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Rhizoferrin - a novel siderophore from the fungus *Rhizopus microsporus* var. *rhizopodiformis*

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Summary. From a strain of *Rhizopus microsporus* var. *rhizopodiformis* a novel siderophore, named rhizoferrin, was isolated by ion-exchange column chromatography, gel filtration and preparative HPLC. Hydrolysis with 6 M HCl and subsequent gas chromatography/mass spectrometry (GC/MS) of the esterified/trifluoroacetylated derivatives indicated that citric acid and diaminobutane were the only constituents. From positive fast-atom-bombardment (FAB) and ion-spray tandem mass spectrometry, a molecular mass of 436 Da and the assignment of several daughter ion fragments could be obtained, which indicated the presence of two citric acid residues and one diaminobutane residue. NMR studies finally confirmed N^1,N^4-bis(1-oxo-3-hydroxy-3,4-dicarboxybutyl)-diaminobutane as the structure of rhizoferrin. The iron-binding property was demonstrated on chromeazurol S plates and its siderophore activity was confirmed by iron transport measurements in young mycelia of *R. microsporus*. While rhizoferrin and also ferrioxamines B and E proved to be effective siderophores, coprogen was a poor siderophore in this fungus.

Key words: Rhizoferrin - *Rhizopus* - Siderophore - Iron transport

Introduction

Several fungal genera seem not to synthesize the commonly found hydroxamate siderophores although the requirement to sequester iron from the environment is obvious (reviewed by Winkelmann 1991). Thus we found that strains of *Rhizopus microsporus* var. *rhizopodiformis* (Mucorales, Zygomycetes; for taxonomic description see Schipper and Stalpers 1984) which are known as agents of mucormycosis, are unable to synthesize hydroxamate siderophores during iron deprivation. Several cases of mucormycosis in dialysis patients have been reported during the recent years suggesting that iron from the host can be utilized by *R. microsporus* especially when desferrioxamine is present (Windus et al. 1987; Segal et al. 1988; Boelaert et al. 1988; Van Cutsem and Boelaert 1989; Abe et al. 1990). Ferrioxamines are siderophores produced by procaryotic *Streptomyces* species. They have so far not been found within the realm of eucaryotic fungi and are also not taken up by a variety of fungi (Winkelmann and Zähner 1973; Wiebe and Winkelmann 1975). However, recent reports suggest that ferrioxamines may be utilized by some fungal groups, such as *Rhizopus* (Van Cutsem and Boelaert 1989) or *Geotrichum* (Mor and Barash 1990). Thus, although ferrioxamines are not synthesized by fungi, they may serve as siderophores in some fungal groups. In the present investigation we confirm, by radioactive labeling, that-iron is transported via ferrioxamines in *Rhizopus* but we also present evidence that *Rhizopus* synthesizes its own siderophore, rhizoferrin (Fig. 1), composed of citric acid and diaminobutane which transports iron even better than the ferrioxamines.

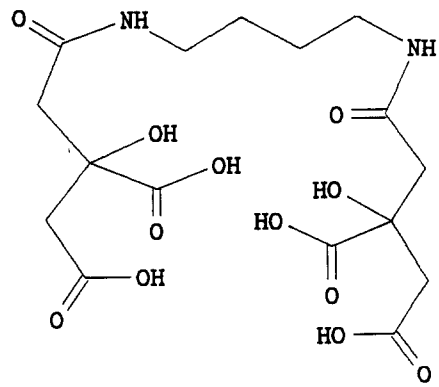

Fig. 1. Structural formula of rhizoferrin

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Materials and methods

Strains and cultivation conditions. The strain *Rhizopus microsporus* var. *rhizopodiformis* (B 58739) was provided by J. R. Boelaert (Brugge, Belgium) and was maintained on yeast/malt/glucose/agar containing, per litre, 4 g yeast extract, 10 g malt extract and 4 g glucose. Conidiospores were harvested by shaking with sterile 0.9% NaCl solution containing 0.1% Tween 80 (Serva, Heidelberg) and washed twice by repeated sedimentation. Conditions of siderophore production were exactly according to earlier protocols (Wiebe and Winkelmann 1975; Konetschny-Rapp et al. 1988). Briefly, asparagine/salts/glucose medium without additional iron salts was inoculated with a spore suspension and incubated on a rotary shaker at 27° C for 10–14 days.

Isolation of rhizoferrin. As no hydroxamate or catecholate siderophores could be detected, we decided to analyze the organic acid fraction of the culture filtrate after two weeks of growth in low-iron medium. The organic acids were isolated according to the method of Tokumitsu and Ui (1974) which we had used earlier for the isolation of organic acids in culture filtrates of *Neurospora crassa* (Winkelmann 1979). The culture filtrate was first passed through a Dowex 50WX8 column (H form) to remove the cations and then, after adjusting the pH to 6.8, through a Dowex 2X8 column (formate form). The organic acid fraction was eluted with 60% formic acid, evaporated to dryness and dissolved in 50 ml distilled water. A further purification was achieved on Bio-Gel P2 (Pharmacia, Freiburg). Rhizoferrin-containing fractions were detected by chromeazurol S plates according to Schwyn and Neilands (1987). The combined fractions were lyophilized yielding a slightly yellow product.

Analytical and semi-preparative HPLC. The crude rhizoferrin fraction was separated by HPLC (Nucleosil C_{18}, 5 µm, 250 × 4.6 mm; Grom, Herrenberg) using a gradient of 3–6% acetonitrile in water and a flow rate of 1 ml/min. Semi-preparative HPLC was performed on a column (250 × 8 mm) of Nucleosil C_{18} (5 µm), using a flow rate of 4 ml/min and 3 mg/injection (150 µl). The rhizoferrin peaks were collected and lyophilized yielding a clear white compound.

Gas chromatography and mass spectrometry (GC/MS). Purified rhizoferrin was hydrolyzed with 6 M HCl for 24 h and the hydrolysis products were either silylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Serva, Heidelberg) in pyridine (1:1, by vol.) and analyzed on an SE30 capillary column (80–150° C, 4° C/min) or subjected to esterification and *N*-trifluoroacetylation for subsequent GC/MS (MAT 112 S) measurements. The nitrogen-containing compound was additionally separated by GC on a Duran 50 glass capillary column (20 × 0.3 mm) coated with the chiral phase *N*-propionyl-L-valine-*tert*-butylamide polysiloxan (Chirasil-Val) and detected by a nitrogen-sensitive detector as described previously (Deml et al. 1984).

Mass spectrometry. Positive FAB spectra were recorded on a Varian-MAT 711 instrument coupled with an SS 200 data system. The FAB mass spectra were measured in a thioglycerol matrix. The temperature of the ion source was 35° C. Ion spray mass spectra (LC/MS) were recorded on a Sciex API III triple-quadropole mass spectrometer with 2400-Da mass range equipped with an ion spray ion source (Sciex, Toronto, Canada) under similar conditions as described previously (Berner et al. 1991a).

Spectroscopic measurements. ^{1}H-NMR spectra were recorded on a Bruker AM 500 instrument at 500 MHz (^{1}H-NMR) in H_2O/D_2O (9:1) at 300 K (size 64 K). Assignments were made by comparison with known compounds.

Transport measurements. Spores were grown for 12–24 h in low-iron asparagine/salts/glucose medium until short mycelia were seen under the microscope. The mycelial suspension was incubated with ^{55}Fe-labeled rhizoferrin, ferrioxamine B, ferrioxamine E and coprogen (2 µM, 4 kBq/nmol) at 27° C in a rotary shaker. Samples (0.5 ml) were removed at intervals, filtered through cellulose nitrate membrane filters (Sartorius, Göttingen) and rinsed with 10 ml cold 0.9% NaCl solution. The radioactivity was measured in a liquid scintillation counter and amounts calculated as nmol/mg dry mass.

Results

Ion-exchange chromatography of the culture filtrate yielded a crude product which contained a significant iron-binding activity when assayed on chromeazurol S-agar plates. Further purification on Bio-Gel P2 yielded a slightly yellow material which still contained two major peaks when separated by HPLC on a C_{18} reversed-phase column (Fig. 2). Using a semi-preparative C_{18} column (250 × 8 mm), the two main peaks at $t_R = 11$ min and $t_R = 16$ min were separated, lyophilized and tested for their iron-binding activity. The main peak at $t_R = 11$ min contained a significant iron-binding activity while the second peak at $t_R = 16$ min contained only a very small activity. Therefore, the main peak at $t_R = 11$ min (rhizoferrin) was isolated by semipreparative HPLC on a C_{18} column and yielding a highly purified white product. The electronic absorption spectrum of rhizoferrin (Fig. 3) showed absorption in the region of 200 nm. After adding ferric chloride, a shoulder at 280 nm appeared, indicating the presence of a ligand-to-metal-ion charge transfer band. The isolated rhizoferrin was then subjected to acid hydrolysis for 24 h in 6 M HCl. The dried hydrolysate, and also a chloroform extract at pH 9, was silylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine. Gas chromatography on an SE30 capillary column yielded two hydrolysis products of which citric acid was identified

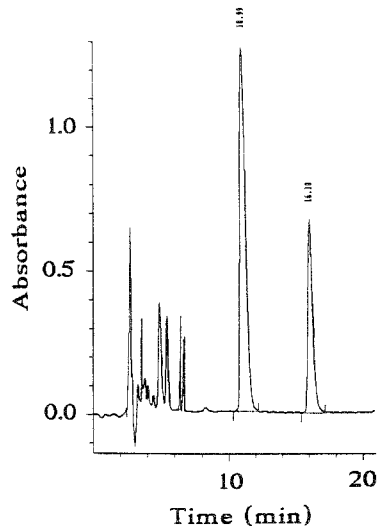

Fig. 2. HPLC chromatogram of the crude rhizoferrin fraction isolated by ion-exchange chromatography and gel filtration. The main peak at $t_R = 11$ min represents pure rhizoferrin. Semi-preparative column: RP 18 (Nucleosil, 5 µm, 250 × 8 mm), gradient: 3–6% acetonitrile in water, flow rate: 4 ml/min. Detector wavelength: 210 nm

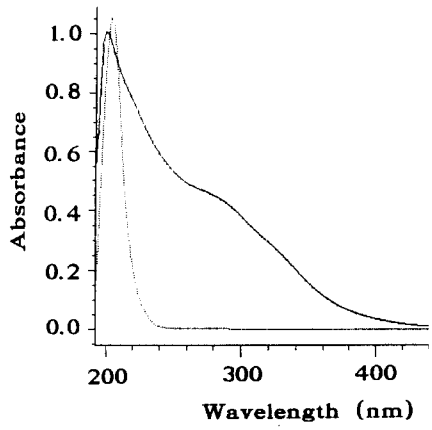

Fig. 3. Electronic absorption spectrum of rhizoferrin (...) and ferric rhizoferrin (——)

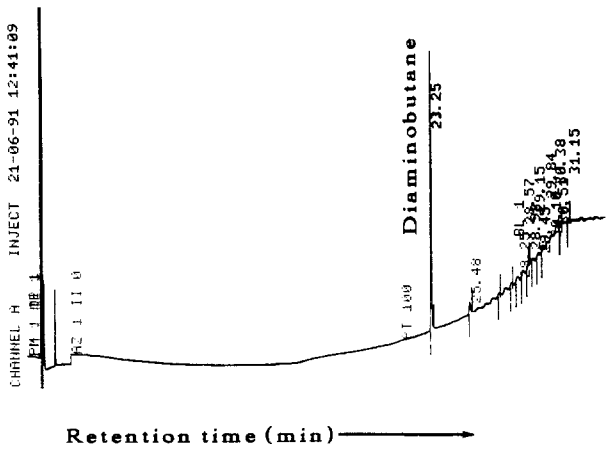

Fig. 4. Gas chromatogram of the *N*-trifluoroacetylated hydrolysis products from rhizoferrin. Column: Chirasil-Val (20 × 0.3 mm). Temperature program: 80–190° C, 3-min isotherm and then 4° C/min, carrier gas H_2, nitrogen-sensitive detector

by its retention time (data not shown). In addition trifluoroacetylation and GC separation on an *N*-propionyl-L-valine-*tert*-butylamide polysiloxan column equipped with a nitrogen-sensitive detector yielded diaminobutane (t_R = 23.25 min) as the only nitrogen-containing constituent (Fig. 4). GC/MS analysis confirmed the presence of diaminobutane showing a characteristic fragmentation of the trifluoroacetyl derivative: (*m/z*) 69, 97, 112, 140, 154, 168, 182, 211 and 280, as we described earlier for ferrioxamine G_2 in *Hafnia alvei* (Reissbrodt et al. 1990).

Positive fast-atom-bombardment spectra showed a quasi-molecular ion peak (MH^+) at *m/z* = 437, consistent with a molecular mass of 436 Da. Ion-spray mass spectroscopy (Fig. 5A) revealed a main mass peak at *m/z* = 459 which corresponded to MNa^+. Fragmentation of this peak by tandem mass spectrometry (Fig. 5B) yielded fragment ions which were assigned as follows: *m/z* 459 = MNa^+ (parent), *m/z* 285 = MNa^+ − citryl, *m/z* 197 = citrylNa^+, *m/z* 153 = citrylNa^+ − COO, *m/z* 111 = diaminobutaneNa^+. These data indicated that

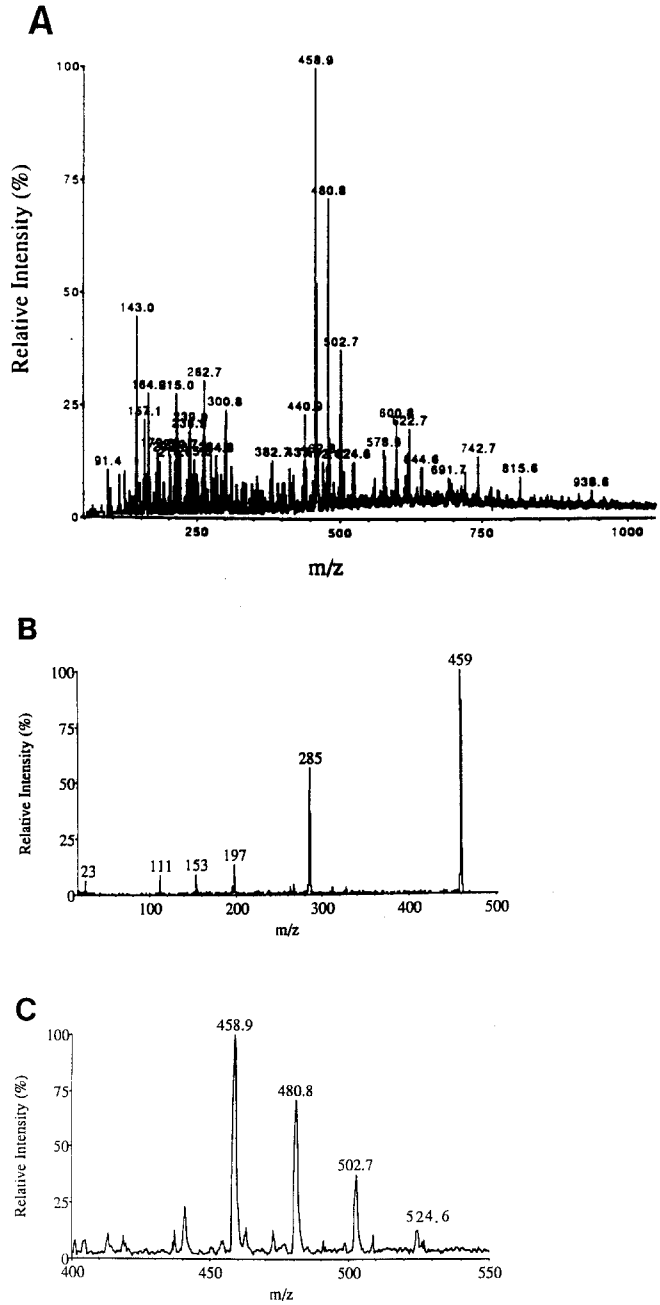

Fig. 5. Ion-spray mass spectra of rhizoferrin. (**A**) Total spectrum; (**B**) tandem mass spectrum of the parent Na adduct (*m/z* 459 MNa^+) and its ion fragments: *m/z* 285 = MNa^+ − citryl, *m/z* 197 = citryl Na^+, *m/z* 153 = citryl Na − COO, *m/z* 111 = diaminobutane Na^+; (**C**) Enlarged section in A

rhizoferrin consisted of one diaminobutane and two citric acid residues. As shown in Fig. 5C, a total of four Na-adduct parent ions (*m/z* 458.9 = M + 1Na^+, *m/z* 480.8 = M + 2Na^+, *m/z* 502.7 = M + 3Na^+, *m/z* 524 = M + 4Na^+) could be assigned, which corresponded to the presence of four carboxyl groups in the rhizoferrin molecule.

NMR spectra were performed to evaluate how the components identified by GC, GC/MS, LC/MS and amino acid analysis are connected. As shown in Fig. 6 and Table 1, the one-dimensional ^{1}H-NMR spectrum

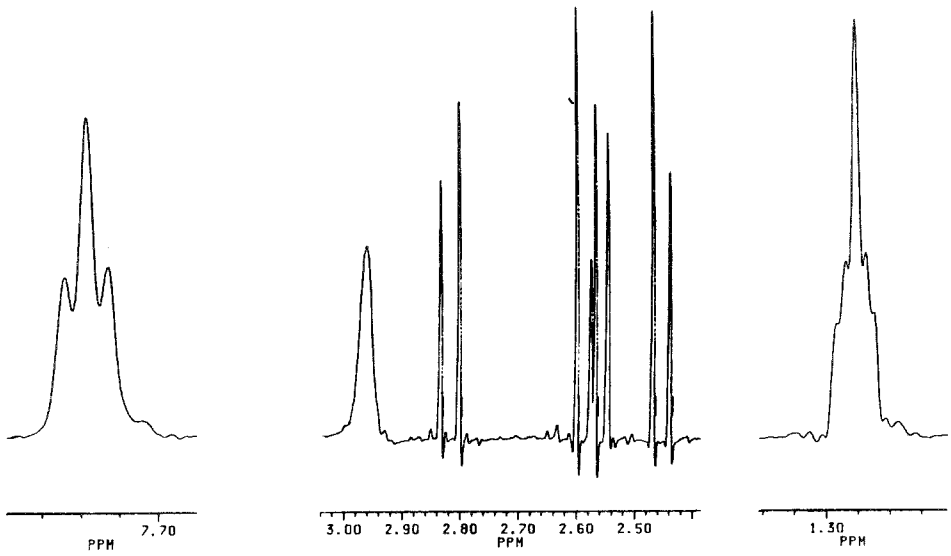

Fig. 6. ^{1}H-NMR spectrum of rhizoferrin in H_2O. For assignment see Table 1

in H_2O shows two double doublets, corresponding to the unit CH_2—X—CH_2. The methylene protons yielded a double doublet from coupling to each of the diastereotopic protons of the adjacent methylene unit. Since there are two systems of doublets, the methylene protons are not equivalent. By comparison of the chemical shifts with literature data, the unit CH_2—X—CH_2 could be identified as citric acid derivatized on one terminal carboxyl group. The signals at 2.96 ppm and 1.28 ppm show partial multiplet structure representing two symmetrical methylene units (integration: 4 H). The chemical shift assigns these signals to the methylene protons on C-1 and C-2 of diaminobutane, respectively. The observed line broadening results from the quadrupole moment of the neighbouring nitrogen nuclei which even affect the β protons on C-2 of the diaminobutane as observed earlier in the ferrioxamines (Reissbrodt et al. 1990; Borgias et al. 1989). The amide protons show a triplet at 7.738 ppm. Since there are no further signals, the ^{1}H-NMR spectrum reveals the total symmetry of the compound even at very high resolution (si = 64 K). From these findings and from the ratio of two moles citric acid/mole diaminobutane, we infer that rhizoferrin must be built from citric acid - diaminobutane - citric acid linked by amide bonds.

Transport experiments with ^{55}Fe-labeled rhizoferrin revealed rapid uptake of labeled iron, indicating siderophore activity of rhizoferrin in this fungus (Fig. 7). A concentration of 2 μM ferric rhizoferrin in the incubation medium revealed iron uptake values of approxi-

Table 1. ^{1}H-NMR data of rhizoferrin

Chemical shift (ppm)	Coupling constant, J (Hz)	Assignment
1.283 (m, 4H)		C-2 methylene protons, diaminobutane
2.439 (d, 1H)	14.45	Cit-(C_q-CH_uH-CONH-)
2.469 (d, 1H)	14.45	Cit-(C_q-CHH_d-CONH-)
2.547 (d, 1H)	14.32	Cit-(C_q-CH_uH-CONH-)
2.568 (d, 1H)	15.99	Cit-(C_q-CH_uH-COOH)
2.576 (d, 1H)	14.32	Cit-(C_q-CHH_d-CONH-)
2.600 (d, 1H)	15.99	Cit-(C_q-$CHHH_d$-COOH)
2.802 (d, 1H)	16.01	Cit-(C_q-CH_uH-COOH)
2.834 (d, 1H)	16.01	Cit-(C_q-CHH_d-COOH)
2.962 (m, 4H)		C-1 methylene protons, diaminobutane
7.738 (t, 2H)		amide protons

C_q = quaternary carbon atom, H_u, H_d represent upfield and downfield proton resonances. m, t, d = multiplet, triplet, doublet

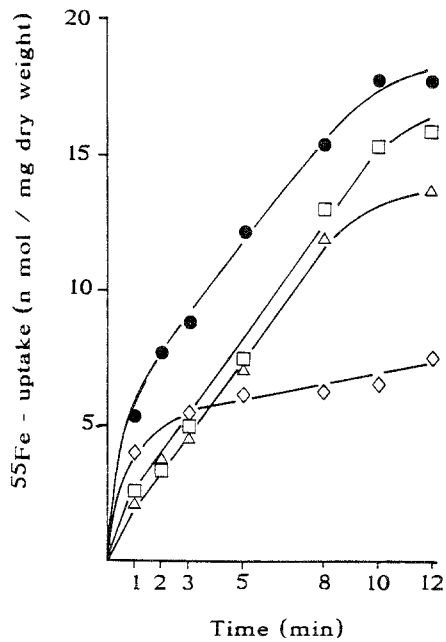

Fig. 7. Time-dependent uptake of ^{55}Fe-labeled siderophores in *Rhizopus microsporus* var. *rhizopodiformis*. Young hyphae grown from conidiospores after 12–24 h of incubation under low-iron conditions were incubated with 2 μM ^{55}Fe-labeled siderophores. At times indicated, 0.5 ml of the cell suspension was removed, filtered and the radioactivity measured. Values are expressed as nmol/min mg dry mass. (●) [^{55}Fe]Rhizoferrin; (□) [^{55}Fe]ferrioxamine B; (△) [^{55}Fe]ferrioxamine E; (◇) [^{55}Fe]coprogen

mately 1.4 nmol min^{-1} (mg dry mass)$^{-1}$ during the initial 1–10 min of incubation. Similar rates but somewhat lower total uptake values were found with ferrioxamine B and ferrioxamine E, both of which revealed comparable kinetics. However, coprogen was a poor siderophore in this fungus, showing an uptake rate of 0.3 mmol min^{-1} mg^{-1} during the linear phase of transport and a low total accumulation of iron (7.5 nmol/12 min), of which a considerable amount may be due to an initial adsorption to the fungal cells.

Discussion

Rhizoferrin, isolated from the fungus *Rhizopus microsporus*, might be the first polyhydroxycarboxylamide siderophore among fungi. Earlier examples of such siderophores are rhizobactin, isolated from *Rhizobium meliloti* (Smith et al. 1985) and staphyloferrin, isolated from *Staphylococcus* (Meiwes et al. 1990; Konetschny-Rapp et al. 1990). Rhizoferrin can be formally derived from staphyloferrin by decarboxylation of the ornithine carboxyl group. However staphyloferrin contains D-ornithine. It would be interesting to know whether D- or L-ornithine is incorporated into rhizoferrin with subsequent decarboxylation or whether the biosynthesis starts from putrescine after prior decarboxylation of ornithine. Diaminobutane (putrescine) is a well known precursor of polyamines in fungi (Tabor and Tabor 1985) and its inhibition will probably also affect rhizoferrin biosynthesis. The susceptibility of *Microsporum* and *Trichophyton* species to suicide inhibitors of polyamine biosynthesis has recently been shown (Boyle et al. 1988; Gruhn and Boyle 1991). Further investigations are underway to study the effect of ornithine decarboxylase inhibitors on rhizoferrin biosynthesis. As the motif of diamines flanked by two carboxylic acid residues is common in both rhizoferrin and ferrioxamines, recognition by a common transport system might be supposed. However, as there are no N-OH groups in rhizoferrin, the diamino backbones of both molecules are structurally different. Our preliminary competition studies revealed (data not shown) that the presence of ferrioxamines did not decrease but rather increased uptake of the ^{55}Fe label from rhizoferrin, which would argue against competitive behaviour between rhizoferrin and ferrioxamines. However, in the light of our recent findings that certain bacterial ferrioxamine transport systems accept *N*-hydroxy-diaminobutane (ferrioxamine G_2, D_2) and *N*-hydroxy-diaminopentane (ferrioxamine B, E) residues and even *retro*hydroxamate derivatives with different interchain length (Berner and Winkelmann 1990; Berner et al. 1991b; Reissbrodt et al. 1990), the existence of a transport system with a relatively broad recognition capacity cannot be excluded. Further studies are required to characterize the iron transport systems of *Rhizopus* and other Zygomycetes in more detail.

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