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Receptor mapping with artificial siderophores

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Abstract - In order to guarantee adequate supply of iron, which is essential for growth, microbial organisms have developed ingenious vehicles, the siderophores. These low molecular weight compounds bind and transport iron from the environment into the cell in a receptor driven mechanism. In an attempt to simulate the properties of one of the siderophoresferrichrome, with artificial binders, tripod-like hydroxamates that bind iron(III) in the same configuration as the natural counterpart with equal growth promotion activity were prepared. *In vivo* tests of these compounds established that three of them approach and even equal the activity of genuine ferrichrome as growth promoters of *Arthrobacter flavescens*. *A. flavescens* is completely dependent on ferrichrome for growth and previously failed to respond to any synthetic compounds. The biomimetic iron carriers described here are thus the first examples that are active towards this mutant and are taken up by a receptor driven mechanism. The implications of these findings in mapping the surface of siderophore receptors are discussed.

INTRODUCTION

Model building with synthetic structures is a valuable method to examine biological phenomena at a molecular level and deduce the underlying principles. Models can serve as "sounding boards" to differentiate between the essential and the superfluous, between the understood, and the still obscure. They enable systematic development of ever superior synthetic analogs till optimal match with the biological systems is reached.

This approach proved successful in the study of ionophore mediated ion transport through biological membranes [1]. It led to the synthesis of artificial ion carriers [2,3], that similarly to the natural counterparts, are capable to selectively bind and transport specific metal ions through lipid membranes. The success of the biomimetic approach in the study of ionophores encouraged us to apply it to a related, but more complex problem, the problem of microbial iron uptake [4].

Continuous supply of iron is essential for the maintenance of living systems. Yet, adequate iron supply is hindered by the low solubility of iron hydroxide in water and by the negligible permeability of cell membranes to charged species. In order to overcome this problem, nature has devised ingenious chemical vehicles, siderophores (Fig. 1), that selectively bind and transport iron from the environment into the cell.

Siderophore mediated iron uptake is not a diffusion controlled process (like ionophore mediated ion uptake), but involves a series of steps that necessitate specific interactions with membranal proteins and membranal receptors [5]. This iron uptake mechanism has been shown to be highly specific and only the natural isomers, but not their enantiomers are active. In the case of enterobactin it is the natural right-handed complex (Δ-cis) that is recognized, while in the case of ferrichrome the left-handed one (Λ-cis) [6].

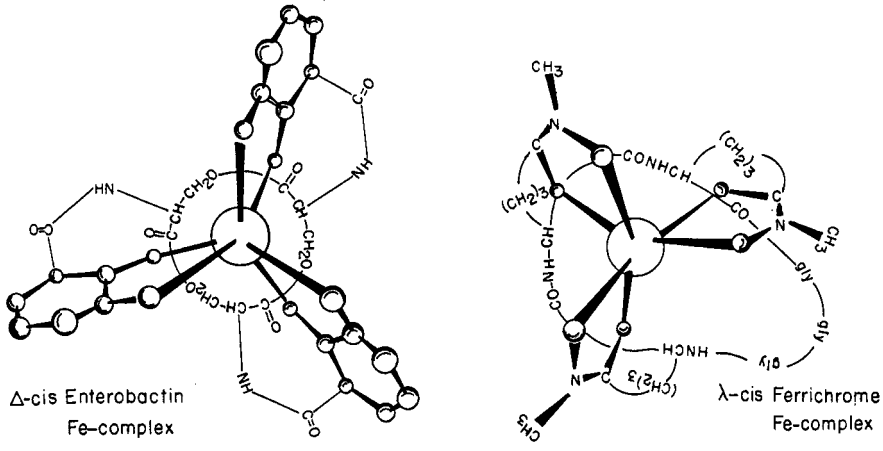

Fig. 1. Examples of natural ferric ion carriers (siderophores).

Cellular iron uptake with artificial molecules necessitates not only the creation of a polar interior to guarantee iron binding, but also simulation of its envelope to allow recognition. In this article we wish to describe a family of chiral, synthetic ferric ion binders that simulate the iron binding and transport properties of ferrichrome both, *in-vitro* and *in vivo*. The *in-vivo* activity of the ferrichrome analogs was measured by their iron induced growth promotion activity towards *Arthrobacter Flavescens*. *Arthrobacter flavescens* possesses a sole receptor for ferrichrome but does not synthesize ferrichrome [7]. It therefore completely depends on externally added ferrichrome for iron uptake and growth.

DESIGN, SYNTHESIS AND PHYSICOCHEMICAL PROPERTIES OF BIOMIMETIC FERRICHROMES

A synthetic molecule has to fulfill several requirements in order to mimic the ion binding and transport properties of genuine ferrichrome: (*i*) form an octahedral ion binding cavity that is suitable for ferric ions, (*ii*) adopt preferentially a left-handed configuration when binding iron [6], and (*iii*) fit to the surface of the microbial receptor.

Structurally, ferrichrome is composed of a hexapeptide ring and three pending side chains that bear each a bidentate hydroxamate group. In this structure the hexapeptide serves as anchor, while the pending side chains create the octahedral cavity suitable for binding iron. The hexapeptide ring may contain different combinations of glycine and serine residues as occurring in the ferrichrome related siderophores ferricrocine or ferrichrysine, but the location of the side arms and their binding sites appears invariable.

Considering the permitted variability of the hexapeptide ring in the ferrichrome family of siderophores, this domain was conceived of secondary importance and allow its replacement by different structures. The hexapeptide ring of ferrichrome was thus substituted by a C_3- symmetric triscarboxylic acid as anchor. C_3-symmetry is not uncommon in natural siderophores as exemplified by enterobactin, the most potent ferric ion binder. This substitution also greatly facilitates synthesis as it does not necessitate the tedious preparation of cyclic peptides. Extension of such readily available C_3-symmetric molecules by natural amino acids followed by hydroxamate groups was anticipated to provide artificial, tripod-like iron binders that could be systematically modified until optimal biological activity is reached (Fig. 2).
The use of amino acids of either the natural L- or unnatural D-configuration was conceived to allow control of the metal complex configuration as either *Λ*-cis or *Δ*-cis. In addition, variations of the nature of the amino acid, i.e. its side chain, was thought to enable systematic shaping of the molecules envelope. In these binders the directionality of the hydroxamate groups has intentionally been inverted relative to that in ferrichrome. This greatly facilitates synthesis, but was anticipated to be of little biological relevance, since the activity of the retroisomer of ferrichrome is indistinguishable from that of ferrichrome [8].

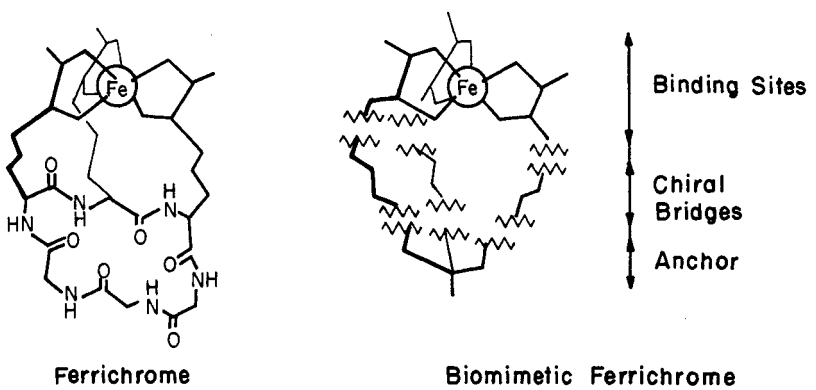

Fig. 2. Principle design of synthetic ferrichromes.

Following this principle design, a family of C_3-symmetric, tripod-like ferric ion binders was synthesized and examined.

The synthesis of these binders (Fig. 3) was accomplished according to schemes reported earlier [9]. It essentially involved coupling of the tricarboxylic acid anchor with the respective hydroxamato amines. Spectrophotometric titration of the ferrichrome analogs with $FeCl_3$ in aq. MeOH established 1:1 ligand : ion stoichiometry for all representatives. Their relative binding efficiencies were determined by competition experiments. Equimolar mixtures of achiral **1** and either chiral **2**, **3** or **5** were incubated with **1** equivalent of $FeCl_3$ in aq. MeOH and the percentage of chiral complex determined by CD. These measurements established relative binding efficiencies for **1** : **2** : **3** : **5** as being 1 : 0.8 : 0.3 : 1.1 respectively. The binding efficiency of these binders are thus all of the same order of magnitude, although there are some variations. Among the chiral binders, the derivatives of the secondary amides leu (**2**) and ala (**5**) are more efficient than the tertiary amide pro (**3**).

All chiral ferric complexes assembled from L-amino acids were found to show preferential left-handed (*Λ*-cis) configuration, as does ferrichrome itself (Fig. 4). There were however differences in the isomeric purity of these complexes as evident from their $\Delta\epsilon$ values (Table).

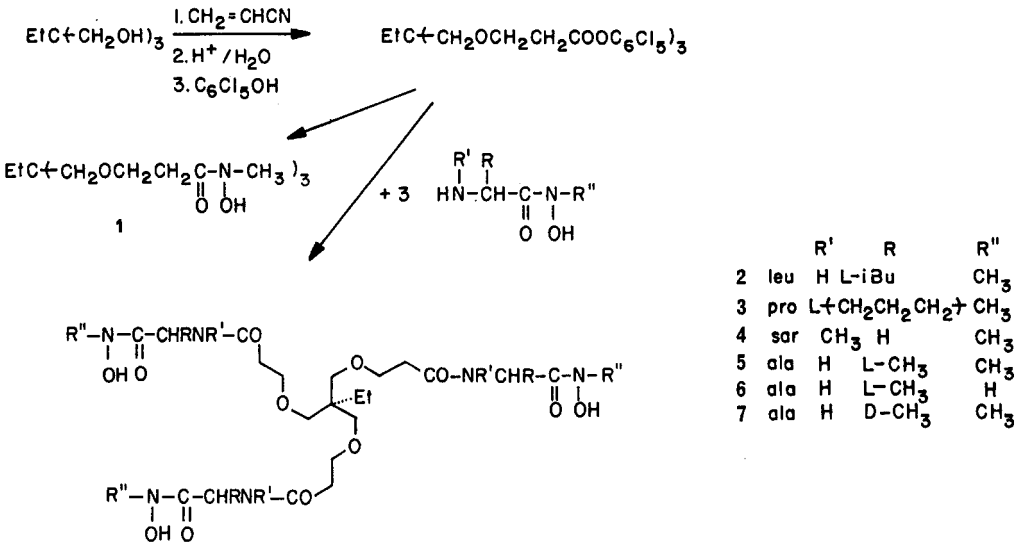

Fig. 3. Synthesis of ferrichrome analogs.

TABLE. UV/VIS and CD spectra of ferric hydroxamates

Compound	UV/VIS[a,b] λ_{max}(nm)	ϵ	CD[a,c] λ_{ext}(nm)	$\Delta\epsilon$
1	430	2530		
2	424	2500	365,413,450	-6.8,0.0,+3.4
3	428	2550	378,430,465	-4.3,0.0,+1.27
4	430	1920		
5	422	2380	370,420,455	-5.2,0.0,+2.0
6	422	2340	370,420,456	-5.1,0.0,+2.06
7	422	2430	370,420,455	+5.2,0.0,-2.0

[a] 0.3 mM solutions of Fe^{3+} complexes in MeOH + 0.1 N aq. NaOAc (8+2).
[b] UV/VIS spectra were recorded on a Hewlett Packard 8450A Diode Array Spectrophotometer.
[c] CD spectra were recorded on a Jasco J-500c Spectrapolarimeter.

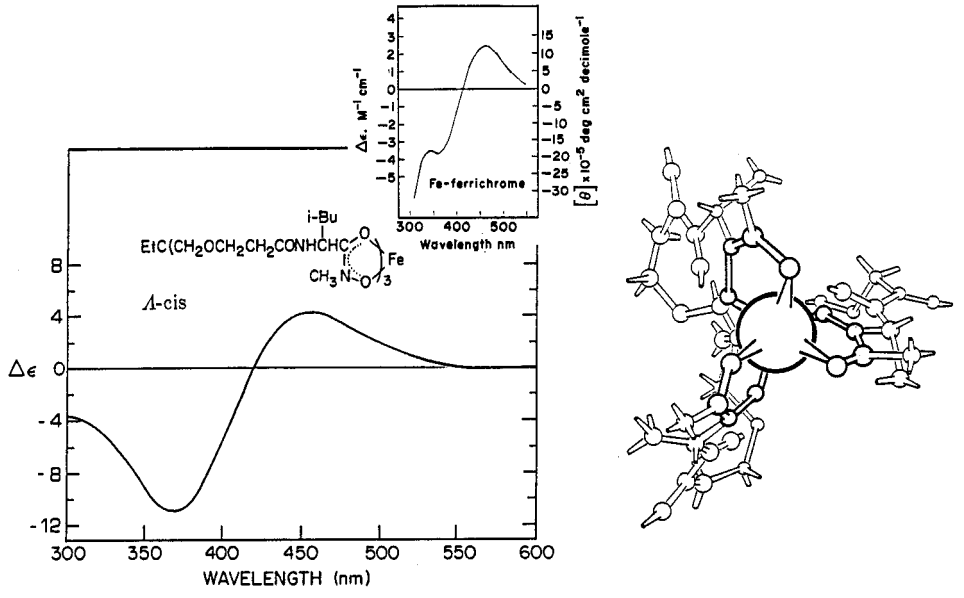

Fig. 4. CD Spectra of ferric complexes of synthetic ferrichrome **2** (in $CDCl_3$) and of ferrichrome (insert).

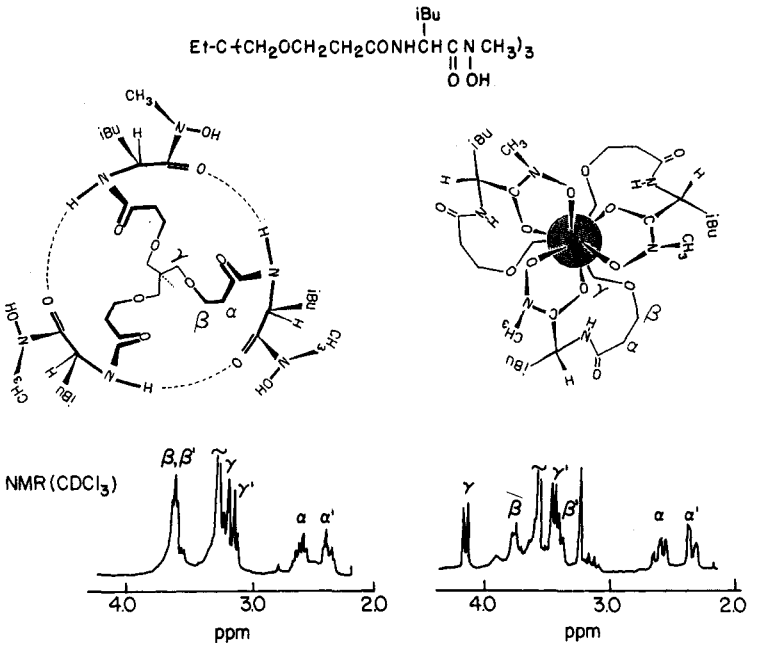

Fig. 5. Part of the ^{1}H-NMR Trace of Synthetic ferrichrome **2** and of its Ga^{3+}-complex.

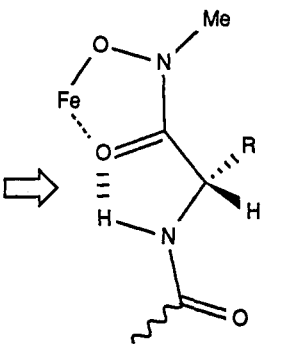

Fig. 6. H-Bonded metal complex.

Specifically, the leu and ala derivatives **2** and **5** showed higher isomeric purity than the pro derivative **3**. That the $\Delta\epsilon$ values are a good measure for isomeric purity was confirmed by NMR examination of the respective Ga^{3+}-complexes. Both the Ga^{3+}- complex of **2** and of **3** were prepared and their NMR spectra recorded. While the Ga^{3+}-complex of **2** showed a single set of signals, that of **3** showed a complex pattern of signals. The Ga^{3+}-complex of **2** (Fig. 5) is thus isomerically pure like the Fe^{3+}- complex of **2** (Table and Fig. 4). On the other hand, the Ga^{3+}-complex of **3** consists of a mixture of isomers, like the weakly dichroic Fe^{3+}-complex of **3**.

The larger isomeric purities of the metal complexes of the leu (**2**) and ala derivatives (**5**) relative to that of the pro derivative (**3**) is attributed, among others, to the possibility of H-bonding of the amide NH to the hydroxamate oxygen in the leu (**2**) and ala (**5**) derivatives which stabilizes a specific configuration of the complex (Fig. 6). Such a type of H-bond has been shown to occur in enterobactin [10] as well as in enterobactin-like compounds [11,12] and to stabilize the complexes once formed. The occurrence of the same type of intramolecular H-bonds in the ferrichrome analogs is supported by the higher complexation efficiencies of **2** and **5** (both of which can form H-bonds) relative to **3** (which can not form such bonds).

BIOLOGICAL TESTING OF BIOMIMETIC FERRICHROMES

In order to examine to which extent the artificial ferrichromes are recognized by microbial organisms and simulate the biological activity of genuine ferrichrome, their growth promotion activity towards *Arthrobacter Flavescens* was tested (All the biological tests were performed by Professor T. Emery at Utah State University). *Arthrobacter flavescens was selected* as test system since it does not produce ferrichrome, but possesses ferrichrome receptors. It therefore completely depends on externally added ferrichrome for growth. Until now, no synthetic iron(III) carrier, except the retrohydroxamate analog of ferrichrome, has shown any growth promotion activity, even at extremely high concentrations [8].

The first compounds tested on *Arthrobacter flavescens* were the parent molecule **1** and the leu derivative **2**. Binders **1** and **2** were found to act as growth promoters of this bacterium with 1% of the efficiency of ferrichrome. *These compounds thus proved to be the first synthetic compounds* to show any activity at all. We therefore felt encouraged to follow this lead and aimed at improving the performance of these binders by further modifications. The first modification was aimed at providing a carrier of decreased lipophilicity, considering the fact that ferrichrome is rather hydrophilic. Replacement of leu in **2** by pro to give **3** serves this purpose. Carrier **3** proved significantly superior to the leu derivative **2** as growth promoter (Fig. 7), reaching 80% of the activity of ferrichrome. The superiority of the pro derivative (**3**) relative to the leu derivative (**2**) excludes the possibility of a passive iron-uptake mechanism by diffusion and was conceived as suggestive of receptor driven uptake. Further modifications were accordingly aimed at reducing the bulkiness of the side arms to closer simulate the envelope of ferrichrome and fit the surface of the membranal receptor. The proline residue in **3** was thus replaced by sarcosine and alanine to give **4** and **5** respectively. And indeed, chiral ala derivative **5** proved to fully match ferrichrome as growth promoter, while achiral **4** reached 85% of the natural siderophore's activity. In order to trace the importance of the terminal methyl group in these binders, it was replaced in the active **5** by hydrogen to give compound **6**. Binder **6** showed drastically reduced activity of less than 1%.

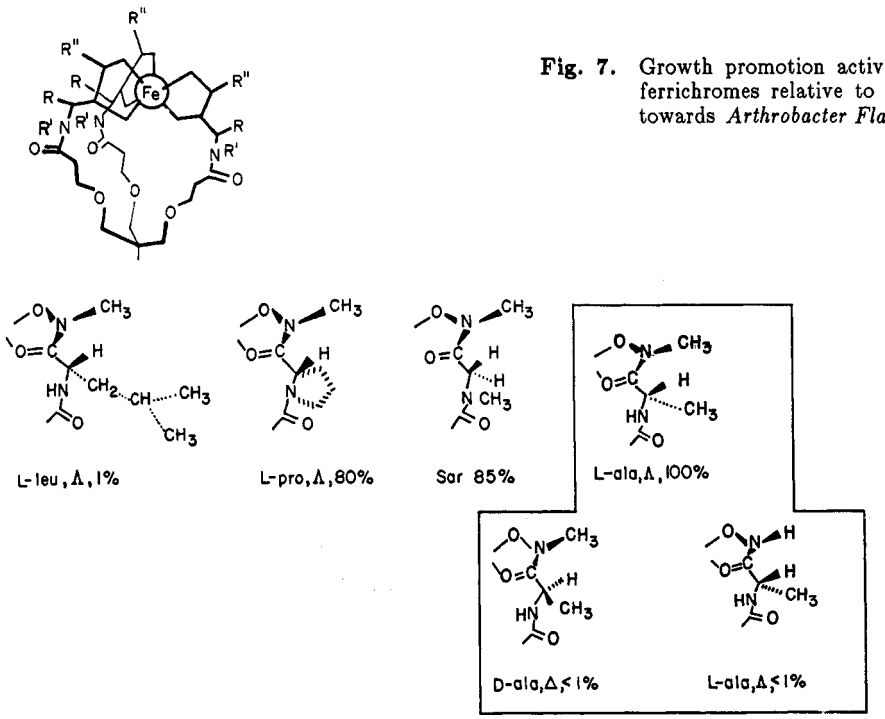

Fig. 7. Growth promotion activity of synthetic ferrichromes relative to ferrichrome towards *Arthrobacter Flavescens*.

A priori, several parameters are likely to govern the observed activities such as ion binding efficiency, fit to the receptor and iron release rate. Since the thermodynamic and kinetic stability (Note a) of the synthetic iron carriers falls within the same order of magnitude, these parameters are unlikely to account for the observed scale of activities. A receptor recognition mechanism is in agreement with the 100-fold increase of activity when replacing bulky, lipophilic leu in **2** by smaller, less lipophilic ala in **3**. Optimal fit is also in line with the similar activities of ala (**5**) and pro (**3**) considering the fact that ala has most frequently been found to replace pro in proteins during the evolutionary process [13]. In order to confirm the occurrence of receptor driven iron uptake, carrier **7**, the enantiomer of carrier **5**, was synthesized and examined. Carrier **7** containing D-ala instead of L-ala showed less than 1% activity. Yet, the extent of optical purity of the different ferric ion complexes is not directly related to their growth promotion activity. The chiral pro derivative (**3**) shows practically the same activity as the achiral sar derivative (**4**). This behavior suggests that cellular uptake of the favored isomer is slower than equilibration between the right and left handed coordination isomers.

The striking similarity of ferrichrome and biomimetic L-ala derivative (**5**) in respect to growth promotion and chiral discrimination suggests, that both chelates act by the same mechanism. The biomimetic carriers may therefore serve as probes to map the ferrichrome receptor.

They allow us to define two major domains (Fig. 8) for siderophore-receptor interactions: the exposed side of the ferric complex and its lateral envelope. The former is highly sensitive to chemical modifications where replacement of the terminal methyl (**5**) by hydrogen (**7**), or inversion of configuration (**6**)

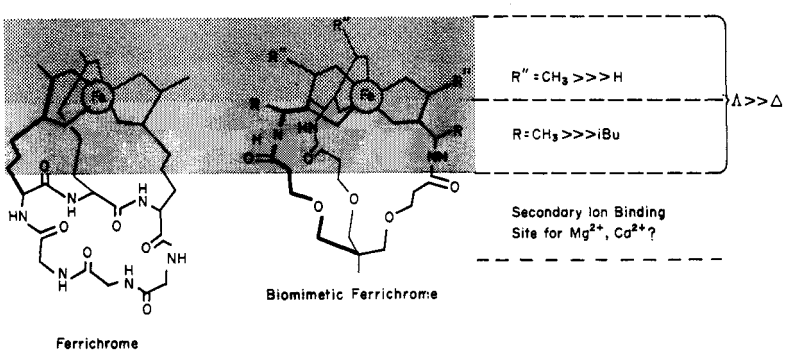

Fig. 8. Domains of receptor recognition in synthetic ferrichromes.

Note a: Iron release from the synthetic iron complexes in MeOH-0.1N NaOAc (8-2) by EDTA was followed spectrophotometrically by measuring the decrease of the Fe^{3+}-absorption at 430 nm. These experiments showed the following series of Fe^{3+}-release rates: **1** > **5** > **2**~**3**, with **3** having a rate constant of $k = 1.8 \times 10^{-2}$ l.mol^{-1}sec^{-1}.

reduces activity to less than 1%. The second domain is more adaptable if it is kept compact: replacement of ala in **5** by pro or sar has little effect, while replacement by leu causes significant drop of activity. These observations suggest that ferrichrome approaches the receptor through the exposed domains of its iron complex. This behavior is reminiscent of that of enterobactin recently described by Raymond [14]. The nature of the anchor in the ferrichrome analogs seems to be of little relevance for recognition, although there appears to be an optimum as to the overall length of the carrier (as evident when comparing **1** with **4**). This point might be very important for the transformation rather than the recognition process.

CONCLUSIONS

The synthetic siderophores described here proved to provide useful probes for identifying the domains relevant for recognition, and for tracing the surface of the siderophore receptors. In addition, these binders promise to also serve as versatile tools in the study of iron uptake mechanisms in other organisms. Interestingly enough, the scale of activities of this family of binders differed from organism to organism. While iron uptake in certain fungi was accelerated most effectively by the most lipophilic of the series, iron uptake in corn roots was most susceptible to the least lipophilic. Experiments are currently in progress to establish the mode of action of these compounds in different organisms in relation to possible diffusion processes, receptor driven or reductase initiated iron uptake.

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REFERENCES

[1] C. More and B.C. Pressman, *Biochem. Biophys. Res. Comm.* **15,** 562-567 (1964); B.C. Pressman, *Proc. Natl. Acad. Sci. USA* **53,** 1076-1083 (1965); B.C. Pressman, *Fed. Proc.* **32,** 1698-1703 (1973); Yu. A. Ovchinnikov and V.T. Ivanov, *Tetrahedron* **31,** 2177-2209 (1975) Yu. A. Ovchinninkov and V. T. Ivanov, *The Proteins* **5,** pp. 295-306, H. Neurath and R. L. Hill. Ed., 3rd Ed., Academic Press, New York (1982).

[2] J.M. Lehn, *Science* **227,** 849-856 (1985).

[3] D.J. Cram, *Science* **240,** 760-767 (1988).

[4] J.B. Neilands, *Structure and Bonding* **58,** 1-24 (1984). T. Emery, *Met. Ions Biol. Syst.* **7,** 77-126 (1978). K.N. Raymond, G. Muller and B.F. Matzanke, *Top. Current Chem.* **123,** 49-102 (1984). R.C. Hider, *Structure and Bonding* **58,** 25-87 (1984).

[5] G. Winkelmann and V. Braun, *FEMS Microbiol. Letters* **11,** 237-241 (1981). B.F. Mutzanke, G.I. Muller and K.N. Raymond, *Biochem. Biophys. Res. Commun.* **121,** 922-930 (1984). J.P Adjimani and T. Emery, *J. Bacteriol.* **170,** 1377-1379 (1988).

[6] D. Van der Helm, J.R. Baker, D.L. Eng-Wilmot, M.B. Hossain, and R.A. Loghry, *J. Am. Chem. Soc.* **102,** 4224-4231 (1980). T. Emery, *Biochemistry* **5,** 3694-3701 (1966).

[7] B.F. Burnham and J.B. Neilands, *J. Biol. Chem.* **236,** 554-559 (1960).

[8] T. Emery, L. Emery, and R.K. Olsen, *Biochem. Biophys. Res. Commun.* **119,** 1191-1197 (1984).

[9] Y. Tor, J. Libman and A. Shanzer, *J. Am. Chem. Soc.* **109,** 6518-6519 (1987).

[10] M. Llinas, D.M. Wilson and J.B. Neilands, *Biochemistry* **12,** 3836-3843 (1972).

[11] Y. Tor, J. Libman and A. Shanzer, *J. Am. Chem. Soc.* **109,** 6517-6518 (1987).

[12] T.J. McMurray, M.W. Hosseini, T.M. Garrett, F.E. Hahn, Z.E. Reyes and K.N. Raymond, *J. Am. Chem. Soc.* **109,** 7196-7198 (1987).

[13] M.O. Dayhoff, R.M. Schwartz and B.C. Orcutt, in "Atlas of Protein Sequence and Structure", (Vol 5, Suppl. 3) (Dayhoff, M.O. ed.), 345-352, National Biomedical Research Foundation (1978).

[14] K.N. Raymond, M.E. Cass and S.L. Evans, *Rec. Trav. Chim. Pays-Bas* **106,** 250 (1987). D.J. Ecker, L.D. Loomis, M.E. Cass and K.N. Raymond, *J. Am. Chem. Soc.* **110,** 2457-2464 (1988).