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Isolation and identification of hydroxamate siderophores of ericoid mycorrhizal fungi

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Three ericoid mycorrhizal fungi were grown in pure culture under iron deprivation: (i) the ascomycete *Hymenoscyphus ericae*, a characteristic endophyte of ericaceous plants on acid soils; (ii) the hyphomycete *Oidiodendron griseum*, an ericoid mycorrhizal fungus which is also a soil-borne fungus able to colonize wood; and (iii) an endophyte of the calciculous ericaceous plant *Rhodothamnus chamaecistus*. All three fungi produced several hydroxamate siderophores which were isolated in the ferric form by adsorption to Amberlite XAD-2, gel chromatography on Sephadex LH20 and by HPLC on a C_{18} reversed-phase column. Siderophores were identified by (i) co-chromatography with known fungal siderophores, (ii) ion spray mass spectrometry after semi-preparative HPLC and (iii) analyzing their electrophoretic behavior. While *H. ericae* and *O. griseum* were similar in producing ferricrocin as their principal siderophore, the endophyte of *R. chamaecistus* produced mainly fusigen.

Keywords: iron chelates, siderophores, mycorrhizal fungi, HPLC separation, ion spray mass spectroscopy

Introduction

With very few exceptions, almost all families of vascular plants contain species which form at least one type of mycorrhiza (Newman & Reddell 1987). The major kinds of mycorrhiza are the vesicular–arbuscular mycorrhiza, the mycorrhiza of the Ericales, the orchidaceous mycorrhiza and the ectomycorrhiza (Harley & Smith 1983, Moser & Haselwandter 1983). Within the Ericales, the ericoid mycorrhiza is the most prominent form. This mycorrhiza type occurs worldwide and dominates within the Ericaceae. It is also found in the Empetraceae and Epacridaceae (Read 1983). It is characterized by hyphal complexes which are formed within the cells of the root cortex by septate fungal hyphae which extend into the soil. On deep organic heathland soils, plant communities with ericoid mycorrhiza predominate (Read 1984).

Based on a bioassay with *Aureobacterium flavescens* JG-9 (previously named *Arthrobacter flavescens* JG-9) it was shown earlier that ericoid mycorrhizal fungi are capable of producing hydroxamate-type siderophores under pure culture conditions (Haselwandter *et al.* 1988, Schuler & Haselwandter 1988). In addition, it has been demonstrated that the pH, as well as supplementation of the nutrient medium with L-ornithine, have an effect upon siderophore biosynthesis (Federspiel *et al.* 1991). However, structures of these siderophores have not been elucidated so far. Hence, the objective of this study was to isolate and identify the main siderophores produced by typical ericoid mycorrhizal fungi. Thus, this report not only confirms the earlier findings of the occurrence of hydroxamate siderophores among mycorrhizal fungi, but also presents data on their structural identity with known fungal siderophores by using semipreparative HPLC and ion spray mass spectroscopy.

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Materials and methods

Ericoid mycorrhizal fungi

For the pure culture studies we selected two fungal strains which are typical for ericaceous plants growing on acid soils and one strain isolated from a calcicolous ericaceous plant: (i) the ascomycete *Hymenoscyphus ericae* (Read) Korf et Kernan, which is the perfect state of a characteristic endophyte commonly isolated from a wide range of ericaceous plants (Read 1974); (ii) the hyphomycete *Oidiodendron griseum* Robak, which was reported by Burgeff (1961), Couture *et al.* (1983) and Dalpe (1986) to form typical ericoid mycorrhiza in addition to being known as a common soil-borne fungus and a colonist of wood (Domsch *et al.* 1980). For comparative purposes we have included an endophyte which was isolated from the calcicolous ericaceous plant *Rhodothamnus chamaecistus* (L.) Rchb. growing on nearly neutral calcareous soil (Rendzina, pH 6.5). By re-inoculation of this endophyte into sterile seedlings of the original host, where it again formed the typical ericoid mycorrhiza, it was shown that this isolate is an ericoid mycorrhizal fungus (Haselwandter & Read 1983).

Culture conditions

The fungi were sub-cultured four times in Hagem's nutrient solution (Modess 1941) on a gyratory shaker (200 rpm) at 25 °C for 4 days. The fourth sub-culture was used to inoculate each of four 500-ml conical flasks containing 250 ml low-iron medium (= LIM1, Szaniszlo *et al.* 1981), deferrated with Chelex 100. The hydroxamate siderophores were isolated after 10 days of incubation in LIM1 under the same culture conditions as described above.

Siderophores

The following fungal siderophores were used for comparison during HPLC (listed with increasing retention times): tetraglycylferrichrome, ferricrocin, ferrichrome, ferrichrome C, asperchrome D1, coprogen, asperchrome B1, ferrirubin, ferrirhodin, triacetylfusarinine C. Most of these siderophores have been isolated earlier by our group from various fungal species. The asperchromes were kindly provided by Professor D. van der Helm (University of Oklahoma, Norman, USA). The structures of fungal siderophores have been compiled in a recent comprehensive review (Jalal & van der Helm 1991).

Isolation of siderophores

The culture filtrate obtained from 1 l nutrient medium was supplemented with $FeCl_3 \cdot 6H_2O$ (330 $mg\,l^{-1}$) and the solution was stirred until a brown colour had developed. The siderophores were adsorbed onto Amberlite XAD-2 (Sigma, München, Germany), washed with three volumes of distilled water and desorbed with one volume of methanol Gel filtration on a Sephadex LH20 column in methanol resulted in a further purification.

HPLC separation

The total hydroxamates were separated on a semipreparative C_{18} reversed-phase column (Nucleosil 7 μm, 8 × 250 mm) using a gradient of acetonitrile (6–40% within 19.2 min) and 10 mM ammonium acetate pH 3, according to Konetschny-Rapp *et al.* (1988). The separated siderophores were collected, lyophilized and analyzed by ion spray mass spectroscopy. Purified samples of fungal siderophores which had been identified earlier were used as a standard mixture for assignment and co-chromatography during HPLC (Figure 1).

Electrophoresis

Charged siderophores were separated by electrophoresis on Cellogel strips using 0.2 M pyridine/acetic acid pH 5.0 according to Diekmann & Zähner (1967), using a field strength of 150 V for 1 h and ferrioxamine B as a reference compound.

Ion spray mass spectroscopy

As identification by comparing retention times is often not reliable, the compounds were additionally isolated on a semi-preparative column (Nucleosil C_{18}, 7 μm,

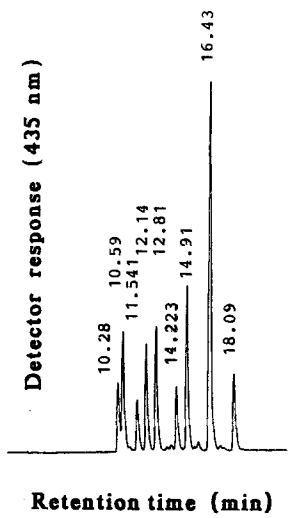


Figure 1. HPLC separation of a standard mixture of fungal siderophores on a semipreparative column (8 × 250 mm, Nucleosil 7 μm) using a gradient of acetonitrile (6–40% within 19.2 min) in 10 mM ammonium acetate pH 3, a flow rate of 3 ml/min, and a sample volume of 50 μl. The assignment of siderophores is as follows: 10.28 min (tetraglycylferrichrome), 10.59 min (ferricrocin), 11.03 min (ferrichrome or ferrichrysin, not shown), 11.54 min (ferrichrome C), 12.14 min (asperchrome D1), 12.81 min (coprogen), 14.22 min (asperchrome B1), 14.91 min (ferrirubin), 16.43 (ferrirhodin), 18.09 min (triacetylfusarinine C).

8 × 250 mm) and subsequently analyzed by ion spray mass spectroscopy using a Sciex API III triple quadrupole mass spectrometer with 2400 Da mass range equipped with an ion spray source (Sciex, Toronto, Canada) under similar conditions as described earlier (Berner *et al.* 1991, Drechsel *et al.* 1991).

Results

Hymenoscyphus ericae

As shown in the HPLC chromatogram in Figure 2, this fungus produces three hydroxamate-type siderophores: ferricrocin (II) as the principal siderophore as well as minor amounts of ferrichrome (I) and ferrichrome C (III). As estimated from the area percentage of HPLC chromatograms, ferricrocin amounted to about 83% of the total hydroxamates in the XAD-2 extract (Table 1). Ion spray mass spectroscopy (Figure 3a) of the total siderophores revealed three molecular ion adducts: $m/z = 771$ (MH^+), $m/z = 793$ (MNa^+) and $m/z = 809$ (MK^+), confirming the presence of ferricrocin (770 Da). The enlarged section of the mass spectrum between $m/z = 725$ and $m/z = 825$ (Figure 3b) shows two quasi-molecular ions (MH^+, $m/z = 741$, and MNa^+, $m/z = 763.6$) which additionally confirm the presence of ferrichrome (740 Da). As ferrichrome and ferrichrysin are indistinguishable during HPLC on reversed-phase materials (Konetschny-Rapp *et al.* 1988), ion spray mass spectroscopy allowed a clear identification in favor of ferrichrome. The small peak at $m/z = 755.2$ (Figure 3b) was assigned to MH^+ of ferrichrome C (754 Da).

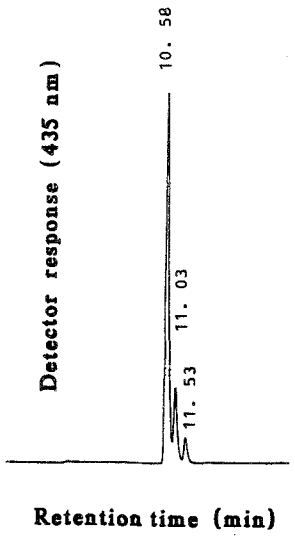
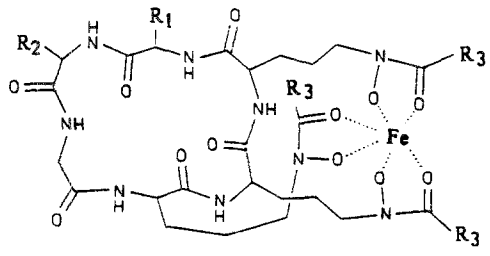


Figure 2. HPLC separation of hydroxamate siderophores produced by *H. ericae*. Assignments: 10.58 min (ferricrocin), 11.03 min (ferrichrome), 11.53 (ferrichrome C). Separation conditions are as described for the standard mixture in Figure 1.



I	Ferrichrome	$R_1 = R_2 = H$		$R_3 = CH_3$
II	Ferricrocin	$R_1 = H$	$R_2 = CH_2OH$	$R_3 = CH_3$
III	Ferrichrome C	$R_1 = H$	$R_2 = CH_3$	$R_3 = CH_3$

Oidiodendron griseum

HPLC separation of the total hydroxamates of this mycorrhizal fungus revealed three clearly detectable peaks and several minor peaks (Figure 4a). The peak at 12.78 min which is present in siderophore extracts not purified by gel filtration was found to be riboflavin and not coprogen (12.16 min, see Figure 1). As shown in Figure 4b, riboflavin is easily eliminated from the bulk of siderophores by gel chromatography on Sephadex LH20 in methanol. The main peak at 10.60 min (Figure 4a) was assigned to ferricrocin (80%) while the peak at 11.53 min represents ferrichrome C. Thus, the excreted siderophores from *O. griseum* were similar to those of *H. ericae* with the exception that ferrichrome C reached a higher proportion and ferrichrome was even absent in the culture filtrate of *O. griseum*. In addition, some minor peaks appeared in the total extracts of *O. griseum* which have not been analyzed further.

Endophyte of Rhodothamnus chamaecistus

The HPLC pattern of siderophores from this fungus (Figure 5) differed significantly from that of the other two typical ericoid mycorrhizal fungi *H. ericae* and *O. griseum*. The main peak at 8.07 min was isolated by semi-preparative HPLC and subsequently analysed by ion spray mass spectroscopy (Figure 6a) The observed quasi-molecular ion peaks ($MH^+ = m/z$ 780.9; $M2H^{2+} = m/z$ 391) are characteristic of fusigen (779 Da). The identity with fusigen (IV) was confirmed by its electrophoretic migration to the anode at pH 5 which was approximately 2.5 times the distance of ferrioxamine B containing only one positive charge/molecule. Fusigen, also called fusarinine C, has been reported

Table 1. Percentage of characteristic siderophores produced by ericoid mycorrhizal fungi

Siderophore	*Hymenoscyphus ericae*	*Oidiodendron griseum*	Endophyte of *Rhodothamnus chamaecistus*
Fusigen	–	<1	58
Ferricrocin	83	80	2
Ferrichrome	11	–	6
Ferrichrome C	5	19	–

Values are given as the area of the HPLC peaks as a percentage of the total area.

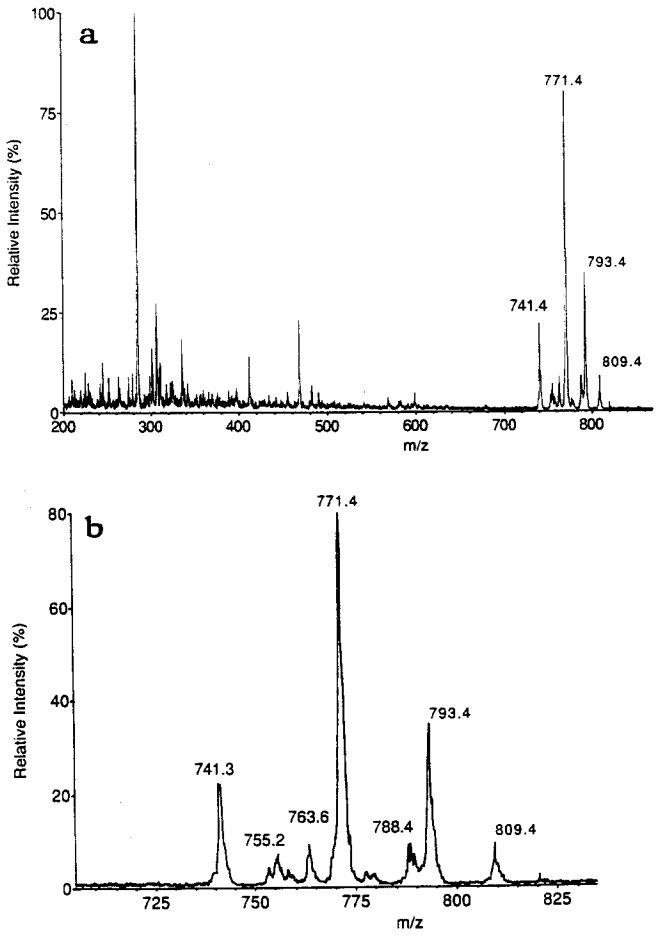


Figure 3. Ion spray mass spectra of the principal siderophores of *H. ericae*. (**a**) Total spectrum showing the quasi-molecular ion peaks of ferricrocin (m/z = 771.4 MH^+, m/z = 793.4 MNa^+ and m/z = 809.4 MK^+). (**b**) Enlarged section of the above spectrum. Assignments: m/z = 741.3 (MH^+) and m/z = 763.6 (MNa^+) ferrichrome and m/z = 755.2 (MH^+ ferrichrome C. Conditions are as described in Materials and Methods.

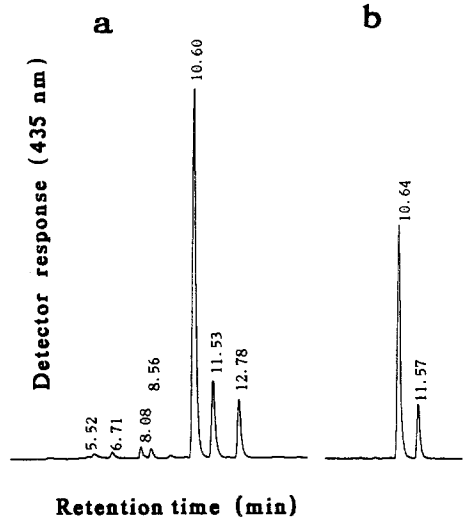


Figure 4. HPLC separation of siderophores of *O. griseum*. Assignments of peaks: (**a**) 10.60 min (ferricrocin), 11.53 min (ferrichrome C), 12.78 min (riboflavin). Removal of riboflavin on a Sephadex LH20 column resulted in only two peaks as shown in (**b**). The minor peaks between 3 and 9 min have not been identified but seem to include coprogen B and fusigen. Separation conditions are as described for the standard mixture in Figure 1.

earlier to be a typical hydroxamate siderophore of *Fusarium* species (Diekmann & Zähner 1967, Sayer & Emery 1968). While the two minor peaks at 10.62 min and 11.06 min could be assigned to ferricrocin and ferrichrome, respectively, the peak at 14.30 min was tentatively assigned to monoacetylfusigen B (839 Da) showing a characteristic quasi molecular ion peak (MH^+) at m/z = 840 in ion spray mass spectra (Figure 6b).

Discussion

This is the first report on the identification of siderophores from mycorrhizal fungi, although earlier reports using biological tests with *A. flavescens* JG-9 have already suggested the excretion of hydroxamate siderophores by ectomycorrhizal fungi (Szaniszlo *et al.* 1981). There is also some evidence that *Glomus* species, forming vesicular–arbuscular

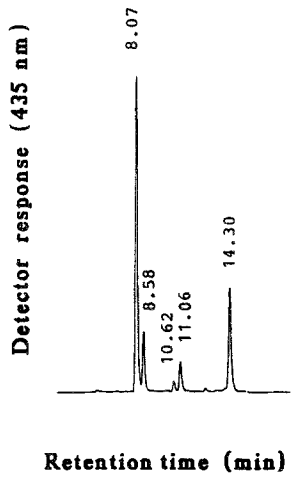


Figure 5 HPLC separation of siderophores excreted by the endophyte of *R. chamaecistus*. Assignments of peaks: 8.07 min (fusigen), 8.58 min (unknown), 10.62 min (ferricrocin), 11.06 min (ferrichrome), 14.30 min (monoacetylfusigen B). Conditions are as described for the standard mixture in Figure 1.

mycorrhizae, may produce hydroxamate siderophores (Cress *et al.* 1986). Ericoid mycorrhizal fungi have also been shown earlier to synthesize hydroxamate siderophores (Schuler & Haselwandter 1988, Federspiel *et al.* 1991). In a continuation of this work we report here on the isolation and chemical structure of the siderophores from ericoid mycorrhizal fungi grown under pure culture conditions. From the results obtained in the present investigation, it is clear that the cyclic hexapeptide siderophore, ferricrocin, is the most characteristic siderophore of ericoid mycorrhizal fungi. On the other hand, fusigen, an ester-type siderophore of the fusarinine class predominates in the endophyte of *R. chamaecistus*.

Ericaeous plants and the ericoid mycorrhiza form dominate on acid soils with a high organic matter content. Although the solubility of ferric iron is increased at low pH, the high organic matter content of heathland soils might again reduce the available iron due to its metal binding properties (Mortensen 1963). Therefore, even under acidic soil conditions, the need to produce siderophores seems to be obvious, as the function of siderophores is not only to solubilize but also to transport iron into the interior of the fungus (Winkelmann 1991a,b). It is interesting to note that the two typical ericaceous mycorrhizal fungi growing on acid soils produce a ferrichrome-type siderophore (ferricrocin) as the principal iron chelator while the endophyte of *R. chamaecistus* synthesizes the ester-type siderophore

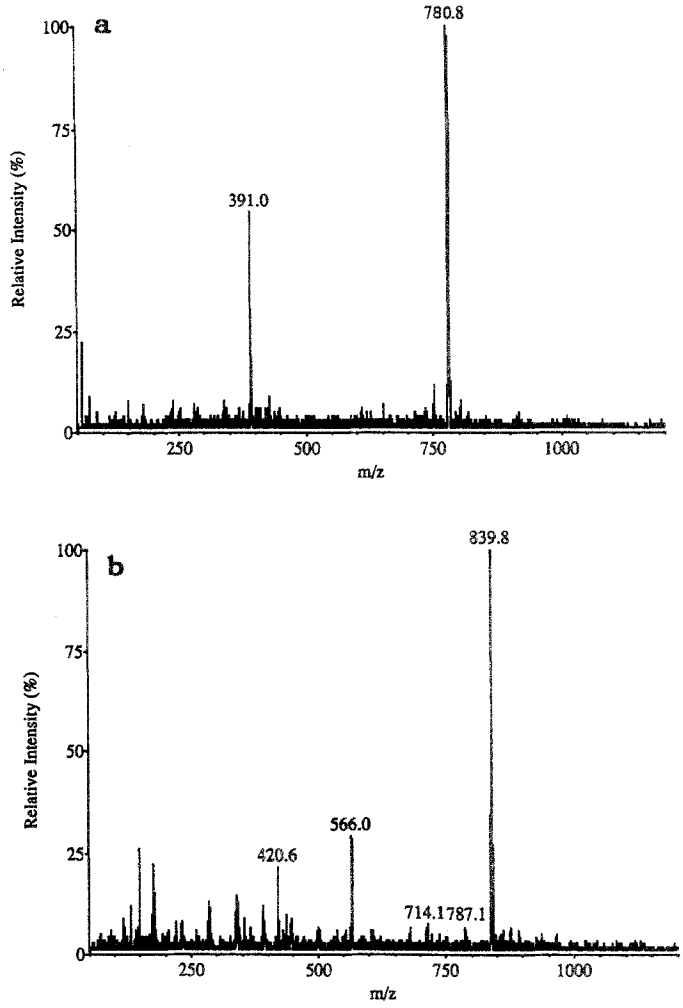
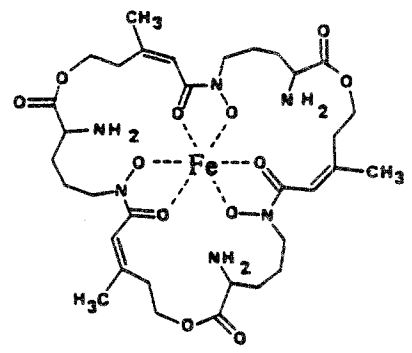


Figure 6. Ion spray mass spectra of the principal siderophores from the endophyte of *R. chamaecistus*. The major components (peaks 8.07 min and 14.30 min in Figure 5) were isolated by semi-preparative HPLC and identified by ion spray mass spectroscopy. (**a**) The quasi-molecular ion peaks (MH^+, m/z = 780.8, and $M2H^{2+}$, m/z = 391) are consistent with the molecular mass of fusigen (779 Da). (**b**) MH^+, m/z = 839.8 was tentatively assigned to monoactylfusigen B.

IV



Fusigen

fusigen, although the host plant prefers a calcareous soil. Whether or not this has any ecological significance or whether this merely reflects a chemosystematic relationship to related fungal genera remains to be solved.

Recently, Shaw *et al.* (1990) reported that roots of *Calluna* infected with mycorrhizal fungi absorbed significantly higher amounts of iron over a given time than those which were uninfected. It has been postulated by these authors that this effect is likely to be attributable to the ability of the mycorrhizal fungi to produce siderophores. This hypothesis, however, needs to be confirmed, in particular as in these experiments relatively high doses of iron were applied. However, in the light of the present findings, it seems probable that the presence of greater amounts of siderophores produced by an iron-stressed mycorrhizal fungus may contribute to an enhanced iron uptake in infected roots compared to non-infected roots.

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