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The carboxylate type siderophore rhizoferrin and its analogs produced by directed fermentation

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SUMMARY

Rhizoferrin is a novel carboxylate-type siderophore which has recently been isolated from *Rhizopus microsporus* and other fungi of the Mucorales (Zygomycetes). The present investigation shows that a variety of rhizoferrin analogs can be produced by directed fermentation. Thus both the diaminobutane backbone and the citric acid side chains of rhizoferrin have been substituted by diamine and citric acid analogs added to the culture medium. The new ligands as well as their iron complexes have been characterized by physicochemical methods. Conditions of precursor incorporation and implications for the biosynthesis of the new siderophores are discussed.

INTRODUCTION

Iron is an essential element for almost every living organism. Based on its redox activity and its ability to form coordination compounds with a variety of different ligands, it is present in a large number of vital enzymes. However, its bioavailability is limited by its low solubility in the normal and physiological pH range.

As an evolutionary response siderophores have evolved. Siderophores are low molecular weight iron chelators produced by bacteria and fungi under iron-limiting conditions [41]. They are excreted into the growth medium, where they form chelate complexes with iron(III), solubilizing the ferric hydroxides. The ferric complexes are taken up into the microbial cells by specific receptors and energy-dependent membrane transport systems.

Siderophores have traditionally been divided into two main groups, based on the chemical nature of their coordination sites. The first group, which includes enterobactin [25,29], regarded until recently as the strongest iron chelator [27], coordinates iron with catecholate hydroxy groups. The second group are hydroxamate ligands, possessing *N*-hydroxylated amide bonds. Examples are the bacterial ferrioxamines [4,23,35] and the fungal ferrichromes [2,8,42]. In cases where donating groups other than aromatic hydroxy functions or hydroxamates are involved, they have been treated as mixed types of siderophores. N, O and S atoms in heterocycles, α-hydroxycarboxylic acids, α-keto-carboxylic acids and carboxylate groups can also participate in the coordination of iron.

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A new type of siderophores has been discovered recently, which contain only hydroxy and carboxy functions as donor groups to iron(III). With a steadily increasing number of representatives, this so called 'carboxylate type' class of siderophores is being established as a third class of siderophores besides catecholate and hydroxamate siderophores.

The first siderophore of this class, rhizobactin, containing three carboxyl and one hydroxyl function, was isolated from a strain of *Rhizobium meliloti* [30]. Another example is staphyloferrin A, containing two citryl residues linked by amide bonds to D-ornithine [14,22]. Staphyloferrin B, likewise produced by numerous staphylococci [13], is not a homolog of staphyloferrin A, but is instead unsymmetrically built with only citric acid in common with staphyloferrin A [10].

In fungi, previously known to produce only hydroxamate type siderophores, a new carboxylate siderophore, rhizoferrin, has been detected [12]. Rhizoferrin functions as the main siderophore of the Zygomycetes. Its production, isolation and structure will be described in this review. Moreover, a directed fermentation has been developed to produce a considerable number of analogs. The constituent building blocks of rhizoferrin, 1,4-diaminobutane and citric acid, could thus be modified in a wide range.

PRODUCTION AND ISOLATION OF RHIZOFERRIN

Rhizopus microsporus var. *rhizopodiformis* has been reported to be the causative agent of mucormycosis in dialysis patients after therapy with the iron chelator desferrioxamin B [6,36]. This is due to the ability of the fungus to utilize the exogenous siderophore ferrioxamine B as an iron supply [5]. This observation led us to examine the fungus' specific siderophore. As a result, a novel carboxylate type siderophore, rhizoferrin, was isolated [12].

Although rhizoferrin (Fig. 1) represents a fairly simple molecule, it may have potential application in biotechnology due to its appreciable metal-binding properties and the ability to be easily degraded by various microorganisms. *Rhizopus microsporus* var. *rhizopodiformis* was grown on a glucose/asparagine/salts medium under iron-limiting conditions [40]. Since neither hydroxamate nor catecholate siderophores could be detected, the organic acid fraction was analyzed. The culture filtrate was first passed through an Amberlite, XAD-2 (ICN, Costa Mesa, CA, USA) column. Then the acidic components were purified by a two step ion exchange procedure with Dowex 50WX8 and Dowex 2X8 and subsequent gel permeation chromatography with Biogel P2 (BioRad, Richmond, CA, USA). Fractions positive on chrome azurole S plates were collected and lyophilized. Final purification was achieved on reversed phase HPLC with a gradient of water/acetonitrile yielding a pure compound.

By this procedure, rhizoferrin could later also be obtained from strains of *Mucor mucedo* and *Phycomyces nitens* (Mucoraceae), *Chaetostylum fresenii* and *Cokeromyces recurvatus* (Thamnidiaceae), *Cunninghamella elegans* and *Mycotypha africana* (Choanephoraceae), *Mortierella vinacea* (Mortierellaceae) and *Basidiobolus microsporus* (Basidioboloceae, Entomophthorales) [33].

STRUCTURE OF RHIZOFERRIN

Rhizoferrin consists of two molecules of citric acid linked to 1,4-diaminobutane through two amide bonds (Fig. 1). The hydrolytic fragments could be determined by derivatization and gas chromatography with mass spectrometric identification. The connectivity of the components was determined by nuclear magnetic resonance spectroscopy. Details of the structure elucidation are described in [12]. Although rhizoferrin appears constitutionally symmetric, it contains two chiral centers in the citric acid residues. The configuration of the quaternary carbon atoms could be determined by CD (circular dichroism) spectroscopy of aqueous solutions of the iron free ligand and comparison with reference compounds of known chirality [11]. It was found that rhizoferrin is naturally produced with R,R-configuration. The implications for biosynthesis will be discussed below.

The iron complex of rhizoferrin showed a metal to ligand charge transfer band at 335 nm which was found for all members of the rhizoferrin family and is thus characteristic for purely carboxylate-type siderophores. The pH stability of the iron complex was estimated by pH-dependent UV spectroscopy. The charge transfer band at 335 nm appeared at pH 4, reached maximum intensity at pH 5.5 and remained unchanged to a pH of 9. This agrees well with the spectrophotometric titrations of rhizoferrin and its iron complex, performed by Rochel [28]. The iron complex of rhizoferrin showed a positive Cotton effect at 340 nm. This is in good agreement with the positive absorption of ferric complexes of the ferrichromes which by comparison with known crystal structures have been assigned a Λ-configuration (the donor atoms are arranged in the shape of a left-handed propeller) around the iron center [37]. The CD spectra of rhizoferrin are thus indicative for a Λ-configuration around the metal center [11]. Acidic solutions of rhizoferrin gradually decompose with formation of two dehydration products, the structures of which have been determined [11]. The first dehydration product, named imido-rhizoferrin, is formed by cyclization of one citryl residue to a succinimidyl derivative (Fig. 2). The chirality of the quaternary carbon atom is retained. The second dehydration product is cyclized on both citryl residues and was therefore named bisimido-rhizoferrin (Fig. 3). Both compounds can be easily detected by capillary electrophoresis in coated capillaries (Fig. 4). In citric acid buffer, 20 mM, pH 2.5, rhizoferrin has a slight net negative charge. This is in agreement with titration experiments of the ligand [28]. Referenced against the neutral marker mesityl oxide, rhizoferrin showed a shorter retention time. Imido-rhizoferrin has fewer negative charges, since it has lost one carboxy group by cyclization to the succinimidyl derivative. Its peak is found between rhizoferrin and the neutral marker. Bisimido-rhizoferrin has one less free carboxy group and is found between imido-rhizoferrin and the neutral marker. The relative amounts of the imido forms increase upon standing in citric acid buffer at pH 2.5. A similar behavior has been observed with all analogs of rhizoferrin described below.

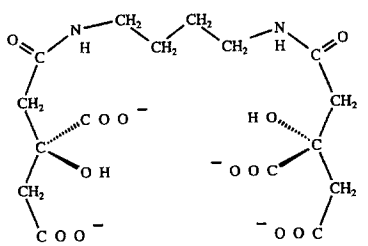


Fig. 1. Structural formula of rhizoferrin.

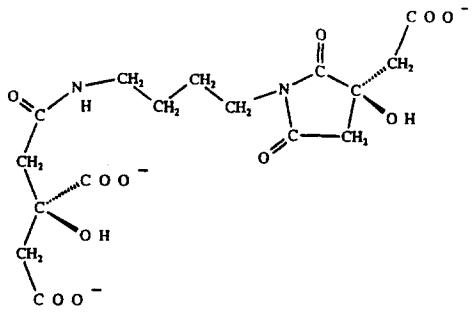


Fig. 2. Structural formula of imido-rhizoferrin.

DIRECTED FERMENTATION OF RHIZOFERRIN ANALOGS

In directed fermentation, building blocks or analogs of constituents of a targeted biomolecule are added to the growth

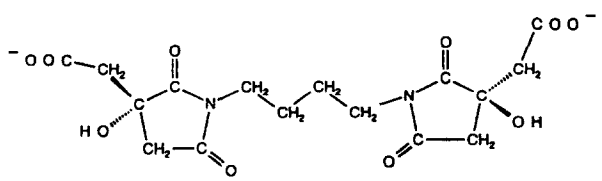


Fig. 3. Structural formula of bisimido-rhizoferrin.

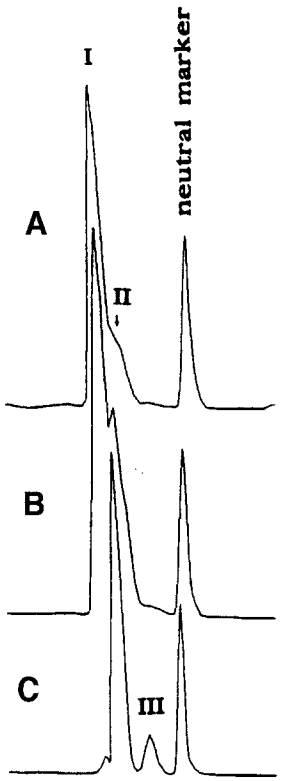


Fig. 4. Capillary electrophoretic separation of rhizoferrin (I), imido-rhizoferrin (II) and bisimido-rhizoferrin (III). (A) Separation of a purified rhizoferrin sample which still contains a small amount of imido-rhizoferrin. (B) Separation of rhizoferrin after incubation in citric acid buffer (pH 2.5 at 25 °C, 1 day), resulting in an increase of imido-rhizoferrin. (C) Separation of a sample of imido-rhizoferrin after incubation for 1 day in citric acid buffer (pH 2.5, 25 °C, 1 day) showing residual amounts of rhizoferrin and bisimido-rhizoferrin. Fused silica capillary, 72 cm × 50 μm, coated with μ-Coat (Applied Biosystems, Weiterstadt, Germany), 15 kV, polarity negative at inlet side, buffer 20 mM citric acid, pH 2.5, detection by UV absorption at 200 nm.

medium of the producing strain. Provided the precursors are taken up into the cell and linked into the biosynthetic pathways, this will result in altered and often increased levels of production of the biomolecule. By this means, isotopically labelled precursors or constitutionally similar constituents can be incorporated into biomolecules and analogs of the naturally occurring biomolecule can be obtained by isolation of the fermentation products.

In the case of rhizoferrin, two components can be modified. First, the chain length of the diamine can be varied and branched diamines and diamines with additional functional groups may be introduced. Second, citric acid may be replaced by similar molecules with fewer or different functional groups, affecting strongly the complexing properties of the resulting siderophore analogs. In both cases, a number of analogs of rhizoferrin have been obtained.

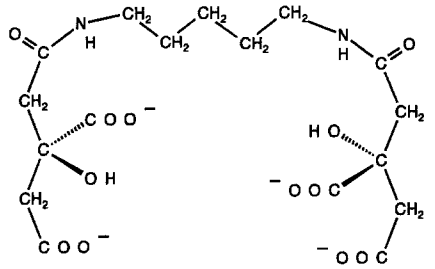
The fermentative production of rhizoferrin was carried out with several producer strains (see above). From these, *Cunninghamella elegans* and *Mucor rouxii* were chosen for an optimization of the fermentation process. Finally *Cunninghamella elegans* was used for production of the analogs described below. Starting with a low rhizoferrin-concentration (about 50 mg L^{-1}), the yield of rhizoferrin was increased to 800 mg L^{-1} in flask cultures. This was achieved by variation of the type of inoculum and the medium composition. The production of rhizoferrin and its derivatives, like other siderophores, occurred simultaneously with biomass formation in the trophophase. As expected, the biosynthesis of the rhizoferrins was strongly regulated by the iron content of the medium. The addition of 10 μM iron(III) decreased production to 10–15%, although growth was not affected.

Diamine analogs of rhizoferrin

Chain elongation as well as chain shortening was achieved by feeding appropriate precursors to the producing strain. Addtion of 1,5-diaminopentane (cadaverine) resulted in the production of an analog with a bridge of five methylene units, named homorhizoferrin (Fig. 5). 1,3-diaminopropane yielded an analog with three methylene units, named norrhizoferrin (Fig. 6). Product formation and yield of both analogs were approximately the same (see Table 1). The structures were confirmed by gas chromatographic detection of 1,5-diaminopentane and of 1,3-diaminopropane in the hydrolyzates of homorhizoferrin and norrhizoferrin, respectively. The calculated masses of the new compounds were confirmed by pneumatically assisted electrospray mass spectrometry and by fast atom bombardment mass spectrometry. The proton and ^{13}C NMR signals of both compounds have been completely assigned [9]. The iron-free ligands of homorhizoferrin and norrhizoferrin have been studied by circular dichroism spectroscopy. The chirality of the citric acid residues manifests itself in the overall chirality of the desferri ligands. At 205 nm, a negative Cotton effect demonstrates the chirality of the asymmetric centers of citric acid in both analogs to be the same as in rhizoferrin, earlier found to be R,R [11].

The CD spectra of the iron complexes at different pH showed a minimum at 310 nm and a maximum at 370 nm, indicating a Λ-configuration around the metal center. The shape of the CD-bands of ferri-homorhizoferrin was approximately the same as for ferri-rhizoferrin, whereas the Cotton effect of ferri-norrhizoferrrin showed only about half that intensity. This correlates with the observation of likewise reduced intensity of the charge transfer band of ferri-norrhizoferrin measured by UV/Vis spectroscopy. These findings indicate a strained geometry of the coordination sites around the iron center in the case of norrhizoferrin, whereas the complexation of homorhizoferrin is apparently not influenced by its elongated diamine bridge. This will be further evaluated by spectrophotometric titration of the ligands and calculation of the complex formation constants of the ferric complexes, as has been done in the case of rhizoferrin [28].

The variation in chain length has been continued by the substitution of the middle methylene group of cadaverine by an oxygen atom. Feeding of the precursor bis-(2-aminoethyl)-ether to cultures of *Cunninghamella elegans*, resulted in the production of an analog named oxahomorhizoferrin (Fig. 7). Product formation was approximately one third of the diaminoalkanes described above. Yield of the new derivative showed a decrease at higher concentration of precursor, indi-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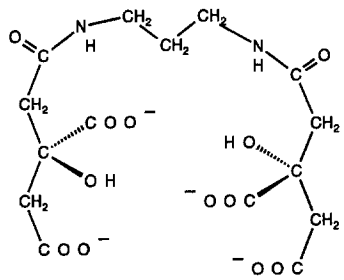
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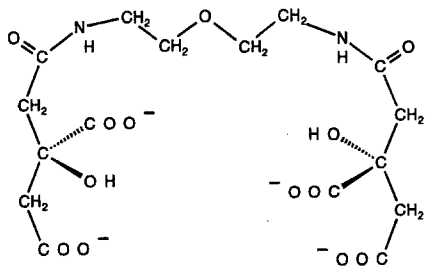
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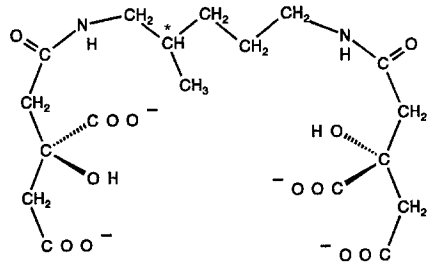
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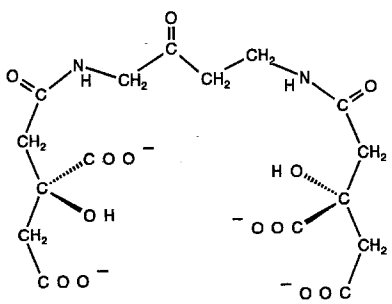
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Figs 5–9. Structural formulae of rhizoferrin derivatives modified in the diamine backbone: (5) homorhizoferrin; (6) norrhizoferrin; (7) oxahomorhizoferrin; (8) 2-methylhomorhizoferrin; (9) 2-oxorhizoferrin.

cating a slight inhibitory effect (data not shown). The identity of the new compound was confirmed by pneumatically assisted electrospray mass spectrometry and by fast atom bombardment mass spectrometry. Bis-(2-aminoethyl)-ether was confirmed in the hydrolyzate by gas chromatography and GC-MS-coupling. Capillary electrophoresis confirmed the acid catalyzed formation of two dehydration products, as demonstrated in the case of rhizoferrin.

Branching of the diamine backbone resulted in considerably smaller production of the corresponding rhizoferrin analog (Table 1). After feeding 1,5-diamino-2-methylpentane (2-methylcadaverine) to cultures of *Cunninghamella elegans*, a new hydrophobic compound was detected in the culture filtrate. It was named 2-methylhomorhizoferrin (Fig. 8) and revealed the highest retention time of all rhizoferrin analogs during reversed phase HPLC. After purification by ion exchange, gel permeation and HPLC, its identity could be confirmed by pneumatically assisted electrospray mass spectrometry and by fast atom bombardment mass spectrometry. 2-Methylcadaverine was identified in the hydrolyzate by gas chromatography and referencing with a standard. ^{1}H and ^{13}C NMR signals could be completely assigned [9]. CD spectroscopy showed R,R-configurated citryl residues and a Λ-configuration around the metal center. Introducing a functionality into the backbone of rhizoferrin will allow the chemical modification and derivatization of the siderophore. Radioactive or nonradioactive labels, fluorescence markers and conjugates with antibiotics can be synthesized. 1,4-Diamino-2-butanone has been described as a potent inhibitor of Ornithindecarboxylase (ODC) in *Aspergillus nidulans* [31] and in Mucorales [20]. Therefore its use as a precursor in directed fermentation of rhizoferrin analogs revealed interesting consequences. Thus, by inhibiting ornithine decarboxylase, the synthesis of rhizoferrin itself was greatly reduced, and the new analog, 2-oxorhizoferrin, (Fig. 9) was preferably synthesized. The final concentration was nearly 500 mg L^{-1} (Table 1). The structure of 2-oxorhizoferrin was proven by gas chromatographic detection of ketoputrescine in the hydrolyzate. The calculated mass was confirmed by pneumatically assisted electrospray mass spectrometry and by fast atom bombardment mass spectrometry. All ^{1}H and ^{13}C NMR signals of 2-oxorhizoferrin have been completely assigned [9]. 2-Oxorhizoferrin is the only analog which is distinguishable in UV spectroscopy from rhizoferrin and all other analogs. The carbonyl group causes an absorption band at 275 nm. Capillary electrophoresis revealed the existence of two imido compounds, due to the unsymmetric diamine. Although these two imido-oxorhizoferrins could also be detected, they have not been preparatively separated by HPLC so far.

Tricarboxylic acid analogs of rhizoferrin

Since the biosynthesis of rhizoferrin was quite tolerant to variations of the bridging diamine compound, we tried to alter the carboxylic acid component of the siderophore as well. In this case an even greater variety of molecules similar to citric acid can be imagined. It may be possible to vary the distance between the complexing groups and the bridging diamine, to vary the geometry and rigidity of the di- or tricarboxylic acid,

TABLE 1

Production formation and yield of rhizoferrin and new derivatives produced by directed fermentation in flask cultures

Precursor (20 mM)	Product formation[a] (%)	New derivative (mg L^{-1})	Rhizoferrin (mg L^{-1})
1,3-diaminopropane	3.0	253	619
1,5-diaminopentane	3.2	288	572
1,4-diamino-2-butanone	5.5	492	87
Bis-(2-aminoethyl)-ether	1.0	88	541
1,5-diamino-2-methylpentane	0.6	53	830
Tricarballylic acid[b]	1.1	92	419
Methyltricarballylic acid[b]	0.5	45	405
Control (no precursor added)	–	–	826

[a] Precursor concentration added to the medium was set at 100%.
[b] Values are given only for the monosubstituted derivatives.

to omit complexing groups or exchange them against different donors.

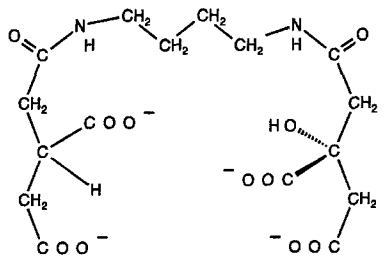
Since the mode of complexation of rhizoferrin is supposed to involve the hydroxy group of the quarternary carbon atom, we started the variations at this point. Desoxy-citric acid (tricarballylic acid) and 3-methyl-desoxy-citric acid (methyltricarballylic acid) are devoid of the donating hydroxy group while retaining the number and positions of the carboxy groups. Addition of tricarballylic acid to the medium resulted in the appearance of a number of new siderophores. The new compounds, positive in the complexation assay with chrome azurole S, were purified by ion exchange and HPLC procedures as described for rhizoferrin. The yield of acid analogs was considerably less than for most of the diamine analogs (Table 1). The principal compound besides rhizoferrin was identified by mass spectrometry and gas chromatography to be monodesoxyrhizoferrin (Fig. 10), where only one citric acid residue was exchanged by desoxy-citric acid. The disubstitution product, didesoxyrhizoferrin (Fig. 11), was present in only very small amounts and could not be separated by reversed phase HPLC from the accompanying imido-rhizoferrin. The structure of monodesoxyrhizoferrin was confirmed by gas chromatography of its hydrolysis products, which contained citric acid and tricarballylic acid in approximately equal amounts. Its ^{1}H and ^{13}C NMR signals could be fully assigned [9].

Only one imido form of monodesoxyrhizoferrin could be detected in capillary electrophoresis. Although the unsymmetric molecule can give two imido forms, no splitting of the peak was observed. The amount of bisimido-monodesoxyrhizoferrin was below the detection limit, even after 7 days at room temperature in citric acid buffer, pH 2.5. From this we conclude that the tricarballylic residue is less prone to cyclization, which might be explained by the reduced acidity of the middle carboxy group. The hydroxy group in α-position to the carboxy group increases its acidity by approximately one unit [28].

The CD spectra of the free ligand of monodesoxyrhizoferrin were recorded in aqueous solutions and showed the characteristic minimum at 205 nm. This Cotton effect, which is related to the absolute configurations of the quaternary carbon atoms in the acid residues, was less intense than for the analogs containing two citric acid residues with R configuration. Compared with the slightly positive effect of S-2-methylbutyric acid at acidic pH, this might imply the presence of tricarballylic amide with S configuration, which has identical absolute configuration as R-citric acid. This is however a preliminary interpretation and has to be substantiated by purification and measurement of didesoxyrhizoferrin which contains two tricarballylic acid residues.

CD spectra of the ferric complex of monodesoxyrhizoferrin, recorded in water in a series of increasing pH, showed differences compared to rhizoferrin. The minimum at 295 nm had a much smaller amplitude as found in rhizoferrin, the maximum at 360 nm had disappeared. This may be due to a less rigid geometry of the pentadentate ligand leading to an equilibrium between Λ and Δ complexes. However, this needs to be confirmed by comparison of the CD data of didesoxyrhizoferrin and additional physicochemical measurements of both ligands.

Feeding 3-methyldesoxy-citric acid to the cultivation medium of *Cunninghamella elegans* gave similar results as in the case of desoxy-citric acid. The main compound produced was the monosubstitution product, monomethylmonodesoxyrhizoferrin (Fig. 12). Dimethyldidesoxyrhizoferrin (Fig. 13) was present in only small amounts and could be separated from imido-rhizoferrin by reversed phase HPLC. Product formation of the new siderophores was even less than in the case of tricarballylic acid (Table 1). The expected masses of both new compounds were confirmed by electrospray and FAB mass spectrometry. Gas chromatography of the hydrolyzate of monomethylmonodesoxyrhizoferrin showed the presence of methyltricarballylic acid and citric acid in equimolar ratio. The ^{1}H and ^{13}C NMR signals of monomethylmonodesoxyrhizoferrin have been completely assigned [9]. The CD data are similar to those of monodesoxyrhizoferrin. The interpretation of the chirality of the acid residues and of the configuration around the metal center will be determined when a sufficient amount of dimethyldidesoxyrhizoferrin is available.



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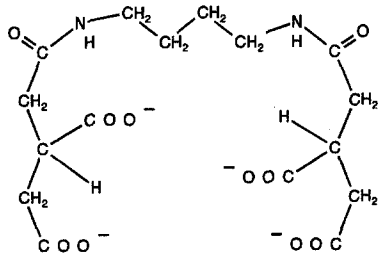

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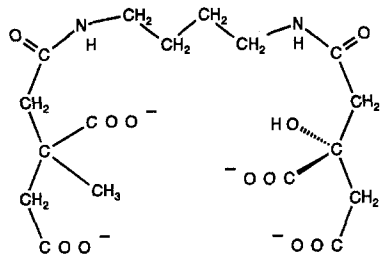

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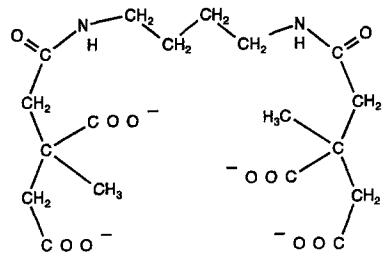

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Figs 10–13. Structural formulae of rhizoferrin derivatives modified in the citryl residues: (10) mono-desoxyrhizoferrin; (11) didesoxyrhizoferrin; (12) monomethylmonodesoxyrhizoferrin; (13) dimethyldidesoxyrhizoferrin.

Biological activity

All of the new rhizoferrin analogs were tested for their ability to act as siderophores. The bioassay was carried out as a growth promotion assay on agar plates incubated with *Morganella morganii* 13 and *Morganella morganii* SBK3 as described earlier [34]. The medium was iron-depleted by the addition of bipyridyl. All rhizoferrin analogs produced growth zones, indicating their ability to compete with bipyridyl in iron complexation and relieve the iron-restricted growth by iron transport into the cells of the indicator strains. The growth zones of the diamine analogs were comparable to or slightly less than the growth zones observed for rhizoferrin itself. The acid analogs showed smaller growth zones.

Inhibitor studies

The synthesis of putrescine in bacteria, fungi and plants proceeds by decarboxylation of ornithine, mediated by ornithine decarboxylase. In bacteria and plants decarboxylation of arginine may also lead to putrescine, involving arginine decarboxylase (ADC) giving agmatine which is then hydrolyzed by agmatine ureohydrolase to produce putrescine. In the fungus *Neurospora crassa*, arginine is converted to putrescine via ornithine, as has been shown by Davis et al. [7]. Also in other fungi studied [21,32], putrescine appears to be exclusively synthesized as a result of ODC activity. In order to provide the participation of ODC in the biosynthesis of rhizoferrin by *Cunninghamella elegans*, we investigated the effect of several well known inhibitors of ornithine decarboxylase. The inhibitors and their mode of action are shown in Table 2.

The most effective inhibitors of rhizoferrin biosynthesis were 2-difluoromethylornithine and 1,4-diamino-2-butanone, the addition of which resulted in a decrease of the concentration of rhizoferrin to about 4% and 8%, respectively. 2-Methylornithine had much less effect on the biosynthesis of rhizoferrin (reduction to about 50%), and 1,3-diaminopropane had no effect. Unexpectedly, the growth of *Cunninghamella elegans* was apparently not influenced by 2-difluoromethylornithine, 2-methylornithine and 1,3-diaminopropane, whereas 1,4-diamino-2-butanone reduced the growth to about 40%. These findings may be explained by the results obtained by Martinez-Pacheco and Ruiz-Herrera [19], who showed that the different effects of 1,4-diamino-2-butanone and 2-difluoromethylornithine on ODC is due to the differential accessibility of the inhibitors. From the results obtained with the four inhibitors of ODC it seems clear that ODC is the enzyme which is responsible for putrescine formation in *Cunninghamella elegans*. Furthermore, the supplementation of the medium with 1,4-diamino-2-butanone and 1,3-diaminopropane resulted in the production of new analogs of rhizoferrin, as described above (2-oxorhizoferrin, norrhizoferrin).

TABLE 2

Ornithinedecarboxylase inhibitors and their mode of action

Inhibitor	Mode of action
2-methylornithine	competitive inhibitor [1]
1,4-diamino-2-butanone	competitive inhibitor [31]
1,3-diaminopropane	inhibitor [26]
2-difluoromethylornithine	mechanism-based inhibitor [24]

Differential effects of D- *and* L-*ornithine on the biosynthesis of rhizoferrin*

As expected, supplementation of the optimized production medium with the presumed putresine precursor L-ornithine resulted in a higher concentration of rhizoferrin (128%). In contrast, the addition of D-ornithine instead of the L-isomer resulted in a reduction of the concentration of rhizoferrin to less than 60%. Further experiments are necessary to permit definitive conclusions whether D-ornithine acts as a (competitive) inhibitor.

Implications for biosynthesis

The variations in chain length of the diamine backbone were very well tolerated. Branching or functionalization in β-position to the amino group of the diamine compounds was also accepted. However it was not possible to introduce α-amino acids. Therefore the biosynthesis of rhizoferrin must be different to the biosynthesis of staphyloferrin A, produced by staphylococci. Neither by feeding of D-ornithine nor by application of inhibitors of ornithine decarboxylase, with and without simultaneous addition of D-ornithine, was it possible to detect even trace amounts of staphyloferrin A in *Cunninghamella elegans*.

The chirality of the citric acid residues was the same in all diamine analogs and the same on both sides of the diamine. This points to a common precursor for both citryl amides in each rhizoferrin analog. The easy interchangeability of the diamine constituents may be interpreted as the diamines being nucleophilic substrates for an activated form of citric acid with defined chirality.

A higher degree of enzyme specifity seems to be involved in the formation of the activated citryl species since analogs of citric acid are more difficult to be incorporated into rhizoferrin analogs than diamines. Competition by citric acid, a key compound of the citric acid cycle, might also explain the comparatively low incorporation of tricarballylic acid and methyltricarballylic acid into the rhizoferrin analog.

It is interesting to note that citryl-CoA, an intermediate of citrate synthetase [15,18] and ATP citrate lyase [16], has S configuration [3,17]. Aminolysis will, by exchange of priority of the substituents, give citryl amides with R configuration. Citryl-1-phosphate, which has been described as a first intermediate of ATP citrate lyase [39], would likewise give R-configured rhizoferrins through nucleophilic substitution by a diamine compound. Tricarballylic acid is also a substrate for ATP citrate lyase [38], tricarballyl-CoA being an intermediate.

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