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Growth promotion of synthetic catecholate derivatives on Gram-negative bacteria

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Derivatives of benzoic acid, glyoxylic acid benzhydrazone, oxanilic acid and *N*-dihydroxybenzylidene-2,4,6-trimethylaminobenzene were investigated as catecholic iron chelators under iron-depleted conditions. Some of the compounds showed strong positive reactions in the universal chemical siderophore assay (CAS): 3,4-dihydroxybenzoic acid, glyoxylic acid 2,3-dihydroxybenzhydrazone, *N*-3,4-dihydroxybenzylidene-2,4,6-trimethylaminobenzene. In particular these compounds also enabled removal of iron from iron-saturated transferrin. Using various siderophore indicator strains (enterobacteriacecae, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* mutants) in bioassays the following growth promotion could be detected: vicinal substituents (e.g. 2,3- or 3,4-) were essential, the carboxyamido group seen in benzoic acids and glyoxylic acid benzhydrazones contributed to a positive reaction as well as the azomethin group (in *N*-3,4-dihydroxybenzylidene-2,4,6-trimethylaminobenzene). 2,3-Dihydroxybenzoic acid and the 2,3-diacetoxy substitute preferably promoted growth of enterobacteriaceae mutants. In contrast, the 3,4- positioned compounds preferably promoted growth of *P. aeruginosa* mutants and *A. hydrophila* SB 22. Glyoxylic acid di(methoxycarbonyloxy)-benzhydrazones (2,3- and 3,4- positioned) including the 2,3-dihydroxy compound preferably enabled growth of the non-fermenters. *N*-3,4-dihydroxybenzylidene-2,4,6-trimethylaminobenzene supplied all mutants of *Salmonella, Escherichia coli, Klebsiella, Morganella, P. aeruginosa* and *A. hydrophila* with iron. Transport of glyoxylic acid 2,3-dihydroxybenzhydrazone depended on *tonB*, and required the involvement of the iron-regulated outer membrane proteins (IROMPs) FepA, Cir and Fiu.

Keywords: bioassays, catecholic iron chelators, growth promotion

Introduction

Naturally occurring and synthetic ferric iron chelators are currently of great interest because of their essential role in microorganisms (Winkelmann *et al.* 1987, Winkelmann 1991, Rabsch & Reissbrodt 1992), plants (Crowley *et al.* 1991), their potential applications in the treatment of iron overload disease (Dionis *et al.* 1991), as part of siderophore antibiotics (Watanabe *et al.* 1987, Curtis *et al.* 1988, McKee *et al.* 1991), as possible anticancer drugs (Tabor & Kim 1991), as biomimetic iron carriers for further use in malaria therapy (Shanzer & Libman 1991) and, very recently, as selective growth factors for nutrient media (Reissbrodt & Rabsch 1993). Different methods have been used to characterize a compound as a ferric iron chelator. Well known methods include determination of binding constants, measurement of mediated ^{55}Fe uptake into cells, removal of Fe(III) from Fe_2-transferrin, reaction on the Chrome Azurol S (CAS) plate and, of course, growth promotion of microorganisms by different tests (e.g. Rabsch & Winkelmann 1991).

In this paper the growth promoting activity of derivatives of benzoic acid, glyoxylic acid benzhydrazone, oxanilic acid and *N*-dihydroxybenzylidene-2,4,6-trimethylaminobenzene to enterobacteriaceae,

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pseudomonads and *Aeromonas hydrophila* as iron chelates will be demonstrated.

Materials and methods

Siderophore indicator strains and bioassays

To check the growth promotion of the catechol compounds in bioassays, a number of siderophore indicator strains (enterobacteriaceae, *P. aeruginosa, A. hydrophila* mutants, *A. flavescens* JG-9) were used (Table 1). All of these strains except *Aureobacterium* (formerly *Arthrobacter*) *flavescens* JG-9 were examined in Tris–succinate medium. *A. flavescens* JG-9 was examined in AM medium.

Bioassays with the *P. aeruginosa* mutants were made using 400 μM of ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDHA), those with *A. hydrophila* and the

Table 1. List of siderophore indicator strains

Indicator strain	Iron related marker	Detection of	Origin
Enterobacteriaceae			
S. typhimurium enb-7	*ent* (class II)	enterobactin, $(DHBS)_{2,3}$, DHBA, ferrichrome, ferrioxamine and other hydroxamate-type siderophores not alcaligin	J. B. Neilands (University of California, Berkeley, CA, USA)
S. typhimurium SR 1001	*ent* (class II) *tonB*	DHBA	W. Rabsch (BGA Wernigerode, Germany)
E. coli AB 2847	*aroB*	enterobactin, $(DHBS)_{2,3}$, DHBA, ferrichrome, coprogen, none of the ferrioxamines	V. Braun (University of Tübingen, Germany)
E. coli IR 112	*aroB* *tonB*	DHBA	V. Braun (University of Tübingen, Germany)
K. pneumoniae KN 4401	*ent, iuc*	most of the phenolate-type and hydroxamate-type siderophores except amonabactin	P. Williams (University of Nottingham, UK)
M. morganii SBK 3	wild-type	enterobactin, amonabactin, aerobactin, not ferrichrome or ferrioxamine	W. Rabsch (BGA Wernigerode, Germany)
P. aeruginosa PAO 6609	*pvd*	enterobactin, amonabactin, ferrichrome, ferrioxamine E, coprogen, pyoverdines	J.-M. Meyer *et al.* (University of Strasbourg, France)
P. aeruginosa K437	*pvd, pyo,* 90 kDa OMP	as the parent PAO 6609, uptake of pyoverdine diminished	K. Poole *et al.* (University of Kingston, Canada)
P. aeruginosa K407	*pvd,* 80 kDa OMP	as the parent PAO 6609, uptake of enterobactin diminished	K. Poole *et al.* (University of Kingston, Canada)
P. aeruginosa FBP-28	14 kDa OMP	as *P. aeruginosa* PAO, uptake of pyochelin diminished	P. Sokol (University of Calgary, Canada)
A. hydrophila SB 22	*amoA*	amonabactin, myxochelin, ferrichrome, ferrioxamines E, B, not coprogen	S. Barghouti *et al.* (University of Mississippi, Jackson, MI, USA)
A. flavescens JG-9	hydroxamate siderophore auxotroph	hydroxamate type siderophores including alcaligin except aerobactin, nannochelin	P.J. Szaniszlo (University of Texas, Austin, TX, USA)

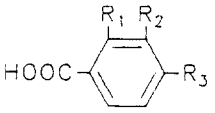
enterobacteriaceae strains except the *tonB* mutants were performed using 220 μM α,α'-bipyridyl-(DIP). In the cases of the *tonB* indicator strains, *Escherichia coli* IR112 and *Salmonella typhimurium* SR1001, 160 μmol of DIP were used for better readings. None of the artificial chelators was added to the AM medium for *A. flavescens* JG-9 since this medium is poor in iron. The bioassays were performed according to Reissbrodt & Rabsch (1988, 1992). Filter paper disks were loaded with 5 μg of the catechol compounds and placed onto the surface of the bioassay plates. Incubation of plates with enterobacteriaceae siderophore indicator strains was at 37 °C, of plates containing all the other indicator strains was at 30 °C; incubation overnight. Only growth zones of more than 15 mm in diameter around the loaded disk were monitored as positive, growth zones smaller than 15 mm were indicated as '±'.

The *E. coli* K-12 mutants listed in Table 7 were kindly provided by K. Hantke, Mikrobiologie II, Universität Tübingen, Germany.

Catecholate-derivatives

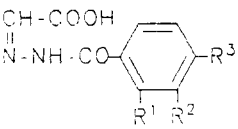
The following catechol chelates were studied:

Benzoic acids



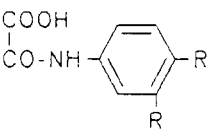
	Compound
R_1 = OH; R_2, R_3 = H	**I**
R_1, R_2 = OH; R_3 = H	**II**
R_1 = H; R_2, R_3 = OH	**III**
R_1, R_2 = $OCOCH_3$; R_3 = H	**IV**
R_1 = H; R_2, R_3 = $OCOCH_3$	**V**

Glyoxylic acid benzhydrazones



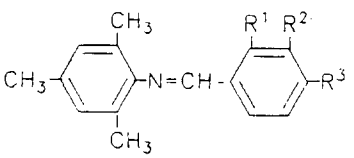
R_1, R_2 = H; R_3 = OH	**VI**
R_1, R_2 = OH; R_3 = H	**VII**
R_1 = H; R_2, R_3 = OH	**VIII**
R_1, R_2 = H; R_3 = $OCOOCH_3$	**IX**
R_1 = H; R_2, R_3 = $OCOOCH_3$	**X**

Oxanilic acids



R= OH	**XI**
R = H	**XII**

N-*Dihydroxybenzylidene-2,4,6-trimethylaminobenzenes*



R_1, R_3 = OH; R_2 = H	**XIII**
R_1 = H; R_2, R_3 = OH	**XIV**

The catecholate compounds used here were synthesized as follows: 2,3-diacetoxybenzoic acid (**IV**) and its 3,4- derivative (**V**) were prepared by common procedures (Beilstein 1927, 1929, 1932). The glyoxylic acid benzhydrazones -3,4-dihydroxy-(**VIII**), -4-hydroxy-(**VI**), -3,4-di(carbonyloxymethyl) (**X**) and -4-carbonyloxymethyl-(**IX**) were prepared from glyoxylic acid and the corresponding benzhydrazides (Heinisch *et al.* 1992). Glyoxylic acid-2,3-dihydroxybenzhydrazone (**VII**), $C_9H_8N_2O_5$, M = 224, m.p. 242–244 °C, was synthesized in analogy to **VIII**, from glyoxylic acid and 2,3-dihydroxybenzhydrazide. The structure and purity of the compounds were confirmed by ^{1}H-NMR-spectra, elementary analysis and thin layer chromatography (TLC). 3,4-Dihydroxy-oxanilic acid (**XI**), $C_8H_7NO_5$, M = 197, m.p. = 218–220 °C (H_2O), was prepared from *N*-(3,4-diacetoxyphenyl)-oxamoylchloride by 30 min heating in 2N HCl, the structure was confirmed by ^{1}H-NMR spectra (Heinisch *et al.* 1993) Oxanilic acid, m.p. = 149–150 °C, (**XII**) was prepared by common procedures (Beilstein). The *N*-dihydroxybenzylidene-2,4,6-trimethylaminobenzenes [-2,4-dihydroxy-(**XIII**) and -3,4-dihydroxy-(**XIV**)] were prepared according to Ulbricht *et al.* (1987). All of them were characterized by TLC, IR and ^{1}H-NMR spectra.

Removal of iron from Fe_2–transferrin and universal chemical assay (CAS)

Removal of iron from Fe_2–transferrin by the catechol-type chelates was investigated according to Ford *et al.* (1988). The molar ratio of Fe_2–transferrin to the individual chelates was 1:300. Application of 50 μg of the catecholates onto the Chrome Azurol S (CAS) plate as the universal chemical assay for the detection and determination of siderophores (Schwyn & Neilands 1987) showed positive reactions (colorless halos of the deferrated dye) after 1 h at room temperature.

Results

Removal of iron from Fe_2–transferrin and CAS test

Some of the compounds showed strong positive reactions on the universal chemical siderophore assay (CAS): 3,4-dihydroxybenzoic acid, glyoxylic acid 2,3-dihydroxybenzhydrazone and *N*-3,4-dihydroxybenzylidene-2,4,6-trimethylaminobenzene reacted very rapidly and effectively. These latter compounds also enabled removal of iron from Fe_2–transferrin. Additionally, removal of iron from Fe_2–transferrin was observed with glyoxylic acid-3,4-dihydroxybenzhydrazone and the 3,4-di(methoxycarbonyloxy) derivative, and with 3,4-diacetoxybenzoic acid and 3,4-dihydroxyoxanilic acid. See Table 2.

Growth promotion by benzoic acid derivatives

Salicylic acid as a monohydroxybenzoic acid (**I**) was

Table 2. CAS reactions of the synthetic catecholate derivatives after 1 h and removal of iron from Fe_2–transferrin

Benzoic acids			Glyoxylic acid benzhydrazones			Oxanilic acids			*N*-dihydroxybenzylidene-2,4,6-trimethyl aminobenzenes		
	CAS	transferrins formed[a]		CAS	transferrins formed[a]		CAS	transferrins formed[a]		CAS	transferrins formed[a]
I	–	weak Fe(N)Tf	**VI**	–	weak Fe(C)Tf	**XI**	(+)	Fe(N)Tf Fe(C)Tf	**XIII**	+	–
II	–	weak Fe(N)Tf	**VII**	++[b]	Fe(C)Tf Tf	**XII**	–	–	**XIV**	+++[b]	Fe(C)Tf Tf
III	++	Fe(N)Tf	**VIII**	+	Fe(C)Tf Tf						
IV	–	Fe(N)Tf Fe(C)Tf	**IX**	–	Fe(N)Tf						
V	–	Fe(N)Tf Fe(C)Tf Tf	**X**	+	Fe(N)Tf Fe(C)Tf Tf						

[a]Detection of following transferrins: Tf = apotransferrin; Fe(C)Tf, Fe(N)Tf = monoferric transferrins with iron either at the C-terminal or N-terminal binding site.
[b]Positive reaction started after 15 min.

unable to support growth of any of the siderophore indicator strains tested, neither enterobacteriaceae nor non-fermenters (Tables 3 and 4). Negative results were obtained with dihydroxybenzoic acids in which the hydroxyl-groups were not in a vicinal position (e.g. 2,6-, 3,5- 2,4-) (data not shown). 2,3-Dihydroxybenzoic acid (**II**) and also 2,3-diacetoxybenzoic acid (**IV**) were able to overcome the limitation of iron within the iron poor Tris–succinate medium and promoted growth of the *E. coli,*

Table 3. Cross-feeding of different siderophore indicator strains by benzoic acid derivatives: enterobacteriaceae

Compound	*S. typhimurium*		*E. coli*		*K. pneumoniae*	*M. morganii*
	enb-7	SR1001	AB2847	IR112	KN4401	SKB3
I	–	–	–	–	–	–
II	+	+	+	+	+	–
IV	+	+	+	+	±	–
III	–	–	–	–	±	–
V	–	+	–	+	+	–

Table 4. Cross-feeding of different siderophore indicator strains by benzoic acid derivatives: non-fermenter and *A. flavescens* JG9

Compound	*P. aeruginosa*				*A. hydrophila*	*A. flavescens*
	PAO 6609	K437	K407	FBP-28	SB22	JG-9
I	–	–	–	–	–	–
II	–	–	–	–	+	–
IV	–	–	–	–	–	–
III	+	+	+	–	+	–
V	+	+	+	+	+	–

Salmonella and *Klebsiella* mutants. 3,4-Dihydroxybenzoic acid (**III** ~ protocatechuic acid) did not feed any of the *E. coli* and *S. typhimurium* strains, and was less active with regard to the *K. pneumoniae* strain. Contrary to the 3,4-dihydroxy compound, 3,4-diacetoxybenzoic acid (**V**) was able to promote growth of the *tonB* mutants and, additionally, of the common siderophore indicator strain *K. pneumoniae* KN 4401. *Morganella morganii* SBK3 did not grow with any of the benzoic acid derivatives tested.

In contrast, a reversed situation was obtained with *P. aeruginosa* and *A. hydrophila* siderophore indicator strains. Here, the 3,4-dihydroxy and 3,4-diacetoxybenzoic acids effectively supplied these non-fermenter strains with iron. While compounds containing the hydroxy and acetoxy groups in the 2,3-position were ineffective with *Pseudomonas* mutants, they stimulated growth of *A. hydrophila* SB22. 2,3-Diacetoxybenzoic acid was not able to support growth of the non-fermenters.

Growth promotion by glyoxylic acid benzhydrazones

All derivatives tested showed growth promotion in *K. pneumoniae* (Tables 5 and 6). The same could be observed in *A. hydrophila* SB 22, except for the 4-methoxycarbonyloxy compound **IX**. Glyoxylic acid 2,3-dihydroxybenzhydrazone (**VII**) promoted growth of the *E. coli* and *S. typhimurium* mutants in a *tonB*-dependent way. The use of various *E. coli* mutants in bioassays has shown that crossing of **VII** through the cell membrane of *E. coli* seems to be dependent—in addition to *tonB*—on the iron-regulated outer membrane proteins (IROMPs) FepA, Fiu and Cir (Table 7). Also, *M. morganii* SBK 3 was fed by this compound. This strong CAS-positive compound was also active in the mutants of non-fermenters. Glyoxylic acid 3,4-dihydroxybenzhydrazone (**VIII**) and its 3,4-di(methoxycarbonyloxy) derivative (**X**) are particularly active in supplying

Table 5. Cross-feeding of different siderophore indicator strains by glyoxylic acid benzhydrazones: enterobacteriaceae

Compound	*S. typhimurium*		*E. coli*		*K. pneumoniae*	*M. morganii*
	enb-7	SR1001	AB2847	IR112	KN4401	SKB3
VI	−	−	−	−	+	−
VII	+	−	+	−	+	+
VIII	−	−	−	−	+	±
IX	−	−	−	−	+	−
X	−	−	−	−	+	−

Table 6. Cross-feeding of different siderophore indicator strains by glyoxylic acid benzhydrazones: non-fermenter and *A. flavescens* JG9

Compound	*P. aeruginosa*				*A. hydrophila*	*A. flavescens*
	PAO 6609	K437	K407	FBP-28	SB22	JG-9
VI	−	−	−	−	+	−
VII	+	+	+	+	+	−
VIII	+	+	+	+	+	−
IX	−	−	−	−	−	−
X	+	+	+	+	+	−

Table 7. Growth response to glyoxylic acid-2,3-dihydroxybenzhydrazone (compound **VII**) given in growth zones

E. coli strain	Relevant receptors			Growth zones (mm)
H1443	*fepA*$^+$	*cir*$^+$	*fiu*$^+$	25
H1728	*fepA*$^+$	−	−	18
H1877	−	*cir*$^+$	−	20
H1875	−	−	*fiu*$^+$	16
H1876	−	−	−	−

iron to all of the *P. aeruginosa* mutants and *A. hydrophila* SB 22. Both the monosubstituted compounds, glyoxylic acid-4-hydroxybenzhydrazone (**VI**) and its 4-methoxycarbonyloxy derivative, were ineffective in *P. aeruginosa* or in the *E. coli* and *S. typhimurium* mutants. Besides *K. pneumoniae* KN 4401, *A. hydrophila* SB 22 seems to utilize the 4-(mono)hydroxy compound **VI** as an iron chelate.

Growth promotion by oxanilic acids

The results obtained with oxanilic acid derivatives were similar. Unsubstituted oxanilic acids were ineffective with the siderophore indicator strains tested. 3,4-Dihydroxyoxanilic acid (**XI**) was active only in *K. pneumoniae* KN 4401 and *A. hydrophila* SB 22.

Growth promotion by N-*dihydroxybenzylidene-2,4,6-trimethylaminobenzenes*

Unusual results were obtained concerning the *N*-dihydroxybenzylidene-2,4,6-trimethylaminobenzenes. The compound containing a vicinal 3,4-dihydroxybenzoylidene group (**XIV**) could override the iron limitation and was able to promote all the siderophore indicator strains tested, except *A. flavescens* JG-9. The 2,4-positioned dihydroxybenzylidene group within this molecule (**XIII**) was unable to feed the strains.

Discussion

Synthetic Fe(III) catecholates have been examined as growth promotion factors in bioassays. While the iron-free chelators are able to remove iron from Fe_2–transferrin and reacted positive on CAS plates (Table 2), the iron chelates showed significant growth promotion of Gram-negative siderophore indicator strains.

In general, none of the catechol-type chelates tested was able to feed *A. flavescens* JG-9 as a known hydroxamate auxotrophic siderophore indicator strain. Salicylic acid, known as part of mycobactins (Ratledge & Chaudrey 1971) or released by *Azospirillum lipoferrum* (Saxena *et al.* 1986) could not promote any of the siderophore indicator strains, neither enterobacteriaceae nor non-fermenters.

2,3-Dihydroxybenzoic acid is an intermediate of the biosynthesis of catecholate-type siderophores in enterobacteriaceae, vibrionaceae, aeromonads and *Yersinia enterocolitica*. It can be released into the culture fluid and detected chemically and by bioassays. 3,4-Dihydroxybenzoic acid, a previously unreported compound, could be detected as an extracellular product of the nitrogen-fixing *Azomonas macrocytogenes* (Westervelt *et al.* 1985). 3,4-DHBA was detected under both iron-deficient and iron-sufficient conditions. It is conceivable that the formation and extracellular release of 3,4-dihydroxybenzoic acid by *A. macrocytogenes* is part of a strategy of iron solubilization. 2,3-Dihydroxybenzoic acid and 3,4-DHBA were detected in an iron-depleted broth culture of the cow pea *Rhizobium* (Jadhav & Desai 1992).

Dihydroxybenzoic acid derivatives containing non-vicinal 2,6-, 3,5- or 2,4-OH groups were unable to form ferric complexes. 2,3-Dihydroxybenzoic acid derivatives (including 2,3-diacetoxy substitutes) preferably promoted growth of enterobacteriaceae mutants (Tables 3 and 4). The results with 2,3-dihydroxybenzoic acid were as expected; however, those for 2,3-diacetoxybenzoic acid were unexpected. Investigations with substituted benzoic acid derivatives (Curtis *et al.* 1988) have shown that blocking of catechol hydroxyls by methylation vitiates the bacteriostatic effect of catechol-substituted cephalosporins. In contrast, both acetoxy groups in the 2,3-position did not affect iron supply to *E. coli* and *S. typhimurium* siderophore indicator strains. A weak diminishing effect was seen in *K. pneumoniae* KN 4401. To decide between a real transport or reduction, hydrolytic cleavage, ligand exchange at the surface and assays with double-labelled $^{55}Fe/^{14}C$ chelates may be necessary.

3,4-Dihydroxybenzoic acid derivatives (including 3,4-diacetoxy substitutes) were preferably active in *P. aeruginosa* and *A. hydrophila* SB 22 mutants (Tables 5 and 6). Utilization of such compounds depends on transport systems in non-fermenters, different from those of enterobacteriaceae (Sokol 1987, Poole *et al.* 1990, 1991, Jurkevitch *et al.* 1992). In general, cross-feeding with the catecholate derivatives (except **III**) was independent of the 90, 80 and exposure of the 14 kDa IROMPs as seen in K437, K407 and FBP-28 mutants.

The other compounds tested here have never been investigated before. Generally, the glyoxylic acid benzhydrazones tested seem to be more active in supplying iron than the benzoic acid derivatives. Glyoxylic acid-di(methoxycarbonyloxy)-benzhydrazones (2,3- and 3,4- positioned), including the 2,3-dihydroxy compound, preferably fed the *P. aeruginosa* and *A. hydrophila* SB 22 mutants.

Probably, *K. pneumoniae* KN 4401 may have a special transport system for this class of compounds. Thus, the requirement of Fep A, Cir and Fiu for iron

uptake by glyoxylic acid 2,3-dihydroxybenzhydrazone (Table 7) corresponded to the results with 2,3-dihydroxybenzoylserine (Hantke 1990).

Results with oxanilic acid derivatives underlined the need of vicinal hydroxyl groups in Fe(**III**) chelates. In contrast to the benzoic acid derivatives, the reversed position of the NH and CO groups in this compound may inhibit recognition and transport of iron chelates via known ferric siderophore routes.

Unusual results were obtained concerning the *N*-dihydroxybenzylidene-2,4,6-trimethylaminobenzenes. The 3,4-dihydroxy compound (**XIV**) containing an azomethin group could alleviate the iron limitation and was able to promote growth of all Gram-negative indicator strains tested. These results are in parallel with strong positive CAS reactions and the ability to remove iron from Fe_2–transferrin. The non-polar 2,4,6-trimethylbenzene hydrophobic backbone might enhance the transport through the cell wall. The 2,4- positioned dihydroxy compound could not feed any of indicator strains. Both the *N*-dihydroxybenzylidene-2,4,6-trimethylaminobenzenes have been proven to be strong inhibitors of lipoxygenases, probably by iron binding within the catalytic center (Nuhn *et al.* 1991). The 3,4-hydroxy compound was superior to the 2,4-hydroxy compound (Bekemeier *et al.* 1989).

M. morganii SBK 3 deviated from other enterobacteriaceae tested. This strain was only fed by very strong catecholates such as glyoxylic acid-2,3-dihydroxybenzhydrazones and *N*-3,4,dihydroxybenzylidene-2,4,6-trimethylbenzene, respectively.

Besides their function in iron transport, these compounds can be used as catecholate moities of β-lactam antibiotics and as potential iron chelators.

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