plants, and Animals*; VCH: New York, 1987.
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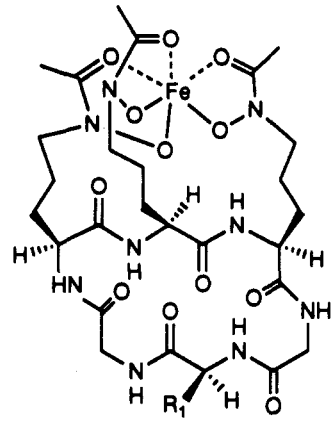
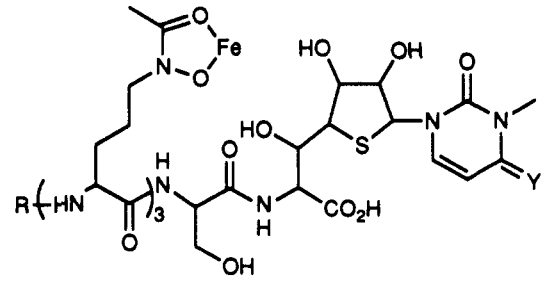
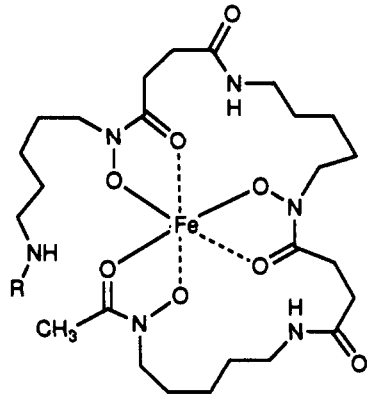
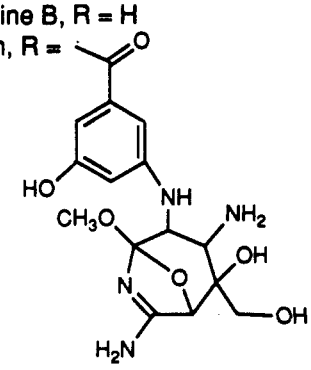


1, ferrichrome, $R_1 = H$
2, ferricrocin, $R_1 = CH_2OH$



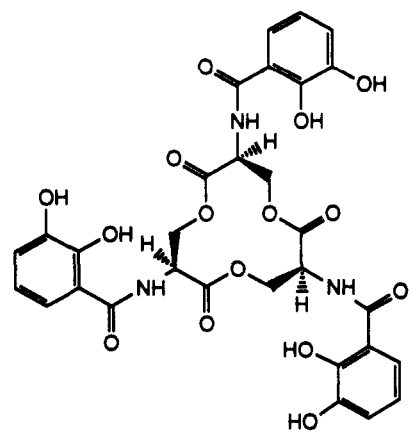
3a, albomycin δ_1, R = H, Y = O
3b, albomycin δ_2, R = H, Y = $NCONH_2$
3c, albomycin ε, R = H, Y = NH



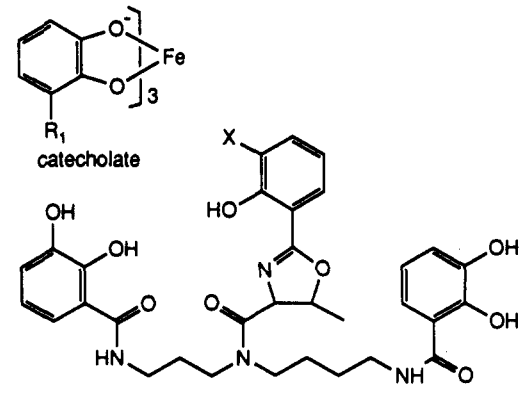


4, ferrioxamine B, R = H
5, ferrimycin, R =

Figure 1. Hydroxamic acid-based siderophores.



6, enterobactin (enterochelin)



catecholate

7, agrobactin, X=OH
8, parabactin, X=H

Figure 2. Catechol-containing siderophores.

be difficult to manipulate during the synthesis of drug conjugates. However, close scrutiny reveals that most siderophores are actually quite simple, consisting of a core or platform to which usually three bidentate ligands are attached. Moreover, evidence suggests that the metal center is the key to microbial recognition, and receptor and transport proteins often do not require the full siderophore structure.[8,9]

The existence of albomycins (**3**) and ferrimycin (**5**), which incorporate both a siderophore component and a toxic agent, prompted attempts to prepare mimics by conjugation of siderophores directly to antimicrobial agents. Early studies included modification of the serine hydroxyl group of ferricrocin (**2**) and the amino group of ferrioxamine B (**4**) derivatives with sulfa drugs. The resulting products had insignificant antibiotic activity.[10] Synthetic replacement of the thioriobosyl group of albomycin with an oxygen analog resulted in loss of antimicrobial activity.[11] Subsequently, a number of groups reported that derivatives of β-lactam antibiotics with single bidentate iron-binding constituents displayed enhanced antibacterial activity, presumably

(8) (a) Matzanke, B. F.; Muller-Matzanke, G.; Raymond, K. N. In *Iron Carriers and Iron Proteins*; Loehr, T. M., Ed.; VCH: New York, 1989; p 1. (b) Raymond, K. N.; Muller, G.; Matzanke, B. F. *Top. Curr. Chem.* **1984**, *123*, 50.

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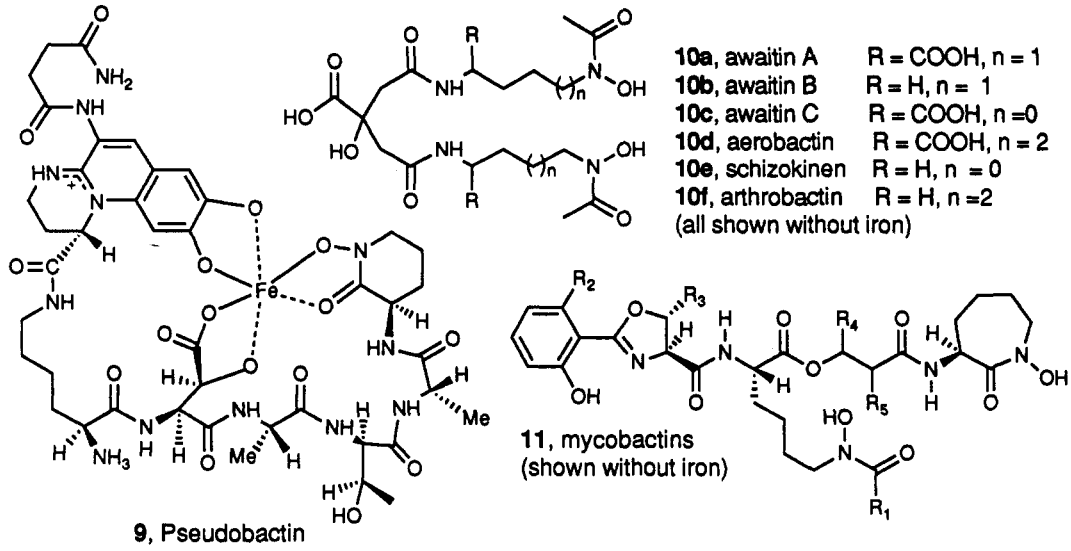
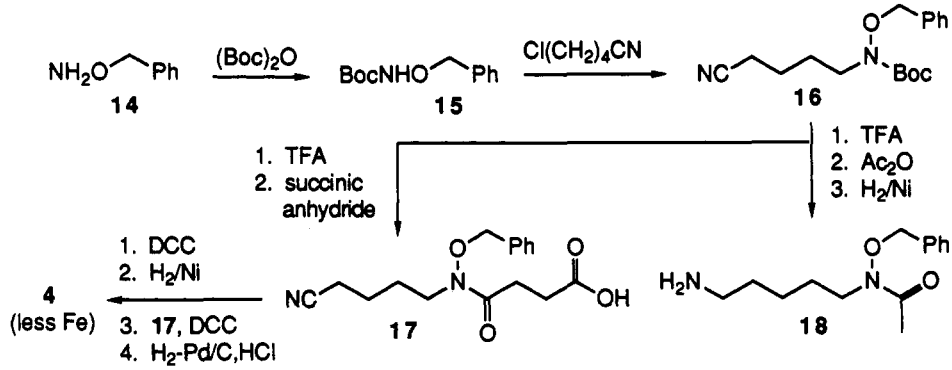


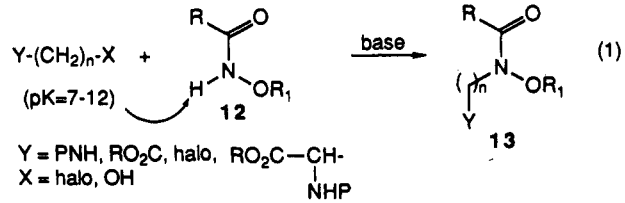
Figure 3. Mixed ligand siderophores.

Scheme I



due to iron transport-facilitated delivery.[12] Still, systematic determination of structure–activity relationships of siderophore–drug conjugates required access to sufficient quantities of siderophore components to determine the minimal structural requirements for siderophore activity, the optimal mode of linkage, and the consequences of varying the attached antimicrobial agent. Representative methods for the synthesis of hydroxamate and catechol iron chelators and components are summarized next.

Biosynthetically, hydroxamate components of siderophores are made by direct oxidation of amines to hydroxylamines and subsequent acylation.[13] Attempts to chemically mimic this process have been frustrated by low yields or overoxidation.[14] Alternatively, reduction of nitroalkanes to hydroxylamines followed by acylation has been more successful.[15] Current emphasis has been to preform the hydroxamate unit and then incorporate it directly into the backbone of a siderophore component utilizing the acidity ($pK \sim 7$–12) of N-unsubstituted hydroxamic acids **12** to facilitate alkylation (eq 1).[16] Protection of the hydroxylamine nitrogen as a carbamate (**12**, R = OR) eliminates competitive carbonyl O-alkylation.[17] The utility of hydroxamate alkylation has been demonstrated by the efficient synthesis of all of the representative (*N*-hydroxyamino)alkane and ω-*N*-hydroxy amino acid iron-chelating components of hydroxamate-based si-



(1)

(12) (a) Ohi, N.; Aoki, B.; Shinozaki, T.; Moro, K.; Noto, T.; Nehashi, T.; Okazaki, H.; Matsunaga, I. *J. Antibiot.* **1986**, *39*, 230. (b) Ohi, N.; Aoki, B.; Moro, K.; Kuroki, T.; Sugimura, N.; Noto, T.; Nehashi, T.; Matsumoto, M.; Okazaki, H.; Matsunaga, I. *J. Antibiot.* **1986**, *39*, 242. (c) Ohi, N.; Aoki, B.; Shinozaki, T.; Moro, K.; Kuroki, T.; Noto, T.; Nehashi, T.; Matsumoto, M.; Okazaki, H.; Matsunaga, I. *Chem. Pharm. Bull.* **1987**, *35*, 1903. (d) Mochizuki, H.; Yamada, H.; Oikawa, Y.; Murakami, K.; Ishiguro, J.; Kosuzume, H.; Aizawa, N.; Mochida, E. *Antimicrob. Agents Chemother.* **1988**, *32*, 1648. (e) Watanabe, N.-A.; Nagasu, T.; Katsu, K.; Kitoh, H. *Antimicrob. Agents Chemother.* **1987**, *31*, 497. (f) Katsu, K.; Kitoh, K.; Inoue, M.; Mitsuhashi, S. *Antimicrob. Agents Chemother.* **1982**, *22*, 181. (g) Nakagawa, S.; Sanada, M.; Matsuda, K.; Hazumi, N.; Tanaka, N. *Antimicrob. Agents Chemother.* **1987**, *31*, 1100. (h) Curtis, N. A. C.; Eisenstadt, R. L.; East, S. J.; Cornford, R. J.; Walker, L. A.; White, A. J. *Antimicrob. Agents Chemother.* **1988**, *32*, 1879.

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(16) (a) Miller, M. J. *Acc. Chem. Res.* **1986**, *19*, 49. (b) Bergeron, R. J.; McManis, J. S. *Tetrahedron* **1989**, *45*, 4939. (c) Bergeron, R. J.; Pegram, J. J. *J. Org. Chem.* **1988**, *53*, 3131. (d) Bergeron, R. J.; McManis, J. S. *Tetrahedron* **1990**, 5881.

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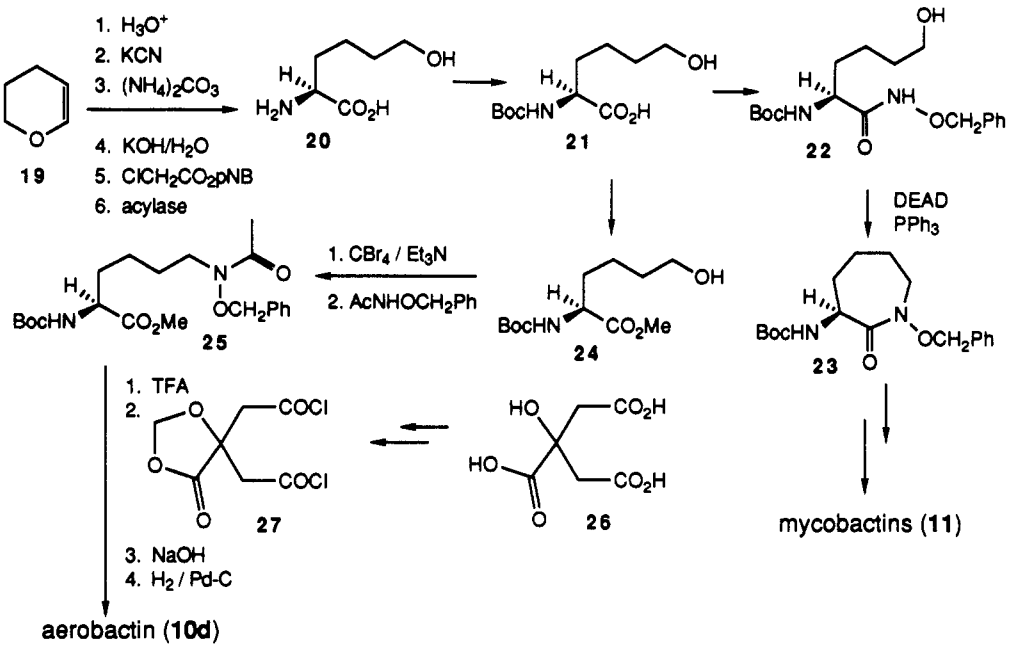
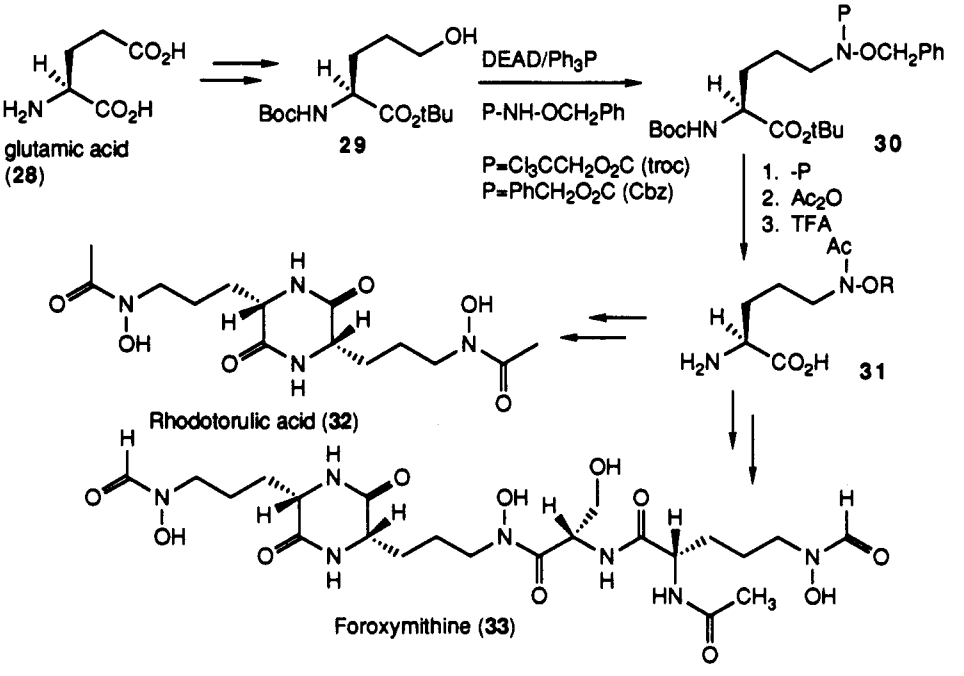
Scheme II



Scheme III



derophores. Extension of this methodology has produced total syntheses of a number of siderophores and analogs. Representative syntheses are summarized in Schemes I–III.

Desferrioxamine B (DFO, 4 less Fe) and analogs were especially attractive synthetic targets since DFO is the only siderophore used clinically. DFO promotes iron excretion in patients with toxic iron overload from transfusional therapy due to thalassemia and related disorders.[1-5] A number of syntheses of DFO have been reported. One approach described by Bergeron (Scheme I)[16c] facilitated syntheses of related natural products desferrioxamine G and E (norcardamine)[16d] and bisucaberin,[16b] as well as the preparation of a variety of important analogs for clinical study. The key to these syntheses is the preparation of the constituent α-amino-ω-(*N*-hydroxyamino)alkane (18) precursors by alkylation of *N*-*t*-Boc-*O*-benzylhydroxylamine (15 to 16).

A number of citrate-based siderophores (e.g., **10a–f**, Figure 3) also contain related α-amino-ω-(*N*-hydroxyamino)alkanes or the corresponding ω-*N*-hydroxy amino acids. Awaitin A (**10a**),[18] schizokinen (**10e**), schizokinen A, arthrobactin (**10f**),[19] and aerobactin (**10d**)[20] have all been synthesized. The synthesis of aerobactin (**10d**, Scheme II) is representative and again demonstrates the utility of direct N-alkylation, in this case for the preparation of the key protected ϵ-*N*-acetyl-ϵ-*N*-hydroxy-L-lysine component **25**. Forms of the same

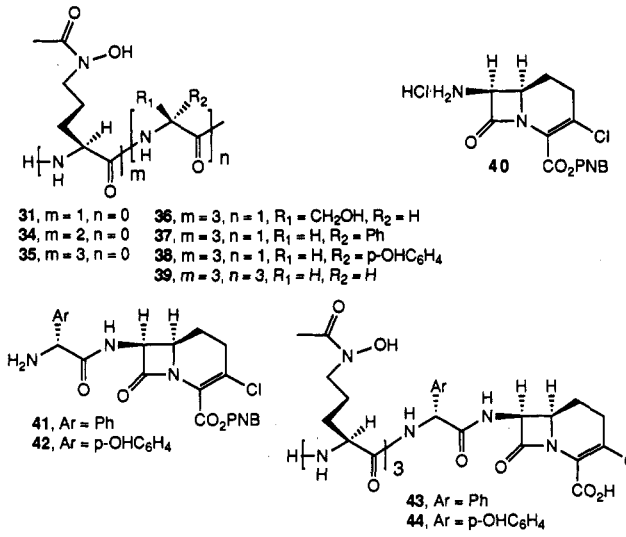
(18) Steinbaugh, B. A. M.S. Thesis, University of Notre Dame, Notre Dame, IN, 1985.
(19) Lee, B. H.; Miller, M. J. *J. Org. Chem.* **1983**, *48*, 24.
(20) Maurer, P. J.; Miller, M. J. *J. Am. Chem. Soc.* **1982**, *104*, 3096.

amino acid are partially responsible for iron chelation in the mycobactins (**11**), essential siderophores for *Mycobacterium tuberculosis* which causes tuberculosis.[21] Slight modification of the linear ω-*N*-hydroxy-L-lysine synthesis allowed preparation of the cyclic component **23** of mycobactin S2.[22]

δ-*N*-Acetyl-δ-*N*-hydroxy-L-ornithine (**31**, R = H) is the primary iron-chelating component of many hydroxamate-based siderophores.[23] The most studied of these, ferrichrome (**1**), is a cyclic hexapeptide containing three contiguous δ-*N*-hydroxy-L-ornithine residues and three glycine residues. A number of syntheses of ferrichrome[24] and analogs[25] have been reported. Early syntheses of ornithine derivative **31** were low yielding and involved either partial reduction of the corresponding ε-nitro amino acid[26] or oxidation and subsequent hydrolysis of ε-imines[27] or nitrones.[28] Isowa[29] utilized *O*-benzyl-*N*-(*p*-toluylsulfonyl)hydroxylamine to introduce the hydroxylamine component in a racemic synthesis of **31**. Subsequent resolution led to the total synthesis of ferrichrome. A more direct synthesis of **31** from L-glutamic acid (**28**) avoided a resolution (Scheme III).[17] This approach allowed the facile preparation of both the free δ-*N*-acetyl-δ-*N*-hydroxyornithine and differentially protected forms of it for use in siderophore and related peptide syntheses.[30] Benz also reported a related synthesis of forms of **31** and albomycin from glutamic acid.[31]

The simplest δ-*N*-acetyl-δ-*N*-hydroxyornithine-based siderophore is rhodotorulic acid (**32**).[32] This cyclic dimer (diketopiperazine) binds ferric ion effectively in a complex containing three rhodotorulic acid molecules and two ferric ions, because it contains only two hydroxamates.[33] Still, it was considered a clinical candidate for the treatment of iron overload, but testing was discontinued because of its oral inactivity, extreme insolubility, and reports of severe pain at injection sites. The glutamic acid route to δ-*N*-acetyl-δ-*N*-hydroxyornithine provided a simple entry to the synthesis of rhodotorulic acid (Scheme III).[34] Modification of this synthetic process led to the preparation of related but unsymmetrical diketopiperazines and to the eventual total synthesis of foroxymithine (**3**),[35] a siderophore with noted angiotensin converting enzyme (ACE) inhibitory activity. These syntheses indicated that, indeed, it is possible to prepare the essential siderophore components in optically pure form and to incorporate the components into siderophore-like structures. With this demonstrated, two key points remained to be considered for the design and synthesis of δ-*N*-acetyl-δ-*N*-hydroxyornithine-based siderophore–drug conjugates: (1) the minimal structural requirements for siderophore recognition and transport and (2) the type of antimicrobial agents and linkers that should be attached to the siderophores or their components.

The minimal structural requirement concern was addressed by synthesizing the parent monomeric δ-*N*-acetyl-δ-*N*-hydroxy-L-ornithine (**31**), the corresponding linear dipeptide **34**, tripeptide **35**, and related extended peptides and determining which would serve as microbial growth stimulants in iron-deficient media.[30] The



parent monomeric δ-*N*-acetyl-δ-*N*-hydroxyornithine (**31**) demonstrated no siderophore-like growth promotion in limited assays. Interestingly, linear dipeptide **34** also was not a growth promoter even though the corresponding diketopiperazine, rhodotorulic acid, is a siderophore. However, zwitterionic tripeptide **35** was nearly as effective a siderophore as ferrichrome (**1**), a neutral cyclic hexapeptide. Thus, consistent with previous studies,[8,9] the most important aspect of recognition and transport appears to be the metal chelation site. Extension of the synthesis and testing of peptide derivatives **36–39** of the linear trimer **35** also reinforced this concept. All of the peptides containing the tri-δ-*N*-acetyl-δ-*N*-hydroxyornithine sequence displayed effective siderophore activity. Therefore, tri-δ-*N*-acetyl-δ-*N*-hydroxyornithine was chosen as the siderophore component in the design of siderophore–antimicrobial agent conjugates. The second point regarding the nature of the antimicrobial agent and its linkage to the peptide-based siderophore components was addressed next.

Initial Design and Syntheses of Siderophore–Antibiotic Conjugates

Since the naturally occurring siderophore antibiotic albomycins (**3**) contain a direct peptide linkage of the

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(22) (a) Maurer, P. J.; Miller, M. J. *J. Am. Chem. Soc.* **1983**, *105*, 240. (b) Maurer, P. J.; Miller, M. J. *J. Org. Chem.* **1981**, *46*, 2835.
(23) (a) For an extensive list, see refs 52–70 in the following: Miller, M. J. *Chem. Rev.* **1989**, *89*, 1563. (b) Matzanke, B. F. In *Handbook of Microbial Iron Chelates*; Winkelman, G., Ed.; CRC: Boca Raton, FL, 1991; pp 15–64.
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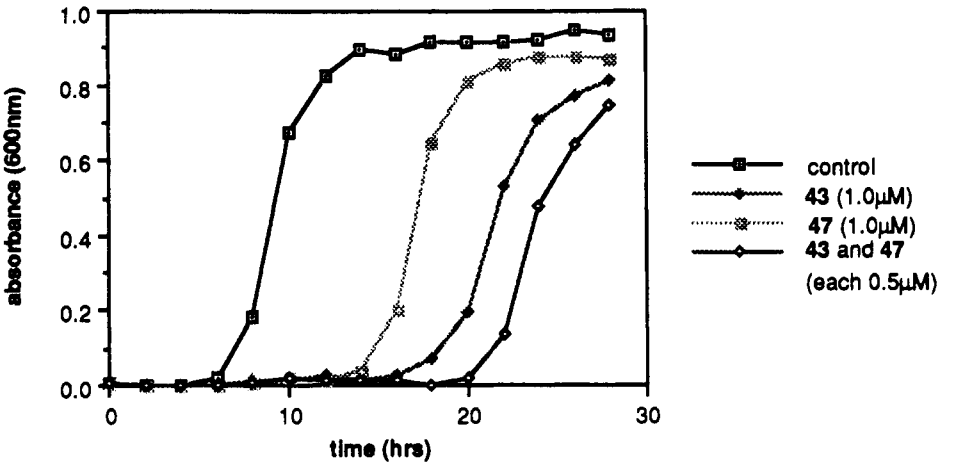

Figure 4. Growth curves of *E. coli* X580 in luria broth in the absence (control) and presence of synthetic siderophore-antibiotic conjugates.

siderophore component to the toxic thioribosyl derivative, peptide siderophore–drug linkages were considered first, with the realization that they might limit the potential and perhaps required release of the drug once the conjugate was transported into a cell. With this in mind, the first choice of the drug was a β-lactam antibiotic since much is known about their mode of action and because they only need to cross the outer cellular membrane of Gram-negative organisms to affect their cytoplasmic membrane-bound peptidoglycan-synthesizing enzyme targets. Moreover, the specificity of iron-assimilation systems in Gram-negative bacteria resides on selective receptors for ferric complexes located on the outer membrane. One concern was that use of the usual sulfur-containing penicillin or cephalosporin derivatives would be incompatible with precedented (Schemes I–III) hydrogenolytic debenzylations of the siderophore component of the conjugates. Fortunately, we were able to obtain a sample of a carbacephalosporin nucleus (**40**), a new class of potent nonsulfur-containing β-lactams,[36] from Eli Lilly and Company. The D-phenylglycyl derivative, Lorabid (**41**), is an especially potent antibiotic which received FDA approval for marketing in 1992.

Tetrapeptides **37** and **38**, with C-terminal phenylglycyl and (*p*-hydroxyphenyl)glycyl residues, typical amino acid side chains of carbacephalosporins, were prepared to determine if attachment of these relatively hydrophobic residues would negate the siderophore activity. Both **37** and **38** were found to be effective siderophores. Protected tri-δ-*N*-acetyl-δ-*N*-hydroxyornithine was then separately coupled to previously prepared carboxyl-protected phenylglycyl and (*p*-hydroxyphenyl)glycyl carbacephalosporins **41** and **42**. Initial standard hydrogenolytic deprotection conditions not only removed the protecting groups but also reduced the essential double bond and removed the chloride substituent in the carbacephalosporin component. This overreduced conjugate had no antimicrobial activity in any of the subsequent biological studies. In fact, it was an effective siderophore (microbial growth promoter), indicating that the phenylglycyl carbacephalosporin nucleus could be assimilated by the iron transport system. Selective hydrogenolysis under acidic conditions gave the desired siderophore–β-lactam antibiotic conjugates (**43** and **44**).[37] The initial results from standard, broad antimicrobial screens of the conjugates were disappointing. Except for a few organisms, the compounds appeared to be devoid of antimicrobial activity even though the parent antibiotic, Lorabid (**41**), displayed remarkable activity in the same assays.

Since bacterial resistance to the albomycins (**3a–c**) is known to occur rapidly by selection or development of strains that are defective in hydroxamate-based iron transport, the development of a similar problem with the synthetic β-lactam conjugates was considered. Thus, a β-lactam hypersensitive strain of bacteria (*Escherichia coli* X580) was chosen for a more detailed study and revealed fascinating results (Figure 4). Careful monitoring of the growth of the organism for up to 36 h indicated that while growth did eventually occur in cultures containing **43**, significant delay of the onset of growth was reproducibly noted. Reincubation of the organisms that eventually did grow in the same culture medium with conjugate **43** resulted in no delay of growth. This suggested that the new organisms were resistant mutants selected from the initial *E. coli*. Furthermore, under iron-deficient conditions, attempted promotion of growth of the new resistant organisms by ferrichrome or related peptide **31** failed, suggesting that the resistance of the mutant was due to the absence of the outer membrane receptor or transport proteins for hydroxamate-based siderophores.

Presumably, the hydroxamate conjugate-resistant strain (HCRS) of *E. coli* utilized non-hydroxamate siderophores to assimilate iron. A separate study determined that enterobactin (**6**) and various other synthetic catechol siderophores stimulated growth of the HCRS under iron-deficient conditions. Thus, analogous catechol-based antibiotic conjugates were anticipated to be effective inhibitors of the HCRS. However, neither enterobactin, the most generally studied catechol siderophore, nor the natural spermidine-based catechol siderophores agrobactin (**7**) and parabactin (**8**) have peripheral functionality for appropriate drug conjugation. Fortunately, reported syntheses of the spermidine-based siderophores and

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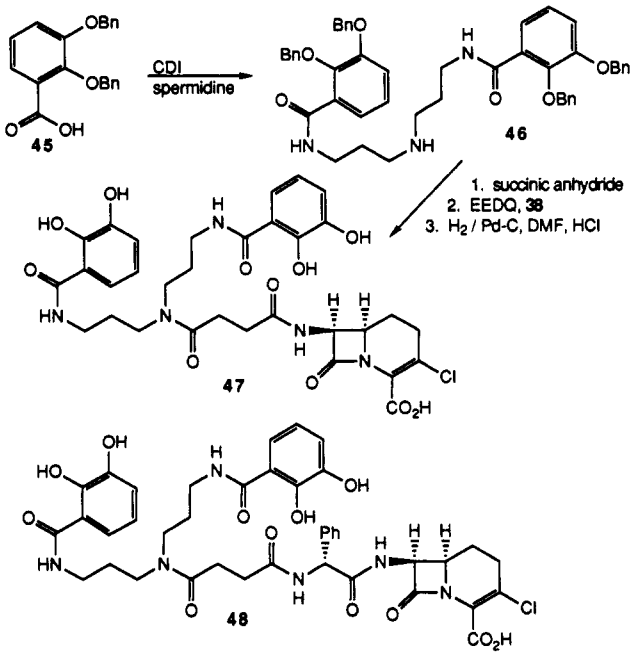
analogs[38-40] were compatible with preparation of drug conjugates. The first catechol-containing carbacephalosporin conjugate (**47**) was designed after the spermexatols, synthetic siderophore analogs with effective microbial growth promotion abilities.[41] To facilitate



the initial syntheses, no spacer or amino acid side chain was incorporated in the design. In fact, **47** contains only two catechol units rather than the usual three bidentate groups common to siderophores. The synthesis of **47** was effected with the key last step again being the careful hydrogenolytic deprotection.[41] As anticipated, incubation of the previously described HCRS *E. coli* with conjugate **47** resulted in inhibition of growth. Moreover, similar addition of **47** to cultures of the original *E. coli* X580 again resulted in delay of growth (Figure 4) with apparent selection of new mutants resistant to the new conjugate and related catechol-based siderophores.

Simultaneous incubation of the parent strain of *E. coli* X580 with both the catechol (**47**) and hydroxamate (**43**) conjugates was also studied.[42] Half the concentration (0.5 μM) of each conjugate was used relative to the separate studies so that the total concentration of β-lactam remained the same. As shown in Figure 4, enhanced antibacterial activity was noted by further delay of growth, as expected. The mutants which eventually did grow apparently lack both the hydroxamate and catechol siderophore receptors needed for uptake of conjugated drugs.

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These preliminary studies suggested that attachment of β-lactam antibiotics to siderophores allows bypassing of the usual porin route and promotes active iron transport-mediated delivery of the antibiotics. It was not yet known if the antibiotics were somehow released from the siderophore component after transport. The rapid selection of resistant strains gave rise to several concerns. First, it was clear that routine MIC (minimum inhibitory concentration) studies, which involve evaluation of inhibition of growth only after a given time, may miss compounds active against parent microbial strains, and thus, many potential lead compounds may not be detected. Second, studies of the detailed mode of action of the conjugates were imperative. Finally, it was important to determine if the siderophore–antibiotic conjugate resistant strains were pathogenic to mammals, since this would compromise the value of the concept of iron transport-mediated drug delivery.

Mode of Action of Siderophore–β-Lactam Antibiotic Conjugates

Both conjugates **43** and **47** generated large zones of inhibition against *E. coli* X580 in separate agar diffusion tests.[43] The resistant strains selected from individual exposure of the *E. coli* to **43** and **47** displayed no cross resistance. The separate affinity of conjugates **43** and **47** for isolated inner membrane penicillin binding proteins (PBPs: the β-lactam sensitive enzymes involved in cell wall biosynthesis) was determined by a competitive assay with ^{125}I-labeled penicillin V.[44] In this manner, hydroxamate conjugate **43** was found to primarily target PBPs 1A/B and 3 while catechol conjugate **47** interacted mostly with PBPs 1A/B and 5/6. The inhibitory activity of each of the conjugates was lost when a β-lactamase-encoding plasmid was introduced into *E. coli* X580. Outer membrane protein (Omp) profile analyses revealed that the hydroxamate conjugate resistant strain (HCRS) lost the expression of FhuA and sensitivity to phages T1 and T5. FhuA is a 78-kDa outer membrane protein used by *E. coli* to recognize ferrichrome and other hydroxamate-based siderophores, even though it does not synthesize ferrichrome.[45] FhuA is also the receptor used by bacteriophages T1, T5, and *ϕ80*. The catechol conjugate resistant strain (CCRS) lost the expression of Cir (74 kDa) and sensitivity to colicin Ia. Cir and Fiu are inducible in response to iron limitation.[46] Although their function is uncertain, these proteins seem to be implicated in a TonB-dependent transport of ferrimonocatechols, a function that could be associated with the recapture of the hydrolytic products of enterobactin. Cir is also the receptor for colicin Ia. This demonstrated the requirement of FhuA and Cir, respectively, for the inhibitory activity of hydroxamate conjugate **43** and catechol conjugate **47**. Interestingly, antibiotic diffu-

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sion assays indicated that 1 μM ferrichrome strongly antagonized the activity of both conjugates against the parent *E. coli* X580 and CCRS, suggesting a possible additional role of FhuA in the activity of catechol conjugate **47**. This was reaffirmed in studies of a double mutant (FhuA-Cir-) which showed a higher level of resistance to catechol conjugate **47**. The susceptibility of the HCRS, lacking the ferrichrome receptor (FhuA-) for the two conjugates, was unchanged in the presence of ferrichrome. *Thus, it was clear that the siderophore–antibiotic conjugates were utilizing iron transport mechanisms and doing so, in the case of 47, by use of two distinct receptors for entry into cells.*

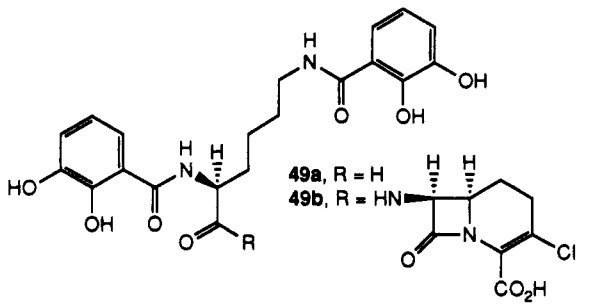
To determine whether the virulence of the conjugate-resistant strains of *E. coli* was altered, *E. coli* X580 and HCRS were cultivated in diffusion chambers implanted in the peritoneal cavity of rats to reproduce iron-restricted in vivo growth conditions. The growth of HCRS was greatly impaired in this cultivation system compared to the parent strain. Furthermore, the bacteria recovered after a 5-h incubation in rats was susceptible to hydroxamate conjugate **43**, suggesting that the resistant organism was not viable, hence probably less virulent in vivo, and only reversion to the parent strain, containing a full complement of iron assimilation mechanisms, allowed growth. Such a reversion would not be detrimental therapeutically since the revertants containing a hydroxamate transport system would be susceptible to the hydroxamate conjugate.

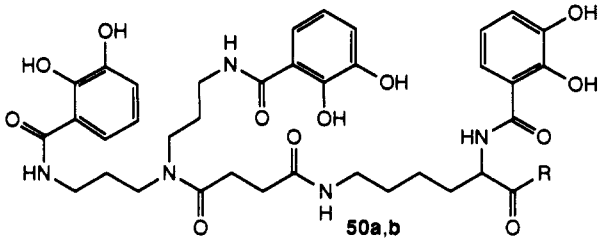
Extended Design and Synthesis of Siderophore–Drug Conjugates

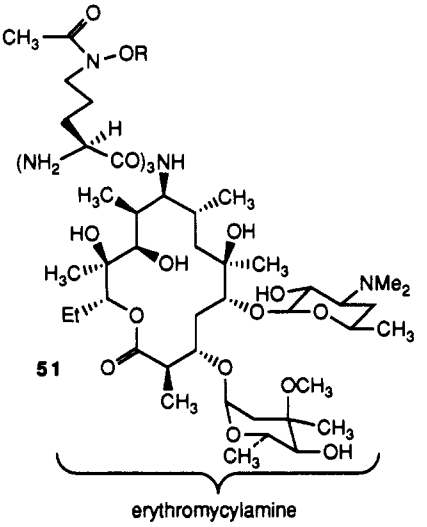
The biological studies of the initial siderophore conjugates prompted synthesis of catechol-containing conjugate **48**, which incorporated the phenylglycyl side chain of the carbacephalosporin.[47] At 10 μM conjugate **48**, complete inhibition of the growth of *E. coli* X580 was observed. At 1 μM, selection of resistant strains was observed again. Detailed studies of these resistant strains have not yet been completed.

The natural bis catechol-containing siderophore bis-(2,3-dihydroxybenzoyl)-L-lysine (**49a**), a presumed siderophore, was isolated from *Azotobacter vinelandii*.[48] Slight modification of the reported synthesis of **49a** and analogs[49] facilitated preparation of carbacephalosporin conjugate **49b**.[50] As observed with conjugate **47**, incubation of *E. coli* X580 and **49b** resulted in significantly delayed growth relative to a control and apparent selection of resistant mutants.

Combination of components of **47** and **49b** led to the synthesis[50] of tricatechol **50a** and conjugate **50b**. In contrast to the previous conjugates, preliminary studies of the incubation of tricatecholate conjugate **50b** with *E. coli* X580 indicated no growth inhibition. In fact, conjugate **50b** and the tricatechol **50a** were both found to be effective *growth promoters* of *E. coli* X580 and two isogenic *E. coli* strains, RW193 and RWB18.[51]







RW193 is capable of using either hydroxamate or catechol siderophores, but does not synthesize enterobactin. RWB18 lacks an outer membrane protein implicated in ferrienterobactin uptake. These results again suggest that the enterobactin receptor is not necessary for uptake of the spermidine-based catechol complexes. More study with *E. coli* and other organisms is required to determine if lack of activity of **50b** is general or species and strain selective, if the decreased solubility or the increased bulk of **50b** is a factor, or if a drug release mechanism is required.

With the indication that some carbacephalosporin conjugates of siderophores may be effective antimicrobial agents, attention was turned to conjugates of drugs that differ from β-lactams structurally and in their mode of action. Erythromycin, a macrolide antibiotic, was chosen since it exerts an antimicrobial effect within cells by interaction with ribosomal RNA,[52] and the preparation of conjugates was anticipated to test the ability of siderophores to fully transport diverse structures intracellularly. Erythromycylamine has an antimicrobial profile similar to that of erythromycin and provided a convenient amine nucleophile for direct

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linkage to the protected tri-δ-*N*-hydroxy-δ-*N*-acetyl-L-ornithine peptide **35** used earlier. Thus, routine coupling and hydrogenolytic deprotection provided conjugate **51**.[53] In initial standard broad antimicrobial screening, conjugate **51** was inactive. However, detailed studies using *E. coli* K-12 indicated that **51** is effective and is specifically recognized and transported by cells containing the ferrichrome receptor TonA (FhuA).[54] This especially remarkable result, in which the drug is larger than the siderophore carrier, may be consistent with a recently postulated gated-porin mechanism.[55]

Future Considerations and Conclusions

This Account has focused on the development of antibacterial conjugates. Perhaps even more important will be the design of species-selective antifungal agents. Immunocompromised patients, such as those with AIDS, are very susceptible to opportunistic fungal infections with strains of *Candida*, *Cryptococcus*, *Pneumocystis*, *Histoplasma*, *Toxoplasma*, *Aspergillus*, and related organisms. Until cures for AIDS and other immunocompromising diseases are found, considerable attention must be given to treating these often fatal opportunistic infections. Iron transport-mediated drug delivery offers a unique possibility to develop species-selective antimicrobial agents to address this problem. A number of reports suggest that catechols and hydroxamates are generated and utilized by *Candida* as siderophores.[56] However, no structural details are known and no assay for siderophore recognition and transport in *Candida* was available. Utilizing the variety of natural and unnatural siderophores and components available from our antibacterial conjugate studies, we recently developed a bioassay for siderophore utilization by *Candida albicans*.[57] Interestingly, the assay indicated that enterobactin and the other catechol siderophores and components described throughout this Account were ineffective; however, ferrichrome, the constituent tri-δ-*N*-hydroxy-δ-*N*-acetyl-L-ornithine (**35**), and several of the extended peptides served as candidal siderophores. The design and synthesis of related antifungal–siderophore conjugates is in progress.

Overall, the synthetic and biological studies described in this Account demonstrate that the concept of iron transport-mediated drug delivery has potential. Furthermore, the conjugates may serve as valuable biological tools for the elucidation of important aspects of microbial iron metabolism by the selection of strains lacking various iron-assimilation machinery. The selection of these "resistant" strains had been considered a major negative factor related to the development of siderophore-mediated drug delivery since early studies with albomycin. The single study indicating that the HCRS selected upon incubation of *E. coli* with conjugate **43** was not pathogenic in vivo needs to be expanded. The prospect of detailed studies of structure–activity relationships of siderophore–drug conjugates holds considerable promise for the development of new therapeutic agents.

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