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Basidiochrome – a novel siderophore of the orchidaceous mycorrhizal fungi *Ceratobasidium* and *Rhizoctonia* spp.

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Abstract

A novel trishydroxamate siderophore, named basidiochrome, was isolated as the principal siderophore from low-iron culture filtrates of *Ceratobasidium* and *Rhizoctonia* species which are known as mycorrhizal fungi associated with orchid roots. Ion-exchange chromatography and preparative HPLC yielded a pure compound which contained two components according to GC–MS analysis: L-N^5-hydroxy-ornithine and 3-methyl-2-*cis*-pentenedioic acid (3-methyl-*cis*-glutaconic acid). FTICR-ESI-MS of both the iron-free and ferric form indicated an elemental composition of $C_{33}H_{47}N_6O_{16}Fe$ (MW = 839) for the ferric form of basidiochrome. The connectivity was further elucidated by 2D-NMR techniques (HSQC, HMBC, COSY, NOESY) indicating that basidiochrome is a novel linear tripeptide consisting of three L-N^5-hydroxy-ornithines each linked to 3-methyl-2-*cis*-pentenedioic acid residues.

Introduction

With at least 24,000 species, the plant family of the Orchidaceae is one of the largest occurring in a wide range of different habitats, from tropical forests to deserts and alpine ecosystems. The life forms orchids produce are manifold, ranging from terrestrial to epiphytic species (Raven *et al*. 2005). A number of orchids such as species of *Phalaenopsis*, *Cattleya*, *Cymbidium*, *Paphiopedilum* and *Odontoglossum* are of horticultural importance, some, like *Vanilla*, are grown commercially for the flavoring agent vanilla, whereas other orchid products are considered to have medicinal properties.

All orchid species undergo a prolonged seedling stage during which they depend on an exogenous supply of carbohydrates provided in nature by mycorrhizal fungi forming the typical orchid mycorrhizas (Smith & Read 1997). Whereas in adult stages most orchids are green and photosynthetically active, about 200 species are achlorophyllous and remain myco-heterotrophic (Leake 1994). A very large number of fungal isolates from orchid mycorrhizas have been referred to the anamorphic form genus *Rhizoctonia* belonging to the teleomorphic basidiomycete genera like *Ceratobasidium*, *Thanatephorus*, and others (Warcup & Talbot 1980; Warcup 1981; Currah *et al*. 1987; 1997).

In general, mycorrhizal fungi strongly affect the mineral nutrition of plants including micronutrient uptake (George *et al*. 1994; Haselwandter & Bowen 1996). Iron is one of the most important mineral nutrients being scavenged through siderophores excreted by almost all aerobic microorganisms under low-iron conditions (Winkelmann 2001, 2002). With regard to plant uptake of iron

the release of siderophores by mycorrhizal fungi may play a role of paramount importance (Haselwandter 1995). Hence we determined the potential of orchidaceous mycorrhizal fungi to synthesize siderophores under iron limiting pure culture conditions. We report here on the isolation and structure elucidation of the main and hitherto unknown siderophore released by different strains of *Rhizoctonia* and *Ceratobasidium* species which we named basidiochrome referring to the basidiomyceteous affiliation of the producing organisms.

Materials and methods

Selection of fungal isolates

For the present study we used the following fungal strains: *Ceratobasidium globisporum*, isolated from *Trichoglottis australensis* (Centralbureau voor Schimmelcultures in Baarn, NL, CBS-no. 569.83), *C. papillatum (=C. cornigerum)* isolated from *Sarcochilus dilatatus* (CBS-no. 570.83), *C. sphaerosporum* isolated from *Pomatocalpa macphersonii* (CBS-no. 571.83), *Rhizoctonia mucoroides* isolated from *Phalaenopsis schilleriana* (= *R. solani* according to Anderson and Stalpers 1994; CBS-no. 116.20), strain DSM-no. 3707 deposited as *R. stahlii* isolated from *Dactylorhiza maculata* (Deutsche Sammlung von Mikroorganismen und Zellkulturen,), *R.* sp. isolated from *Gymnadenia conopsea* (DSM-no. 3713), and an isolate from the alpine orchid *Nigritella nigra* (isolate N1, culture collection of the Department of Microbiology of the University of Innsbruck, Austria). For comparative purposes the fungal endophyte *Rhizoctonia endophytica* var. *endophytica* isolated from healthy *Pinus banksiana* seedlings (CBS-no. 257.60) was included in the study.

Culture conditions and extraction of siderophores

The fungal isolates were sub-cultured in 100 ml low iron medium (LIM-1 medium with 1 g /l of each, L-proline and L-ornithine·HCl; Szaniszlo *et al.* 1981) in 250 ml Erlenmeyer flasks on a gyratory shaker at 120 rev/min and 25 °C. The low iron medium was deferrated using Chelex 100 according to Haselwandter & Winkelmann (1998). At regular intervals (2 days) the CAS assay (Schwyn and Neilands 1987) was applied to determine the siderophore concentration of the culture filtrate. When the maximum of ferrioxamine B equivalents was reached the siderophores were extracted from the supernatant of the cultures following the procedure of Haselwandter and Winkelmann (1998). The hydroxamate siderophores were adsorbed onto XAD-16 (Sigma, Munich, Germany), washed with three volumes of distilled water and desorbed with one volume of methanol. The methanol extract was filtered through a 0.2 μm filter (ANOTOP ^{R} 10, Merck) prior to HPLC analysis. For the selective enrichment of basidiochrome the culture filtrate was passed through a DEAE-52 column and desorbed with 2 M ammonium chloride with subsequent desalting on XAD-16 material.

HPLC separation

HPLC separation of siderophores was performed on a Shimadzu HPLC equipped with two LC-10AT pumps, system controller SCL-10A, autoinjector SIL-10AXL, and UV-VIS spectrophotometric detector SPD-10AV. The siderophores were separated on a C_{18} reversed phase column (ReproSil-pur 120, ODS-3, 5μm, 250 × 4 mm, Dr. Maisch, Ammerbuch, Germany) using a gradient of 6–40% acetonitrile in water (+0.1% TFA) within 20 min, detector wavelength 435 nm. Purification of basidiochrome was achieved by preparative HPLC (LC-8A pumps, Shimadzu) using a Nucleosil 100 C_{18} column (250 × 20 mm, 7 μm) and a flow rate of 5 ml per min. The resulting peaks were collected and the structures confirmed by mass spectrometry and NMR techniques.

Mass spectrometry

FTICR-ESI-MS spectra were recorded in the positive and negative modes on a 4.7 T APEX II FTICR mass spectrometer (Bruker-Daltonics, Bremen, Germany). MS–MS experiments were carried out with argon as collision gas. To enhance mass accuracy, spectra were measured with internal mass standards.

GC–MS experiments

Siderophore samples were hydrolyzed (24 h/110 °C/6 N HCl) and the hydrolysate extracted with ethyl acetate. The aqueous fraction was

derivatized to form the *N*-trifluoroacetyl O-TMS/ethylester derivatives and subsequently analyzed for amino acids by GC–MS (Agilent 6890/5973) on DB5-MS and 2,6-dipentyl 3-butyryl-γ-cyclodextrin/PS255 (30:70) capillaries. The ethyl acetate extract was dried over anhydrous Na_2SO_4, trimethylsilylated with BSTFA/pyridine (1:1) at 60 °C for 30 min and analyzed by GC–MS on a DB5-MS capillary. The EIS-mass spectra obtained were compared with reference spectra from the NIST library. In order to minimize the apparent E/Z isomerism of methylglutaconic acid during hydrolysis, a separate hydrolysis with 1 N HCl for 15 min followed by extraction and derivatization as above was performed.

NMR spectra

NMR spectra were recorded on an AMX 600 NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5 mm triple-resonance probehead with z-gradients. Spectra were obtained in D_2O.

Results

Based upon HPLC analysis in combination with simultaneous injection of reference samples of known hydroxamates (spiking) the main siderophores of some fungal strains could be identified. Under pure culture conditions *Rhizoctonia* sp. isolated from *Gymnadenia conopsea* released ferrirubin as main siderophore. The isolate N1 obtained from the alpine orchid *Nigritella nigra* excreted mainly ferrichrome. However, a prominent siderophore released by six of the eight fungal isolates tested remained unclear. Under the analytical conditions employed this hydroxamate appeared in all the HPLC separations at a retention time which was different from all known fungal hydroxamates. A chromatogram containing the iron complex and the desferri-form is shown in Figure 1. While in 33 day-old cultures of *C. globisporum* ferrirhodin and ferrirubin were often the main siderophores produced as revealed by a characteristic mass of $m/z = 1011[M + H]^+$, in younger cultures (<21 days of incubation) and especially in *Rhizoctonia mucoroides* and *R. stahlii* a hitherto unknown siderophore with $m/z = 840[M + H]^+$ for the ferric form was found. This compound appeared to represent the main

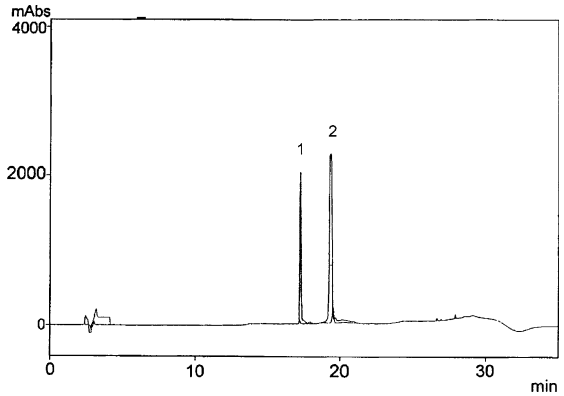
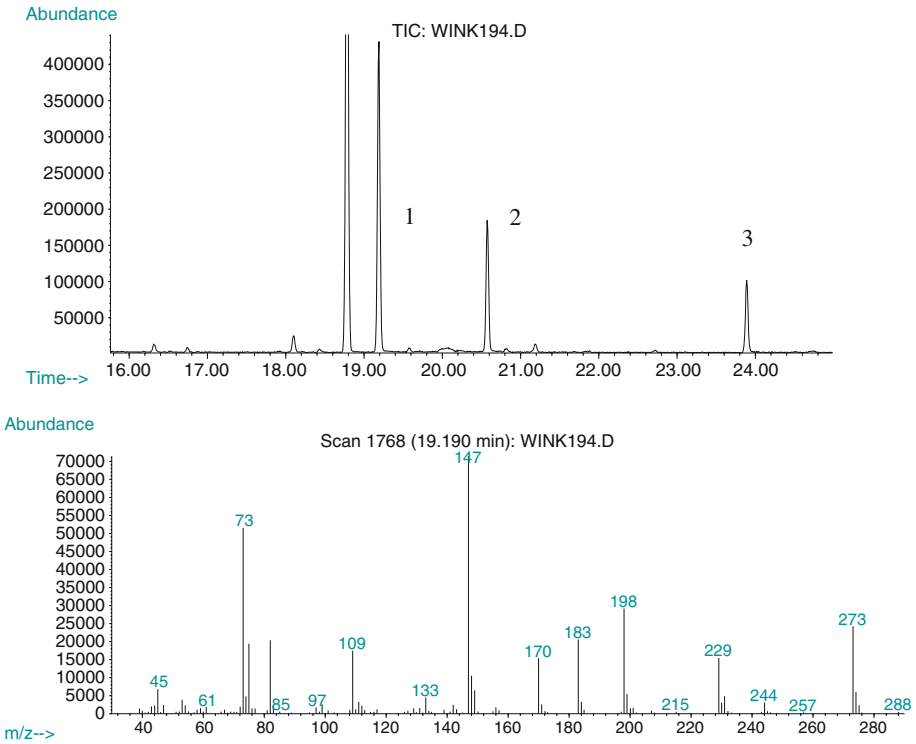


Figure 1. Overlay of HPLC profiles of iron-free basidiochrome (1) and ferric basidiochrome (2) using a reversed phase column (4×250 mm, Nucelosil C_{18}, 5μm) and a gradient (6–40%) of acetonitrile/water (+0.1% TFA) in 20 min. Detector wavelength: 220 nm.

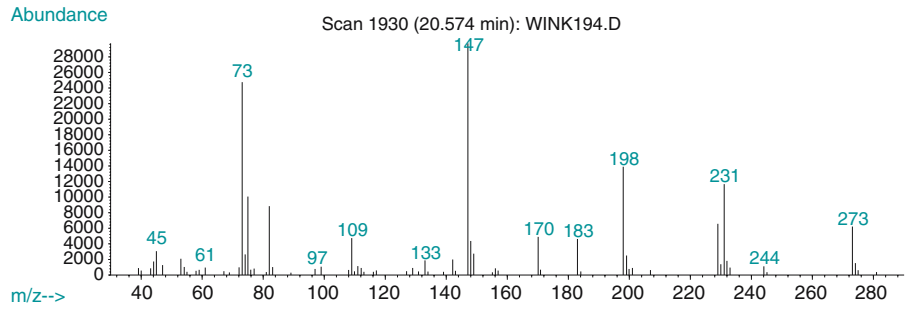
siderophore also in culture filtrates of *C. papillatum* and *C. sphaerosporum*. High resolution FTICR-ESI-MS spectra revealed $m/z = 787.33534$ $[M + H]^+$ for the desferri- and 840.24707 $[M + H]^+$ for the ferric form, corresponding to an elemental composition for the uncharged ferric basidiochrome of $C_{33}H_{47}N_6O_{16}Fe$ with a relative mass error of 0.18 ppm (ferric form) and 0.33 ppm (iron-free form). FTICR-MS was also performed after methylation of desferri-basidiochrome with trimethylsilyldiazomethane. The major product found was the fourfold methylated species with minor products corresponding to up to sevenfold methylation. This is interpreted as indicating the presence of four free carboxyl groups and three *N*-hydroxy groups in the molecule.

Chiral amino acid analysis of the hydrolysate indicated the presence of L-ornithine. In addition, N^5-hydroxy-ornithine was also found upon analysis on the DB5 capillary, a compound which is known to be degraded to ornithine under conditions of HCl hydrolysis. This suggests that the ornithine found is in fact the degradation product from *N*-hydroxyornithine.

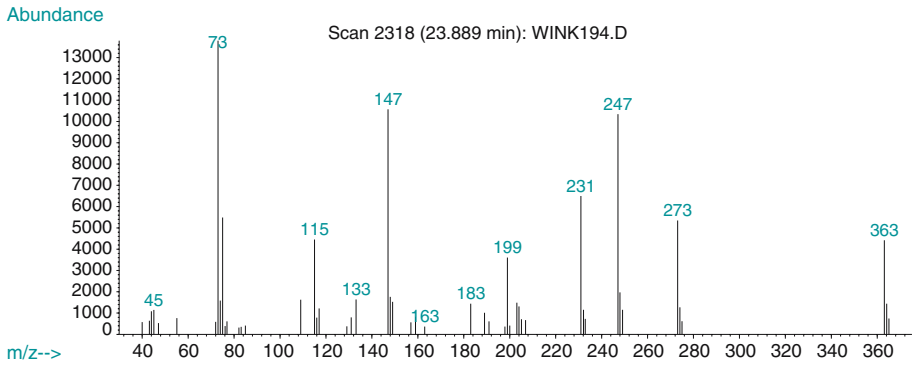
In the ethyl acetate extract, *cis*- and *trans*-3-methyl-2-pentenedioic acid (3-methyl-glutaconic acid) together with 15% of the hydroxylated compound, 3-methyl-3-hydroxy-pentanedioic acid were identified (Figure 2) as their TMS derivatives on the basis of the correspondence of their spectra with reference spectra (Shinka *et al.* 1992). Under mild conditions of hydrolysis, only *cis*-3-methyl-2-pentanedioic acid was found. The results of



Peak 1 (cis-3-methylglutaconic acid (bis-TMS)



Peak 2 (trans-3-methylglutaconic acid (bis-TMS)



Peak 3 (3 hydroxy3-methylglutaric acid (tris-TMS)

Figure 2. GC–MS analysis of silyl derivatives of hydrolyzed iron-free basidiochrome (6 N HCl) showing the GC-separation (top) and the obtained mass spectra of the separated peaks of *cis*-, *trans*-methylglutaconic and hydroxy-methylglutaric acid, respectively (1, 2, 3).

FTICR-MS and GC–MS can be rationalized by proposing basidiochrome to be a non-cyclic (i.e. linear or branched) molecule containing three *N*-hydroxyornithines and three 3-methylglutaconyl moieties. The results of the MS–MS measurements were, however, inconclusive as to the connectivity of these constituents.

From NMR spectroscopy we derived a structure as shown in Figure 3, possessing a sequence of three acylated *N*-hydroxyornithines. The spectra of basidiochrome were recorded in D_2O instead of DMSO due to its better solubitity in aqueous solution. The three ornithyl α-CH (H-2, 2′, 2″) resonances are well separated and the protons (H-6, 6′, 6″) show related chemical shifts in the ^1H NMR as reported by Llinas *et al*. (1977) and Jalal & van der Helm.(1981) . The α-CH protons of the ornithine residue experience a diverse deshielding environment and exhibit separate signals at different chemical shift values, due to the differences in the polarity of the side chains in the peptide backbone. The absolute configuration of ornithine was verified by ^1H NMR data of the basidiochrome. The HSQC spectrum of basidiochrome in D_2O is shown in Figure 4 and the ^1H NMR and ^{13}C NMR data of three ornithine and three 3-methyl-2-pentenedioic acid residues are summarized in Table 1. The hydroxamic acid functions in the siderophore are formed by N^5-hydroxylation and N^5-acylation of the ornithine residue. The NMR data in Table 1 show that the three ornithyl N^5-acylgroups are all related and made of three 3-methyl-*cis*-2-pentenedioic acid units. The NOE experiment performed with the compound revealed that the methylgroups (CH_3-13, 13′, 13″) and the protons (H-9, 9′, 9″) are *cis* to each other. Thus the connectivity of the three N^5-hydroxy-N^5-(*cis*-3-methyl-1-oxo-2-pentenoic acid)-ornithine units in the iron-free basidiochrome could be clearly established by NMR data. The structural formula of ferric basidiochrome (Figure 5) therefore consists of a tripeptide sequence of *N*-hydroxyornithine linked to *cis*-methylglutaconic acid giving three hydroxamic acid residues for binding one atom of iron.

Discussion

When incubated for a long period of time *C. globisporum* produced mainly ferrichrome type siderophores, such as ferrirubin and ferrirhodin.

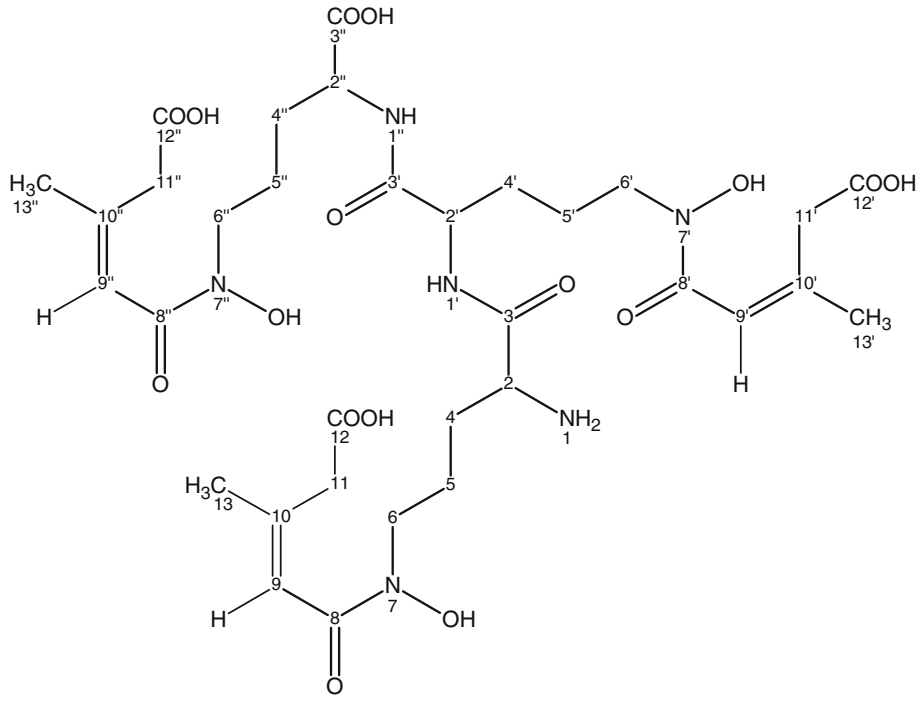


Figure 3. Structure of basidiochrome (iron-free) and the numbering according to the spectral data.

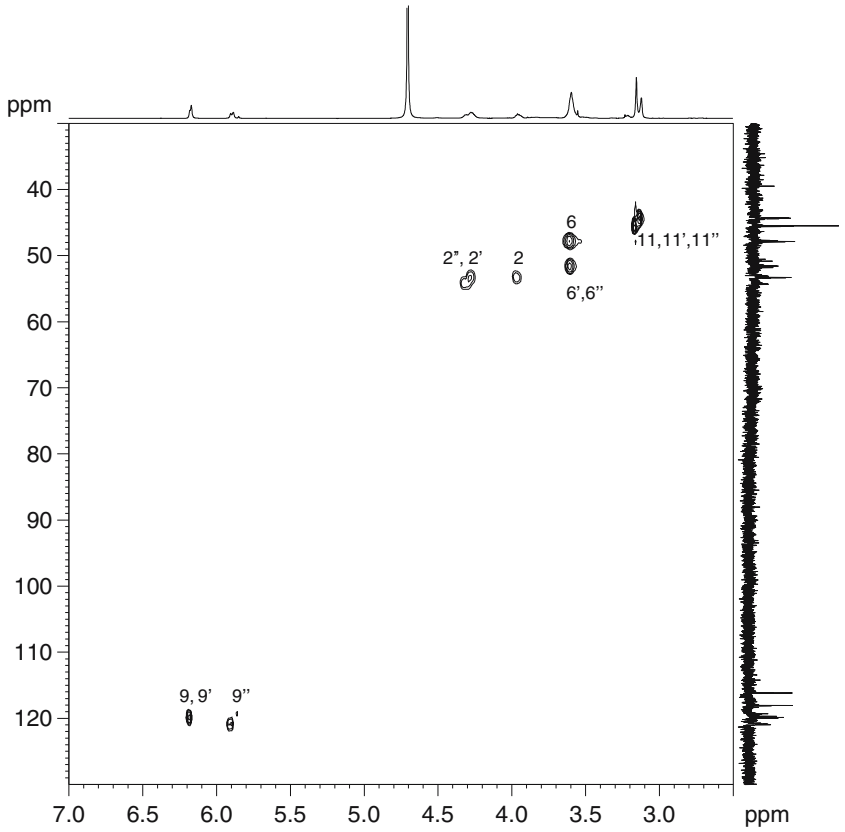


Figure 4. Part of the HSQC-NMR spectrum (600 MHz) of iron-free basidiochrome.

However, younger cultures of this species and culture filtrates of *C. papillatum* and *C. sphaerosporum* contained larger amounts of basidiochrome. This was most obvious in case of the anamorphic *Rhizoctonia* species *R. stahlii* and *R. mucoroides*, where basidiochrome was always the principal siderophore. In contrast to the ferrichrome type siderophores which contain *trans*-anhydro-mevalonic acid residues, basidiochrome possesses methylglutaconyl residues in *cis*-configuration. Thus, basidiochrome has structural features in common with the ferrichromes, such as the triornithine sequence, although the tripeptide sequence ser-ser-gly and the cyclic nature are missing. An analogous linear compound, named des(diserylglycine)-ferrirhodin (DDF), had been previously reported by Jalal *et al.* (1985), where the hydroxamic acid residues are present as anhydromevalonyl residues with an additional glycine residue at the triornithyl backbone. DDF was found as a byproduct among siderophores from *Aspergillus ochraceus* of which ferrirubin was a major siderophore, together with some asperchromes as minor compounds (Jalal *et al.* 1984). Thus, it seems not uncommon among fungi that structurally incomplete ferrichrome-type siderophores are produced and excreted, possibly as precursor products. Certain enzymes in the biosynthetic routes may be missing or inactive. The question remains, however, whether or not these compounds are capable of functioning as siderophores in fungal cells. In that respect it is of interest to note that DDF showed siderophore activity in the producing strain and also in the auxotrophic *Microbacterium flavescens* (Jalal *et al.* 1985).

Basidiochrome is a chemically relatively stable trihydroxamate which unlike the ester-containing fusarinines and coprogens for example, is not easily degraded under alkaline conditions. We therefore suggest that the chemical stability, the resistance to esterolytic cleavage by fungal competitors as reported for coprogens (Hördt *et al.* 2000) and the lower solubility at lower pH near the

Table 1. ^{1}H and ^{13}C chemical shifts of basidiochrome (iron-free) in D_2O (600 MHz).

^{1}H chemical shifts δ[ppm]	^{13}C chemical shifts δ[ppm]	structural group	position
1.64	22.9	CH_2CH_2	5, 5′, 5″
1.76	18.9	CH_3	13, 13′, 13″
1.90	18.8		
1.79/1.65	28.5	CH_2CH_2	4, 4′,4″
3.13	44.2	$=CCH_2$	11, 11′, 11″
3.16	45.3		
3.59	51.7	CH_2N	6, 6′, 6″
3.62	47.7		
3.95	53.1	CHN	2, 2′, 2″
4.28	53.1		
4.31	54.0		
5.89	120.7	CH=	9, 9′, 9″
6.18	119.7		
–	146.3	C=	10, 10′, 10″
	143.4		
–	166.8	NOHC=O	8, 8′, 8″
	169.3		
–	173.4	C=O	3, 3′, 3″
	170.0		
–	175.7	CO_2H	12, 12′, 12″
	175.7		
	175.9		
	175.4		

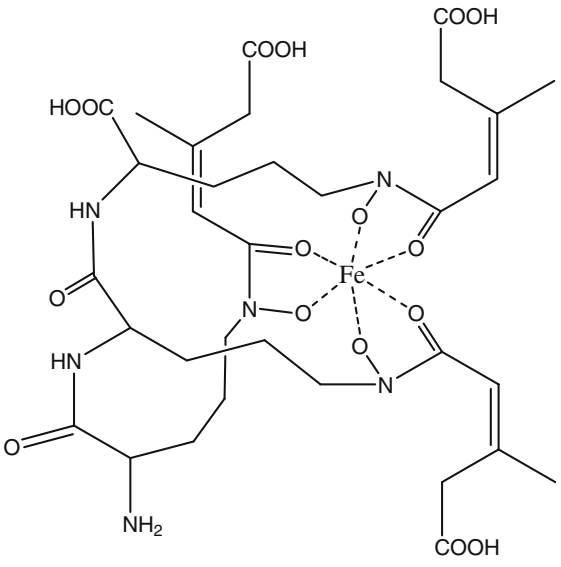


Figure 5. Structure of ferric basidiochrome representing a linear trishydroxamate siderophore containing a tripeptide sequence of L-N^5-hydroxyornithine linked to *cis*-methylglutaconic acid residues.

root surface make basidiochrome a suitable siderophore for orchidaceous mycorrhizal fungi colonizing the plant roots. It has been pointed out earlier that the occurrence and prevalence of specific siderophores in the environment is an important aspect of the ecology of certain organisms (Winkelmann 2002, 2004), suggesting that survival in various ecosystems correlates well with the properties of siderophores produced.

Due to the four carboxyl groups, basidiochrome is an acidic siderophore. While the solubility under acidic conditions is decreased compared to ferrirubin and ferrirhodin, it aids in the solubilization of insoluble oxides of iron. Thus basidiochrome can be anticipated to be less soluble in the rhizosphere and may therefore preferentially adhere to the orchid mycorrhizal root surface, where it may function as an external reservoir of siderophore-bound iron thereby preventing diffusion into the outer rhizosphere. A similar behavior has been observed with ferrichrome A in *Ustilago sphaerogena* where ferrichrome A is excreted under iron deficiency in the early growth phases as an acidic siderophore, followed by the later production of uncharged ferrichrome. Emery and coworkers had pointed out that utilization of iron from ferrichrome A differed from ferrichrome as revealed by EPR specroscopy and suggested a reductive iron uptake mechanism at the membrane for ferrichrome A (Ecker *et al.* 1982). With basidiochrome, however, no iron uptake experiments into the mycorrhizal fungi have so far been performed, but a reductive iron uptake mechanism for basidiochrome might be an option.

It is of interest to note that filtrates of 33 day-old cultures of *C. globisporum* contained mainly ferrirubin/ferrirhodin and basidiochrome only as a minor product. The predominance of basidiochrome in the anamorphic *Rhizoctonia* strains, such as *R. mucoroides*, *R. stahlii* and *R. endophytica* var. *endophytica* is interesting and might be an indication that the metabolism and biosynthesis of the siderophores in these strains deviates from the teleomorphic species. With the exception of the latter, the *Rhizoctonia* anamorphs as well as the teleomorphic *Ceratobasidum* species are all typical orchidaceous mycorrhizal fungi belonging to the Heterobasidiomycota (Roberts 1999). While single-strand conformation polymorphism and mitochondrial ribosomal DNA sequences have already been used for identification

of the mycobionts of a single orchid species (Kristiansen *et al*. 2001), comprehensive molecular data on the phylogeny of the set of species investigated in this study is not available. It would be interesting to see whether a phylogenetic analysis of nucleotide sequence data from the nuclear ribosomal RNA would position the six species synthesizing basidiochrome in one group, and the remaining two isolates in one or two others. In addition it must be pointed out that *R. endophytica*var. *endophytica* releases the same main siderophore as the orchid endophytes. This is noteworthy in particular because this strain was originally isolated from healthy *Pinus banksiana* seedlings, and inoculation experiments suggested that this fungal species is an endophyte of various conifers and angiosperms (Saksena & Vaartaja 1960).

Many isolates of the mycobionts of orchids belong to the anamorphic form genus *Rhizoctonia* in sterile condition, and cover a wide range of teleomorphic fungi belonging to at least five major taxonomic groups of Basidiomycota (Rasmussen 2002). It is of interest to note that also isolates of the well known plant pathogen *R. solani* may form mycorrhizal associations with orchids (Perkins and McGee 1995; Carling *et al*. 1999). According to Ogoshi *et al*. (1983) *R. solani* var. *fuchsiae* represents a binucleate *Rhizoctonia* as well as *R. endophytica* and *R. stahlii*, with *R. endophytica* var. *endophytica* in the same anastomosis group as *C. papillatum* (= *C. cornigerum*). As stated by Andersen and Stalpers (1994) *R. mucoroides* is synonymous with *R. solani*. Hence the findings of this study may also be of interest with regard to a better understanding of pathogenic relationships between fungi and plants.

It is assumed that the mycorrhizal fungi of orchids are involved in uptake of mineral nutrients for the host plants (Smith & Read 1997), but this aspect has not been well investigated and the physiological interactions between the mycorrhizal fungi and adult photosynthetic species are not well understood. For the myco-heterotrophic orchids it is expected that the mycorrhizal fungus continues to supply carbohydrates and mineral nutrients throughout the life-span of the plant (Leake 1994). In analogy to other mycorrhizal systems the potential of orchidaceous mycorrhizal fungi to synthesize siderophores can be anticipated to affect the iron nutrition of the associated plants (Haselwandter 1995).

So far, the chemical structures of the main siderophores released have been described for ericoid mycorrhizal, ectendomycorrhizal and ectomycorrhizal fungal species. Ericoid mycorrhizal fungi produce ferricrocin and fusigen as main siderophores (Haselwandter *et al*. 1992). Ferricrocin was also demonstrated to represent the main siderophore of the ectomycorrhizal fungus *Cenococcum geophilum* (Haselwandter and Winkelmann 2002), the ectendomycorrhizal fungi in the genus *Wilcoxina* (Prabhu *et al*. 1996), and *Phialocephala fortinii* as typical dark septate root endophyte (Bartholdy *et al*. 2001). This paper adds substantial knowledge with regard to the siderophore release by another important group of mycorrhizal fungi, the orchidaceous symbionts.

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