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Mini review

Synthetic siderophores as biological probes

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Summary. Synthetic molecules that mimic the properties of the natural siderophores promise to become powerful tools in the exploration of microbial iron(III)-uptake phenomena. Such molecules can serve as probes to (i) establish the essential structural requirements for biological action, (ii) trace alternative reaction pathways and (iii) compare receptors of different biological origins. In this article a series of synthetic ferrichrome analogs will be described. The strategy adopted for the design and synthesis of these compounds will be outlined and their properties in vitro and in vivo examined. The growth promotion activity of these compounds in *Arthrobacter flavescens* is used to map the ferrichrome receptor surface. Their activities towards *Zea mays* allow us to trace the plants' reductive iron(III) uptake routes. Potential applications of modified ferrichrome analogs for the isolation of ferrichrome receptors, the generation of fluorescent probes and ultimately new families of antibiotic or antifungal agents, will also be indicated.

Key words: Iron-upake — Siderophores — Ferrichrome — Biomimetic siderophores — *Arthrobacter flavescens* — *Zea mays*

Introduction

Vital life processes are governed by specific interactions between molecules, such as those occurring between enzymes and substrates, hormones and receptors, antigens and antibodies. Much research effort has therefore been devoted by biologists, chemists and physical chemists to identify the forces that dictate molecular recognition. Powerful physical techniques such as X-ray and NMR, coupled with computational methods, aided in these investigations and provided structural information on the most complex biomolecules. They also stimulated chemists to reproduce some of the structural characteristics of the natural compounds with synthetic molecules and thereby reproduce the capability of molecular recognition (Cram 1988; Lehn 1988; Kellogg 1982, 1984; Rebek 1987). A new branch of chemistry thus emerged; the branch of biomimetic chemistry (Breslow 1980). Biomimetic chemistry aims to simulate biological processes with synthetic chemical tools. It relies on identifying the essential structural features of the biological systems and on incorporating these very features into the simplest possible molecules.

Microbial iron uptake, and specifically siderophore-mediated iron uptake (Neilands 1984; Raymond et al. 1984; Hider 1984), appears eminently suited to be studied by the tools of biomimetic chemistry. Siderophores are low-molecular-mass iron(III) chelators that are excreted by microorganisms under iron-deficient conditions in order to bind and transport iron from the environment into the cell (see Fig. 1). Siderophore-mediated iron(III) uptake, as elaborated below, is in essence governed by chemical recognition.

For an iron(III) carrier to be biologically active it has to meet two requirements: (i) to bind iron(III) specifically and (ii) to interact favorably with membrane receptors (Fig. 2). This implies the presence of a double-recognition phenomenon: iron binding and receptor matching. Synthetic models that simulate the performance of the natural iron(III) carriers may therfore provide information on the requirements for effetive siderophore–receptor interactions and make available

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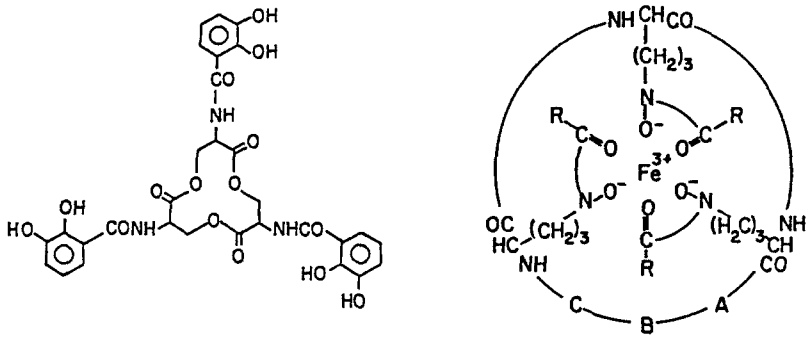


Fig. 1. Representative examples of siderophores. Enterobactin *(left)* and the ferrichromes *(right; A, B, C* = Gly or Ser residues; $R = CH_3$, or $CHC(CH_3)CH_2CH_2OH$ or $CHC(CH_3)CH_2COOH$)

probes to trace the mechanism of siderophore-mediated iron uptake. The pioneering efforts in this field concentrated on the study of enterobactin, the most powerful natural iron(III) binder known. They aimed to identify the origin of enterobactin's outstanding binding and transport properties (Raymond and Carrano 1979; Shanzer et al. 1986) and led to the preparation of synthetic analogs (Harris and Raymond 1979; Tor et al. 1987a), some of which proved to facilitate iron(III) uptake into *Escherichia coli* (Raymond 1984; Ecker et al. 1986, 1988).

In this article we describe our approach towards synthetic ferrichrome analogs. We will first discuss the strategy adopted and the principles applied for the design and synthesis of these compounds. We then examine the activity of these iron(III) binders in two inherently different biological systems, *Arthobacter flavescens* and *Zea mays* (corn roots), while emphasizing their utility as probes, and conclude by briefly speculating on future possibilities.

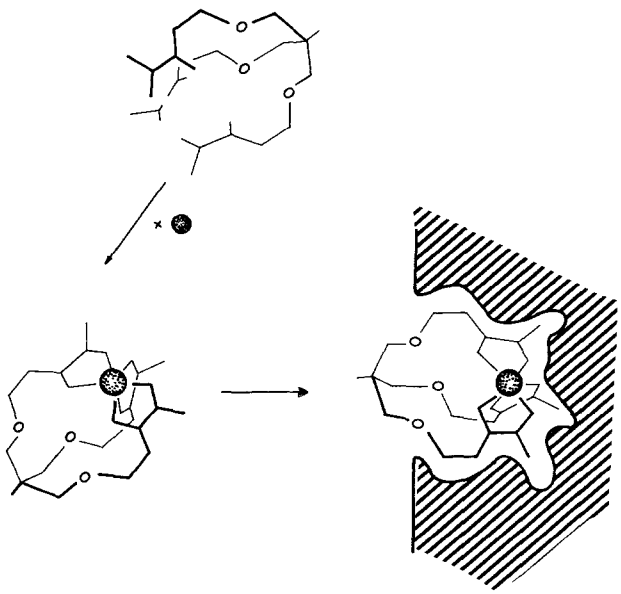


Fig. 2. Effective iron(III) binding and receptor binding as requirements for biological activity

Results and discussion

Ferrichrome was selected as a target siderophore since it is one of the best studied and offers a large opportunity for biological testing (Emery 1971; Leong et al. 1974; Leong and Neilands 1976; Matzanke et al. 1984; Adjimani and Emery 1988). Ferrichrome possesses a non-symmetric tripodal structure and is composed of a hexapeptide ring and three side chains that carry the hydroxamate-ion-binding sites. When binding iron(III), the latter create an octahedral ion-binding cavity, while the hexapeptide ring merely serves as an anchor. *A priori,* two coordination isomers are possible: either right- or left-handed. Since ferrichrome possesses chiral centers, the two isomers are diastereomeric and therefore not energetically equivalent nor equally populated. The left-handed complex is the predominant one in ferrichrome (Emery 1966; Van der Helm et al. 1980) and is the only active isomer. *Enantio*-Ferrichrome, which forms complexes of predominantly Δ-*cis* configuration, is inactive (Winkelmann 1979; Winkelmann and Braun 1981). The predominant *Λ* configuration is shared by all members of the ferrichrome family of siderophores, which all possess a non-symmetric tripodal structure, but differ in the constituents of the hexapeptide ring and the nature of the terminal groups (see Fig. 1).

For a synthetic molecule to simulate ferrichrome it has to fulfill two requirements. Firstly, it has to bind iron(III) specifically in a left-handed configuration and, secondly, it has to create an envelope prone to interact favorably with the ferrichrome receptor. The challenge of the latter task is further highlighted by the lack of structural information on the membrane receptors and implies the search for a key to a largely unknown lock.

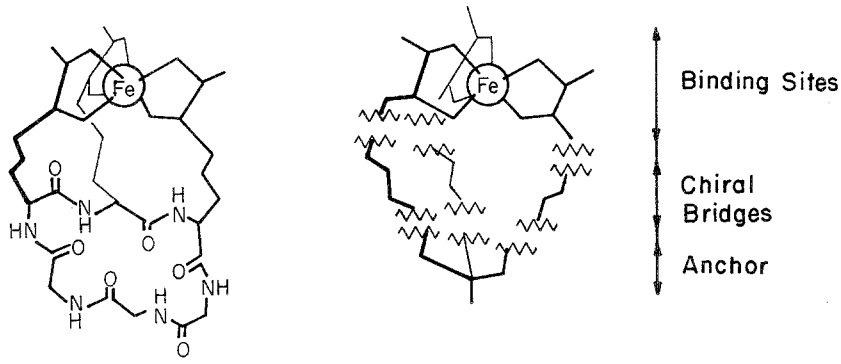


Fig. 3. Natural ferrichrome *(left)* and principle design of synthetic ferrichrome analogs *(right)*

We chose to assemble the ferrichrome analogs from three modular subunits: (i) a C_3-symmetric anchor, (ii) extending chiral bridges and (iii) terminating binding groups (Fig. 3). The substitution of the non-symmetric hexapeptide rings by C_3-symmetric residues was to facilitate synthesis while simultaneously generating a tripodal arrangement fit for octahedral iron(III) binding. The chiral information of the hexapeptide ring was transmitted into the bridges by using chiral amino acids. The latter were also used to direct the absolute configuration of the iron complexes formed towards the desired Λ configuration, and to allow systematic modifications of the molecules' spatial requirements by varying the nature of the amino acid used.

Following this principle two series of tripode-like hydroxamate binders (Fig. 4) were synthesized (Tor et al. 1987b; Shanzer et al. 1988). One was based on a trigonal amine as anchor, the second on a tetrahedral carbon. The synthesis of these binders was performed by condensation of the selected amino acid derivatives, hydroxamate derivatives, with the tripodal trisamine or triscarboxylate. All ligands were found to bind Fe^{3+} in a 1:1 stoichiometry, on the basis of ultraviolet titration experiments, and to form complexes of preferentially left-handed configuration when using L-amino acids, as revealed by their CD spectra[1]. The absolute configuration of these iron(III) complexes was thus identical with that of natural ferrichrome (Emery 1966; Van der Helm et al. 1980).

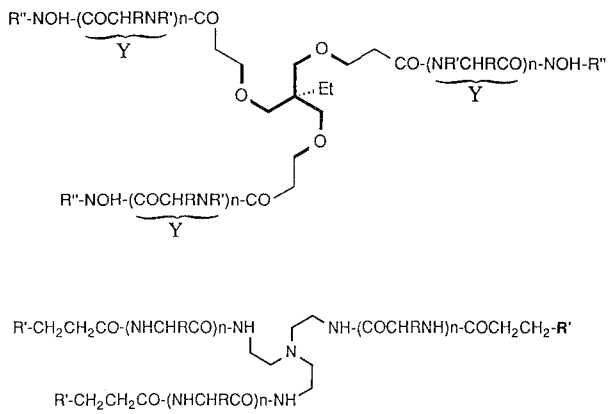


Fig. 4. Synthetic ferrichrome analogs: Carbon-based binders (*top*, Y = amino acid residues, Ile, Leu, Pro, Ala, Sar; $R'' = CH_3$ or H); nitrogen-based binders (*bottom*, $n = 0.1$; R = *i*Bu, $R'' = NOHCOC_4H_4OCH_3$, CONHOH)

In order to test the biological performance of these compounds, *Arthrobacter flavescens* was chosen as model (Burnham and Neilands 1960). This bacterium possesses ferrichrome receptors but does not produce ferrichrome itself. It is therefore completely dependent on externally added ferrichrome, or ferrichrome substitutes, for growth. Addition of the synthetic ferrichrome analogs to the culture medium of this bacterium and measurement of its resulting growth provides a sensitive indicator for the biological effectiveness of these compounds. While the nitrogen-based binders showed no appreciable activity, the carbon-based L-Leu derivative ($R'' = CH_3$) showed 1% of the growth-promotion activity of the natural ferrichrome. We therefore concentrated on the carbon-based derivatives. If the observed activity is of any significance, it should be possible to optimize these structures by further modifications.

Considering the fact that the ion-binding chains in ferrichrome do not possess substituents, we proceeded to reduce the bulkiness of the pro-

[1] Circular dichroism of iron hydroxamates is a most useful tool to determine the absolute configuration of hydroxamate complexes and also to obtain an estimate of their optical purity

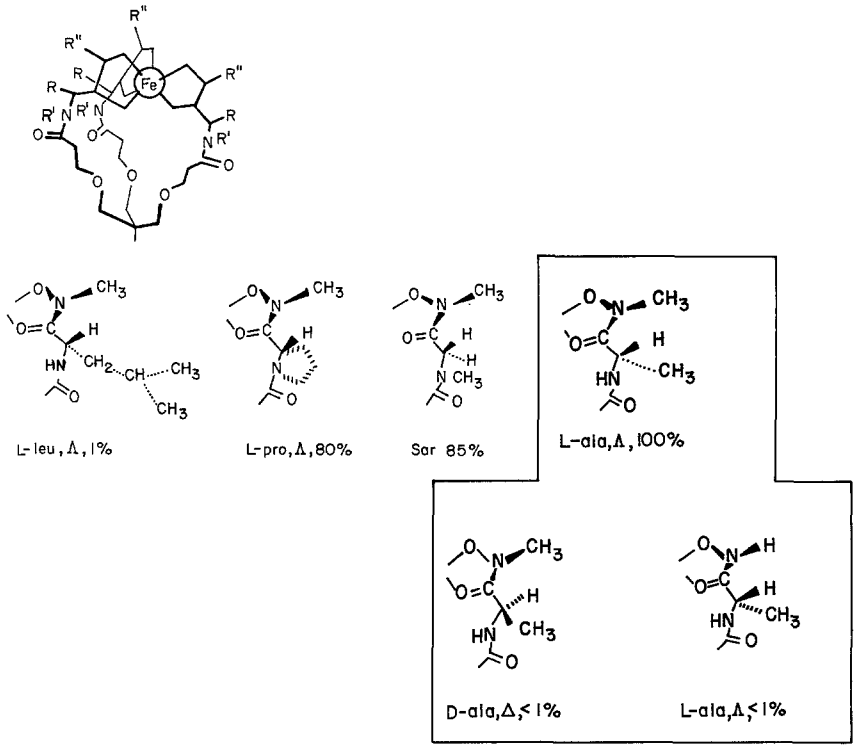
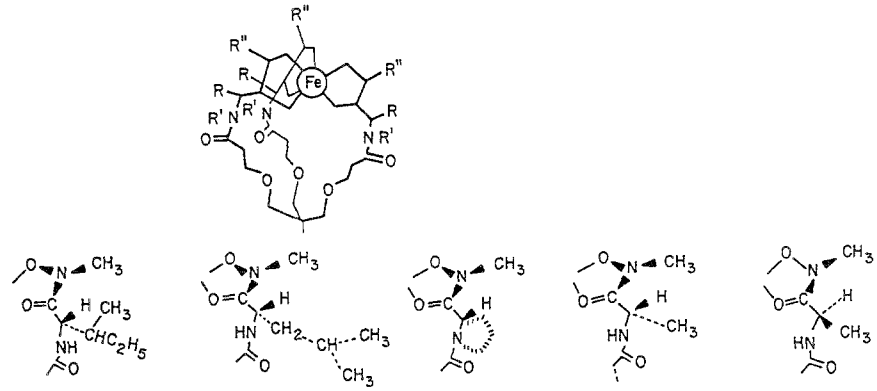


Fig. 5. Relative activity of synthetic ferrichrome analogs as growth promoters of *Arthrobacter flavescens* (% relative of ferrichrome)

jecting amino acids residues and started by replacing L-Leu by either L-Pro or Sar. And indeed, the activity increased from 1% to 80% and 85% respectively (Fig. 5). In order to establish whether the observed effect derived from the 'shrinking' of the amino acid, or from a conformational change caused by replacing secondary amide bonds by tertiary amide bonds, the L-Pro and Sar were substituted by L-Ala. The L-Ala derivative was found fully to equal ferrichrome in its growth-promotion activity towards *A. flavescens*. Although the details of the observed growth-promotion activity are still under investigation (Winkelmann, unpublished results), the considerations given below strongly support the involvement of a membrane receptor.

The higher activity of the L-Ala derivative relative to the more lipophilic L-Leu derivative excludes the possibility of mere diffusion. Moreover, the similar biological activity of the L-Pro and L-Ala derivative is in line with the observation that L-Ala has most frequently been found to replace L-Pro during the evolutionary process (Dayhoff et al. 1978). Further support for the occurrence of a receptor-driven process was obtained when the D-Ala derivative, which forms iron(III) complexes of Δ-*cis* configurations instead of *Λ-cis* configuration, was found to lack any activity. This pronounced chiral discrimination is analogous to that observed with ferrichrome, where the antipode of the natural isomer, *enantio*-ferrichrome, lacked any transport activity in fungi and showed reduced activity in *E. coli* (Winkelmann 1979; Winkelmann and Braun 1981). Moreover, replacement of the terminal methyl group in the synthetic L-Ala derivative by hydrogen decreased the molecule's effectivenes, as did analogous replacements of the terminal methyl group by hydrogen in *retro*-ferrichrome (Emery et al. 1984).

It is appealing to use the observed scale of activity for defining the domains relevant for recognition. Of major importance is the stereochemistry around the metal center, where *Λ* configuration is a prerequisite for biological activity and where a terminal methyl group is significantly superior to a terminal hydrogen. The lateral portion of the molecule allows fine tuning to match the respective biological receptor optimally. The anchor, on the other hand, seems to allow significant modifications. The nitrogen-based derivatives failed to provide active compounds, presumably due to their prevalence in the protonated form. These findings are in line with those reported for enterobactin (Ecker et al. 1986, 1988), where the exposed part of the iron(III)-binding region was suggested to be of relevance for biological activity.



L-ileu, Λ	L-leu, Λ	L-pro, Λ	L-ala, Λ	D-ala, Λ
rel. Fe^{3+} 1.5	1.3	1.2	1.5	1.1
Uptake (blank = 1)				
rel. Ga^{3+} 0.92			1.05	
Uptake (blank = 1)				

Fig. 6. Relative activity of synthetic ferrichrome analogs as ion carriers into corn roots

With a systematic series of biomimetic ferrichrome analogs at hand, we have produced a 'kit' that offers itself as a probe to examine iron-uptake mechanisms in other biological systems. We chose to examine this possibility on *Zea mays* (corn roots), which has been shown to make effective use of ferrichrome as an iron(III) carrier in a highly specific iron(III)-uptake process (Crowley 1987).

Iron-uptake experiments (using radioactive $^{59}Fe^{3+}$) established facilitated iron(III) uptake with the L-Ile and L-Ala derivatives, and to a smaller extent with the corresponding L-Leu and L-Pro derivatives (Ganemore-Neumann et al., unpublished results). In order to establish whether iron(III) uptake by the synthetic compounds is specific and involves the same pathway as that promoted by ferrichrome, we tested its sensitivity to stereochemical effects. Most informatively, the D-Ala derivative proved practically inactive (Fig. 6). This pronounced chiral discrimination in favor of the left-handed isomer supports the involvement of a highly specific chiral recognition site.

A priori, the observed high chiral discrimination may imply either the involvement of (i) a chiral receptor or (ii) a highly stereoselective enzyme. A plausible candidate would be a reductive enzyme. In order to examine this possibility, $^{59}Fe^{3+}$ was replaced by $^{69}Ga^{3+}$ and its uptake monitored. Gallium(III) simulates iron(III) in its coordination to hydroxamate binders and forms hydroxamate complexes of the same overall shape (Borgias et al. 1986). However, gallium(III) hydroxamates can not undergo reduction and can therefore only be taken up via non-reductive processes. None of the most active synthetic iron(III) carriers facilitated gallium(III) uptake. This result suggests that the synthetic-ferrichrome-mediated iron(III)-uptake processes involve as stereoselective enzymatic reduction step.

Conclusions

Synthetic models provide powerful tools for elucidating the intricate mechanisms of microbial iron uptake and assimilation. The biomimetic approach is eminently suited for this task as it provides ever better models through a series of reiterative steps from design and synthesis to biological testing. Should elements other than those initially considered prove relevant, they may be easily introduced by modifications of the model.

The biological activity of the synthetic ferrichrome analogs indicates that there can be more than a single structural solution to fit a given biological receptor. Moreover, it led to a method for distinguishing between two types of molecular domains: those that are responsible for the bonding interactions with the receptor sites and those that seem to be exogeneously positioned and of little importance for recognition. The conservative structural features of the bonding domains allow us to map the surface of biological receptors by applying the principle of complimentary. The almost non-interacting exogeneous domains, on the other hand, lend themselves to chemical modifications in order to gain specific advantages. Attractive possibilities include the attachment of labels that would allow us to probe the iron-uptake pathway, or the anchoring to polymeric supports that would enable membrane receptors to be isolated by affinity chromatography. Considering the high specificity of some of these receptor-driven

iron-uptake processes, synthetic siderophores might well be used as 'Trojan horses' for the introduction of toxic antimetabolites.

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References

Adjimani JP, Emery T (1988) Structural aspects of iron transport in *Mycelia sterilia* EP. 76. J Bacteriol 170:1377–1379

Borgias BA, Barclay SJ, Raymond KN (1986) Structural chemistry of gallium(III). J Coord Chem 15:109–123

Breslow R (1980) Biomimetic control of chemical selectivity. Acc Chem Res 13:170–177

Burnham BF, Neilands JB (1960) Studies on the metabolic function of the ferrichrome compounds. J Biol Chem 236:554–559

Cram DJ (1988) The design of molecular hosts, guests and their complexes. Science 240:760–767

Crowley DE, Reid CPP, Szaniszlo PJ (1987) Microbial siderophores as iron sources for plants. In: Winkelmann G, van der Helm D, Neilands JB (eds) Iron transport in microbes, plants and animals. pp 375–386

Dayhoff MO, Schwartz RM, Orcutt BC (1978) In: Dayhoff MO (ed) Atlas of protein sequence and structure, vol 5, Suppl 3. National Biomedical Research Foundation, pp 345–352

Ecker DJ, Matzanke BF, Raymond KN (1986) Recognition and transport of ferric enterobactin in *Escherichia coli.* J Bacteriol 167:666–673

Ecker DJ, Loomis LD, Cass ME, Raymond KN (1988) Substituted complexes of enterobactin and synthetic analogues as probes of the ferric-enterobactin receptor in *E. coli.* J Am Chem Soc 110:2457–2464

Emery T (1966) Initial steps in the biosynthesis of ferrichrome. Incorporation of $^{\delta}N$-hydroxyornithine and $^{\delta}N$-acetyl $^{\delta}N$-hydroxyornithine. Biochemistry 5:3694–3701

Emery T (1971) Role of ferrichrome as a ferric ionophore in *Ustilago spherogena.* Biochemistry 10:1483–1488

Emery T, Emery L, Olsen RK (1984) Retrohydroxamate ferrichrome; A biomimetic analogue of ferrichrome. Biochem Biophys Res Commun 119:1191–1197

Harris WR, Raymond KN (1979) Ferric ion sequestering agents, 3. J Am Chem Soc 101:6534–6541

Hider RC (1984) Siderophore mediated absorption of iron. Struct Bonding 58:25–87

Kellogg RM (1982) Bioorganic modelling. Top Curr Chem 101:111–145

Kellogg RM (1984) Chiral macrocycles as reagents and catalysts. Angew Chem Int Ed Eng 23:782–794

Lehn J-M (1988) Supramolecular chemistry — scope and perspectives. Angew Chem 100:91–116

Leong J, Neilands JB, Raymond KN (1974) Coordination isomers of biological iron transport compounds III. Biochem Biophys Res Commun 60:1066–1071

Leong J, Neilands JB (1976) Mechanisms of siderophore iron transport in enteric bacteria. J Bacteriol 126:823–830

Mutzanke BF, Muller GI, Raymond KN (1984) Hydroxamate siderophore mediated iron uptake in *E. coli*; stereospecific recognition of ferric rhodotorulic acid. Biochem Biophys Res Commun 121:922–930

Neilands JB (1984) Methodology of siderophores. Struct Bonding 58:1–24

Raymond KN, Carrano CJ (1979) Coordination chemistry and microbial iron transport. Acc Chem Res 12:183–190

Raymond KN, Mueller G, Matzanke BF (1984) Complexation of iron by siderophores. A review of their solution and structural chemistry and biological function. Top Curr Chem 123:49–102

Rebek J (1987) Model studies in molecular recognition. Science 235:1478–1484

Rebek J (1988) Recent progress in molecular recognition. Top Curr Chem 149:189–210

Shanzer A, Libman J, Lifson S, Felder CE (1986) Origin of the Fe^{3+}-binding and conformational properties of enterobactin. J Am Chem Soc 108:7609–7619

Shanzer A, Libman J, Lazar R, Tor Y, Emery T (1988) Synthetic ferrichrome analogues with growth promotion activity for arthobacter flavescens. Biochem Biophys Res Commun 157:389–394

Tor Y, Libman J, Shanzer A, Lifson S (1987) Biomimetic ferric ion carriers. A chiral analog of enterobactin. J Am Chem Soc 109:6517–6518

Tor Y, Libman J, Shanzer A (1987) Biomimetic ferric ion carriers. A chiral analog of enterobactin. J Am Chem Soc 109:6518–6519

Van der Helm D, Baker JR, Eng-Wilmot DL, Hossain MB, Loghry RA (1980) Crystal structure of ferrichrome and a comparison with the structure of ferrichrome A. J Am Chem Soc 102:4224–4231

Winkelmann G (1979) Evidence for stereospecific uptake of iron chelates in fungi. FEBS Lett 97:43–46

Winkelmann G, Braun V (1981) Stereoselective recognition of ferrichrome by fungi and bacteria. FEMS Microbiol Lett 11:237–241

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