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SYNTHETIC FERRICHROME ANALOGUES WITH GROWTH PROMOTION ACTIVITY FOR ARTHROBACTER FLAVESCENS

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Summary: Two families of trihydroxamic acid analogues of ferrichrome were chemically synthesized and tested for biological activity with Arthrobacter flavescens. Compounds using a tertiary amine as anchor showed little activity. Several compounds using tetrahedral carbon as anchor showed activity approaching or equalling that of the natural siderophore, ferrichrome. The biological activity is discussed in relation to physical and chemical properties of the analogues.

Continuous supply of iron is essential for the growth of virtually all cells. Adequate iron supply is often hindered by the low solubility of ferric hydroxide, and by the negligible permeability of charged species through biological membranes. Nature has developed ingenious chemical vehicles, siderophores, that overcome this barrier by selectively binding and transporting iron from the environment into microbial cells (1). Cellular uptake of many of these siderophore-iron complexes has been shown to be highly specific. For example, only the natural isomer of ferrichrome, but not its enantiomer, is effectively recognized (2). This specificity depends upon recognition by membrane receptors. In this article we describe the synthesis of a family of chiral iron(III) chelates, three members of which approach and even equal the activity of ferrichrome as growth promoters of Arthrobacter flavescens. Comparison of activities also allows the identification of the essential structural parameters for biological activity and offers an approach to the mapping of the receptor binding site. A. flavescens was selected as test organism because it has an absolute requirement

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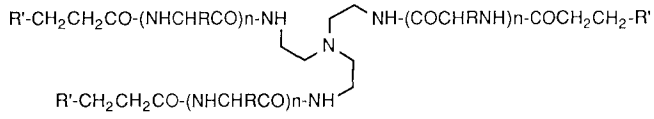
for ferrichrome for growth (3), and no synthetic iron carriers, except the retrohydroxamate analogue of ferrichrome (4), have shown any significant growth promotion activity except at extremely high concentrations.

RESULTS AND DISCUSSION

Our synthetic ferric ion complexes were built from three units: (i) a C_3-symmetric molecule as anchor (triamine for family A, or tricarboxylate for family B), extended by (ii) amino acids as bridges and terminated by (iii) hydroxamate groups as binding sites. Amino acids of L-configuration were selected to ensure preferential left-handed configuration of the ferric ion complexes (5, 6), and to allow systematic modifications by amino acid replacement.

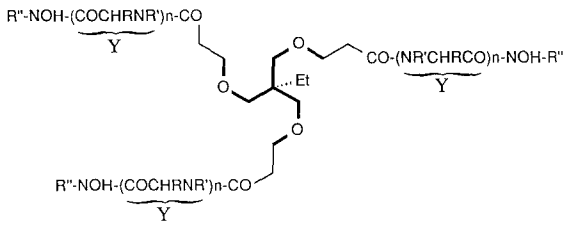
Our biomimetic carriers differ from asymmetric ferrichrome by using as anchor C_3-symmetric tris-amine in family A (Fig. 1) or tris-carboxylate in family B (Fig. 2) instead of a non-symmetrically substituted hexapeptide ring. The inversion of the hydroxamate sequence in type B relative to ferrichrome was anticipated to be of little relevance since the activity of the retro-isomer of ferrichrome is indistinguishable from that of ferrichrome (4).

The synthesis of family A and family B compounds was achieved by the schemes described earlier for the derivatives A2 (5) and B2 (6). All compounds were found to bind iron(III) in a 1:1 stoichiometry. The configuration of the iron(III) complexes of the L-amino acid derivatives of both families was found by circular dichroism (CD) to be preferentially left handed (Λ-cis) as for ferrichrome itself (7). However, there were differences in the optical purity of the complexes, i.e. the extent of preference of the Λ-cis



Code	n	R	R'
A1	0	-	N(OH)CO-p-$C_6H_4OCH_3$
A2	1	i-Bu	N(OH)CO-p-$C_6H_4OCH_3$
A3	0	-	CON(OH)H

Fig. 1. Structures of the family A tris-hydroxamate ligands. The leu in A2 is of L-configuration.



Code	n		Y	R"
B1	0		-	CH_3
B2	1	L-leu	(R=i-Bu,R'=H)	CH_3
B3	1	L-pro	(R-R'=-$CH_2CH_2CH_2$-)	CH_3
B4	1	sar	(R=H,R'=Me)	CH_3
B5	1	L-ala	(R=Me,R'=H)	CH_3
B6	1	D-ala	(R=Me,R'=H)	CH_3
B7	1	L-ala	(R=Me,R'=H)	H

Fig. 2. Structures of the family B tris-hydroxamate ligands. Y represents the amino acid residues.

configuration, relative to the Δ-cis configuration, as measured by the $\Delta\epsilon$ values of their dichroism (Table 1).

The first compounds tested as growth promoters of A. flavescens were the parent molecules A1 and B1 and the leucyl derivatives A2 and B2 of each system (Table 1). No member of the A series showed significant activity. This was not due to the bulky **p**-methoxyphenyl group since replacement of this group in A1 by H to give A3 did not improve effectiveness. Therefore, type B derivatives were selected for further studies. The next modification was aimed at providing a carrier of decreased lipophilicity, considering the fact that ferrichrome is rather hydrophilic. Replacement of leu in B2 by pro to give B3 served this purpose. The activity of complex B3 was significantly greater than the leu derivative B2 as growth promoter of A. flavescens, reaching 80% of the activity of ferrichrome. The superiority of the pro derivative B3 relative to the leu derivative B2 excludes the possibility of a passive iron-uptake mechanism by diffusion and is suggestive of receptor driven uptake. Further modifications were accordingly aimed at reducing the bulkiness of the side arms to better match the surface of the receptor relying on the fact that the binding arms of ferrichrome are unsubstituted. Pro in B3 was thus replaced by sar and ala to give B4 and B5, respectively. Indeed, chiral ala derivative B5 proved to fully match ferrichrome as growth promoter, while achiral B4 reached 85% of the natural siderophore's activity. The structure of B5 is compared

Table 1: Growth promotion activity and spectral properties of ferrichrome analogues

	Activity percent[a]	Absorption maximum, nm	Extinction coefficient[b,c]	CD[b,d]
A1	1	426	3550	-
A2	0.06	426	3250	337, 407, 450 -250, 0.0, +2.00
A3	0.1	428	2760	-
B1	1	430	2530	-
B2	1	424	2500	365, 413, 450 -6.8, 0.0, +3.4
B3	80	428	2550	378, 430, 465 -4.3, 0.0, +1.27
B4	85	430	1920	-
B5	100	422	2380	370, 420, 455 -5.2, 0.0, +2.0
B6	<1	422	2430	370, 420, 455 +5.2, 0.0, -2.0
B7	<1	422	2340	370, 420, 456 -5.1, 0.0, +2.06

[a]Growth promotion activity of the synthetic compounds for A. flavescens was determined in comparison with ferrichrome (100%) using the penicillin disc method (Luckey, M., Pollack, J.R., Wayne, R., Ames, B.M. and Neilands, J.B., J. Bacteriol. 111, 731-738 (1972).
[b]0.3 mM solutions of Fe(III) complexes in methanol and 0.1 N aqueous sodium acedtate (8:2).
[c]Spectra were recorded on a Hewlett Packard 8450A diode array spectrophotometer.
[d]CD spectra were recorded on a Jasco J-500c spectropolarimeter. The first line gives wavelengths of extrema, the second line the values of $\Delta\epsilon$.

to ferrichrome in Fig. 3. Replacing the methyl end group of B5 by H to give B7 drastically reduced the activity to less than 1%.

Properties such as ion binding strength and iron release rate may influence biological activity, . The relative binding strengths of the chiral complexes B2, B3, and B5 were determined by incubating each of them with equimolar amounts of achiral B1 and $FeCl_3$ in methanol and 0.1 N aqueous sodium acetate (8:2) and measuring the concentration of the chiral Fe complexes of B2, B3 and B5 by CD. These experiments established the following series of relative complexation strengths: B1 : B2 : B3 : B5 :: 1 : 0.8 : 1.1 : 0.65. The rates of iron release into EDTA in the same solvent were followed spectrometrically by measuring the decrease of the Fe(III)-hydroxamate absorption at 430 nm. The relative release rates were found to be: B1 > B5 > B2 > B3,

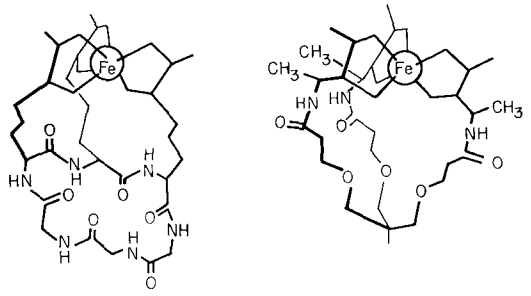


Fig. 3. Schematic representation of the ferric complexes of natural ferrichrome (left) and the symmetric biomimetic ferrichrome B5 (right) of identical growth factor activity.

with B3 having a rate constant of 1.8×10^{-2} liter mol^{-1} sec^{-1}. Since the thermodynamic and kinetic stability of the B family of iron carriers are of the same magnitude, these parameters are unlikely to account for the observed differences. A receptor recognition mechanism is also suggested by the 100-fold increase of activity upon replacing bulky, lipophilic leu in B2 by smaller, less lipophilic ala in B3. Receptor recognition is also in line with the similar activities of ala B5 and pro B3 considering the fact that ala has most frequently been found to replace pro in proteins during the evolutionary process (8). The occurrence of receptor-driven iron uptake was further confirmed by the fact that the enantiomer, B6, showed less than 1% of the activity of the L-ala derivative B5. 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, B3, shows practically the same activity as the achiral sar derivative, B4. 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 B5 with respect to growth promotion and chiral discrimination suggests that both chelates act by the same mechanism. The biomimetic compounds B may therefore serve as probes to map the ferrichrome receptor. They allow us to define two major domains for siderophore-receptor interactions: the exposed side of the ferric complex and its lateral envelope. The former is highly sensitive to chemical modifications since replacement of the terminal methyl (B5) by hydrogen (B7), or inversion of configuration (B6), reduces activity to less than 1%. The second domain is more adaptable if it is kept compact: replacement of ala in B5 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 Ecker et al. (9). The nature of the anchor in the ferrichrome analogues seems to be of little relevance for recognition, although there appears to be an optimum as to the overall length of the carrier as is evident when comparing A1 with A2 or B1 with B4. This point might be important for translocation rather than the recognition process. Experiments examining this possibility are currently in progress.

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