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High-performance liquid chromatography of siderophores from fungi

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Summary. A reversed-phase HPLC separation of iron(III) chelates of 16 representative fungal siderophores including ferrichromes, coprogens and triacetylfusarinine C was established in order to investigate siderophore production of fungi. For comparison purposes, the widely used bacterial siderophore ferrioxamine B was included. Culture filtrates of the fungi *Penicillium resticulosum, Fusarium dimerum, Aspergillus fumigatus* and *Neurospora crassa* were quantitatively analyzed for the presence of known and unknown siderophores after growth in low-iron culture media and adsorption on XAD-2 columns using this HPLC separation system. Photodiode array detection allowed the distinction between siderophores and non-siderophores. According to their ultraviolet/visible spectra, a further classification of the siderophores into four types due to the number of anhydromevalonic acid residues per molecule (0-3) was possible.

Key words: Siderophores — HPLC separation — Identification — Fungi

Introduction

Fungal siderophores are low-molecular-mass iron-complexing compounds of the trihydroxamate class, biosynthesized and excreted during iron limitation (Neilands 1952). Three functions have been assigned to fungal siderophores: (i) the solubilization of iron; (ii) the transport of iron to the membrane surface (Carrano and Raymond 1979) or the transport of the whole siderophore across the cytoplasmic membrane (Emery 1971; Winkelmann 1974); (iii) a function as an intermediate iron store within mycelia and conidiospores (Matzanke and Winkelmann 1981; Matzanke et al. 1987a, b). A comprehensive treatise on iron transport in microbes, plants and animals has been published recently (Winkelmann et al. 1987).

High-performance liquid chromatography (HPLC) is a fast and often-applied technique for the screening of microbial metabolites from culture media. But up to now no comprehensive application of this method has been reported for the control of the total siderophore production in culture filtrates of fungi. A long-term, preparative-scale separation of siderophores by reversed-phase low-pressure liquid chromatography (Jalal et al. 1984) and a preparative-scale HPLC of ferricrocin (Fiedler 1981) have been described.

This paper demonstrates the separation of 17 representative trihydroxamate iron(III) siderophores (Fig. 1) by reversed-phase HPLC and the use of this procedure for the detection and quantification of siderophores in XAD eluates from low-iron cultures of fungi.

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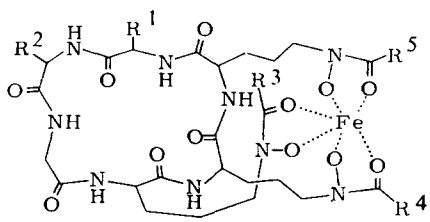
Materials and methods

Cultures and maintenance. Four different fungi were selected for siderophore analysis: *Penicillium resticulosum* CBS 150.45 and *Fusarium dimerum* Penz. var. pusillum Wr., CBS 254.50 were from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Neurospora crassa 74A (wild type) was from the Fungal Genetic Stock Center, Department of Microbiology, University of Kansas Medical Center. *Aspergillus fumigatus* Tü 142 was from the stock of the Institute of Biology, Tübingen. The cultures were maintained on agar slants containing per litre: 4 g yeast extract, 10 g malt extract and 4 g glucose. Conidiospores were harvested with 0.9% NaCl solution containing 0.1%

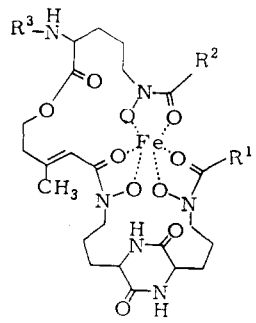
a



	R^1	R^2	R^3	R^4	R^5
Ferrichrome	H	H	CH_3	CH_3	CH_3
$(Gly)_4$ Ferrichrome *	H	H	CH_3	CH_3	CH_3
Ferricrome C	H	CH_3	CH_3	CH_3	CH_3
Ferricrocin	H	CH_2OH	CH_3	CH_3	CH_3
Ferrichrysin	CH_2OH	CH_2OH	CH_3	CH_3	CH_3
Asperchrome D1	CH_2OH	CH_2OH	A	CH_3	CH_3
Asperchrome B1	CH_2OH	CH_2OH	CH_3	A	A
Ferrirubin	CH_2OH	CH_2OH	A	A	A
Ferrirhodin	CH_2OH	CH_2OH	B	B	B
Ferichrome A	CH_2OH	CH_2OH	C	C	C
DDF **	-	-	B	B	B

* 4 Gly instead of 3 Gly in ferrichrome
** DDF = des(diserylglycyl)-ferrirhodin is a ferrirhodin without the -Ser-Ser-Gly- sequence.

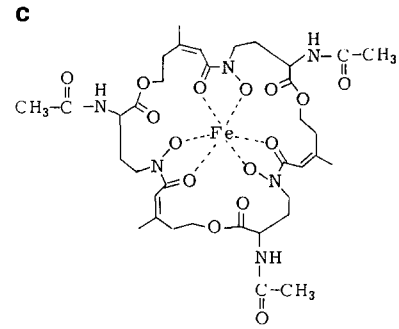
b



	R^1	R^2	R^3
Coprogen B	A	A	H
Coprogen	A	A	$-CO-CH_3$
Neocoprogen I (Fe-isotriornicin)	CH_3	A	$-CO-CH_3$
Neocoprogen II	CH_3	CH_3	$-CO-CH_3$

A = N^5-(*trans*- 5-hydroxy-3-methylpent-2-enoyl)-
B = N^5-(*cis*- 5-hydroxy-3-methylpent-2-enoyl)-
C = N^5-(*trans*- 4-carboxy-3-methylpent-2-enoyl)-

c



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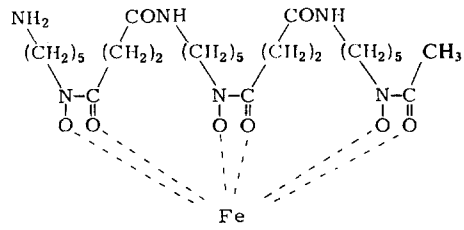

Fig. 1a–d. Structural formulae and numbering of the siderophores separated by HPLC: **a** Ferrichromes and derivatives; **b** coprogens; **c** triacetylfusarinine C (fusigen); **d** ferrioxamine B

Tween 80. The low-iron medium was as described earlier (Wiebe and Winkelmann 1975).

Siderophores. The siderophores (Fig. 1) used for comparison purposes were isolated and purified according to known procedures. Coprogen was isolated from low-iron cultures of *Neurospora crassa* 74A (Keller-Schierlein and Diekmann 1970, Wong et al. 1983). Coprogen B was from *F. dimerum* Penz. var. pusillum Wr. CBS 254.50 (Diekmann 1970). Ferrichrome, tetraglycyl-ferrichrome and ferrichrome C were isolated from *Neovossia indica* as described (Deml et al. 1984). Ferricrocin was isolated from *A. viridi-nutans* CBS 127.56 (Diekmann and Krezdorn 1975). Ferrichrysin was isolated from *A. melleus* Tü 42. Ferrirubin was isolated from *P. variabile* CBS 385.48 and triacetylfusarinin C (triacetylfusigen) was purified from cultures of *P. javanicum* v. Beyma CBS 341.48. Ferrirhodin was kindly provided by W. Keller-Schierlein, ETH Zürich, Switzerland. The asperchromes D1, B1 and des(diserylglycyl)-ferrirhodin, isolated from *A. ochraceus* gold (Jalal et al. 1984b) as well as the neocoprogens I and II, isolated from *Curvularia lunata* (Hossain et al. 1987) were gifts from D. van der Helm, Department of Chemistry, University of Oklahoma at Norman, USA. Ferrichrome A isolated from *Ustilago sphaerogena* was a gift from J.B. Neilands, Department of Biochemistry, Berkeley, Calif., USA. The purity of these standard siderophores was analysed by HPLC.

Equipment. The separations were established on an HPLC system from Waters (Eschborn, FRG) consisting of a multisolvent delivery system 600, a 712 WISP autosampler, a variable-wav-

elength detector LC spectrometer Lambda Max model 481, and a data module 740. In order to obtain the ultraviolet/visible spectra of the components, standards and culture samples were also run on an HP 1040M HPLC chromatograph with photodiode array detector HP 1040M (Hewlett-Packard, Waldbronn, FRG). Three types of columns (Grom, Ammerbuch, FRG) were used: column C_{18} (250 × 4.6 mm I.D.) was packed with 5 µm Nucleosil C_{18}, column C_8 (250 × 4.6 mm I.D.) with 5 µm Spherisorb Octyl and column diol (125 × 4.6 I.D.) with 7 µm Nucleosil Diol. Each of them was supplied with a pre-column (10 × 4.6 mm I.D.) (I.D. = internal diameter).

Standard siderophore mixtures. Equal volumes of 1 mM aqueous standard solutions of the pure siderophores were combined to give the standard mixtures. The exact concentration of the individual solutions were determined by spectroscopy using the absorption coefficients summarized in Table 1. The standard solution of ferrichrome A had a concentration of only 0.32 mM because of its low solubility in water. Mixture I included all of the 17 siderophore components listed in Table 1, mixture II contained no ferrioxamine B. Mixture III only consisted of tetraglycyl ferrichrome and neocoprogen II.

Preparation of samples from the culture media. Aliquots of cultures (10–50 ml) were filtered to remove mycelia. After adding ferrous sulfate under stirring, the brown culture filtrate was passed through an Amberlite XAD-2 column (Serva, Heidelberg, FRG). After washing with three volumes of distilled water, siderophores were desorbed with one volume methanol. The eluates were concentrated to a volume of about 1 ml and then diluted with water to give a final volume of exactly 5 ml test solution. Before injection this solution was centrifuged and filtered with an HV filter (0.45 µm, Millipore).

Separation of standard mixtures. Mixture I was chromatographed on column C_{18} and mixture II was separated on column C_8. The sample volume for the reversed-phase separations was 30 µl. The solvents for gradient elution contained 10 mM phosphate buffer, pH 3 and acetonitrile. For chromatography on column diol 5-µl samples of mixture III were applied and eluted with a water–acetonitrile gradient: 80–50% of acetonitrile in 10 min and then 50% for a further 10 min. Thus about 2 nmol or 1.5 µg of each compound was separated. The flow rate was set at 1 ml/min and the absorbance of the effluent was monitored at 435 nm. The peaks were identified by coinjection of the standard mixture and the corresponding pure component.

Examination of the culture samples. Samples of suitable volumes from XAD-2 eluates of fungal cultures were chromatographed under the same conditions as described for standard mixture I using the optimized gradient. Identification of the peaks was carried out by comparing the retention times and ultraviolet/visible spectra with those of the standard mixture and by the coinjection procedure. Quantification of siderophores was performed by comparing the area values with those of the standard calibration graphs.

Table 1. Siderophores of the standard mixture

Peak	Siderophore	Formula	M_r	$\varepsilon/\lambda_{max}$ (M^{-1} cm^{-1}/nm)	t_R on column (min)		
					A	B	C
1.	Coprogen B	$C_{33}H_{51}O_{12}N_6Fe$	779.6	2754/440[a]	4.34	5.22	
2.	Ferrioxamine B	$C_{25}H_{45}O_8N_6Fe$	613.4	2800/428[b]	8.59		
3.	$(Gly)_4$-ferrichrome	$C_{29}H_{45}O_{13}N_{10}Fe$	797.6	3500/424[c]	10.59	6.57	15.74
4.	Neocoprogen II	$C_{27}H_{41}O_{11}N_6Fe$	681.5	2612/422[d]	10.59	6.57	15.28
5.	Ferricrocin	$C_{28}H_{44}O_{33}N_9Fe$	770.6	2460/434[b]	13.01	7.66	
6.	Ferrichrysin	$C_{29}H_{46}O_{14}N_9Fe$	800.6	3020/430[b]	13.95	8.14	
7.	Ferrichrome	$C_{27}H_{45}O_{12}N_9Fe$	740.5	2895/425[b]	13.95	8.14	
8.	Ferrichrome C	$C_{28}H_{14}O_{12}N_9Fe$	754.6	—	15.81	9.35	
9.	Neocoprogen I	$C_{31}H_{47}O_{12}N_6Fe$	751.6	2730/428[d]	16.70	11.08	
10.	Asperchrome D1	$C_{33}H_{52}O_{16}N_9Fe$	886.7	—	21.12	13.56	
11.	Coprogen	$C_{35}H_{33}O_{13}N_6Fe$	821.7	2820/434[b]	23.44	14.74	
12.	Asperchrome B1	$C_{37}H_{58}O_{16}N_9Fe$	940.8	—	27.91	16.07	
13.	Ferrirubin	$C_{41}H_{64}O_{17}N_9Fe$	1010.9	3388/450[e]	29.82	16.68	
14.	DDF[f]	$C_{33}H_{53}O_{19}N_6Fe$	797.7	—	30.60	21.19	
15.	Ferrichrome A	$C_{41}H_{58}O_{20}N_9Fe$	1052.8	3360/440[b,g]	31.00	16.86	
16.	Ferrirhodin	$C_{41}H_{64}O_{17}N_9Fe$	1010.9	3802/445[e,g]	35.06	17.90	
17.	Triacetylfus. C	$C_{39}H_{57}O_{15}N_6Fe$	905.8	—	39.31	19.34	

t_R = retention times on column *A* (Nucleosil C_{18}), *B* (Spherisorb Octyl C_8), *C* (Nucleosil Diol). For gradient programs see Fig. 2 and materials and methods, separation of standard mixtures

[a] Diekmann (1973)
[b] Wong et al. (1983)
[c] Deml et al. (1984)
[d] Hossain et al. (1987)
[e] Keller-Schierlein (1963)
[f] DDF = des (diserylglycyl)-ferrirhodin
[g] Determined in methanol

Results

HPLC separation of the siderophore standards

Besides the 16 fungal trihydroxamate siderophores, we also included the bacterial trihydroxamate siderophore ferrioxamine B because of its use as a reference compound. All 17 siderophores were first chromatographed on a C_{18} column using phosphate buffers of varying pH between 2 and 5. The ionizable siderophores coprogen B, ferrioxamine B and ferrichrome A, having free amino and carboxy groups, were found to give sharp peaks only below pH 3.5. The zwitterionic des(diserylglycyl)-ferrirhodin was eluted as a sharp peak at all pH values investigated. Therefore we adjusted the buffer at pH 3 for the following gradient optimization. The eluate of the 17 siderophores was detected at 435 nm since most siderophores exhibit absorption maxima between 420 nm and 450 nm with absorption coefficients around 3000 (± 500) M^{-1} cm^{-1}. Detection at this wavelength facilitates the determination of siderophores in culture filtrates. The optimized separation is shown in Fig. 2a. The peaks are assigned to the siderophores listed in Table 1 by coinjection of the standard mixture I and each single component. The 17 siderophores gave 15 detectable peaks; only tetraglycyl-ferrichrome, neocoprogen II and also the couple ferrichrome/ferrichrysin were not separated on the C_{18} column.

Therefore, the separation of siderophores has also been elaborated on a C_8 column (Fig. 2b), which generally gave shorter retention times, but with less efficient separation coefficients. In particular, the first components eluted show relatively broad elution profiles and the retention times were less reproducible when compared to the C_{18} column. On column C_8 ferrioxamine B could be eluted only as a very broad peak after 15 min. Its increased retention compared to the C_{18} column has already been reported by Cramer et al. (1984).

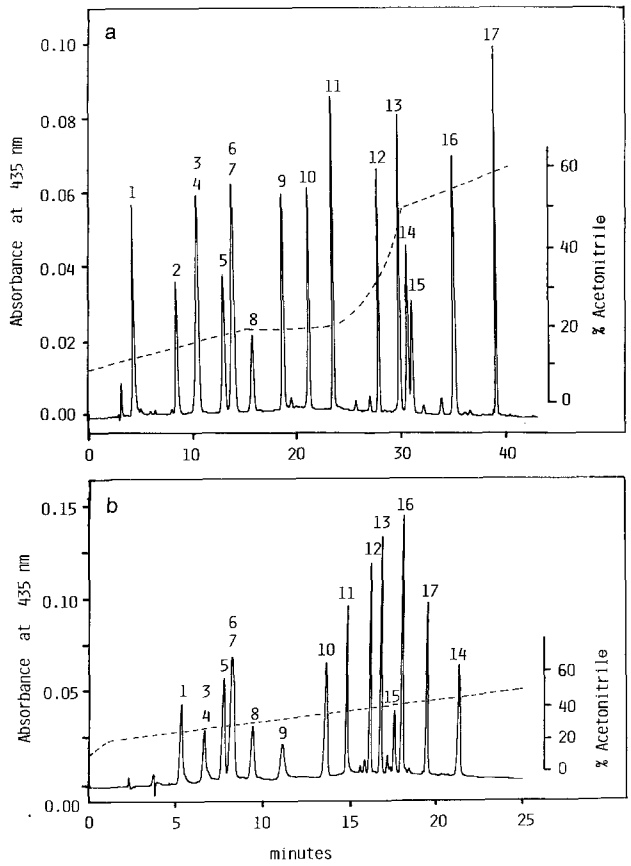

Fig. 2a, b. Separation of standard mixture **(a)** I on Nucleosil C_{18} and **(b)** II on Spherisorb Octyl C_8 using a gradient of 10 mM phosphate buffer pH 3 (solvent A) and acetonitrile (solvent B). *Flow rate:* 1 ml/min, sample volume 30 µl. Assignment see Table 1. *Elution programs:* **a** The percentage of B was first increased linearly 10%–20% in 15 min, then using concave gradient No. 9 20%–50% in 15 min and further raised linearly 50%–60% in 10 min. After this the portion of B was reduced to 10% in 5 min. The equilibration delay was 20 min resulting in a cycle time of 65 min. **b** A linear 10%–20% gradient of B in 1 min was followed by a linear 20%–50% increase of B in 24 min and reduction of B to 10% in further 5 min. Cycle time was 50 min

Table 2. R_f values of siderophores on silica gel 60 thin layers

Siderophores	R_f in solvent		
	I	II	III
Triacetylfusarinin C	0.52	0.61	0.25
Ferrichrome C	0.45	0.49	0.13
Ferrirhodin	0.38	0.43	0.32
Ferrichrome	0.36	0.38	0.09
Ferricrocin	0.32	0.34	0.11
Ferricrysin	0.32	0.36	0.12
Neocoprogen II	0.26	0.34	0.08
Asperchrome D1	0.25	0.29	0.15
Neocoprogen I	0.22	0.30	0.10
Coprogen	0.22	0.30	0.15
Asperchrome B1	0.22	0.28	0.27
Hexahydroferrirhodin	0.21	0.27	0.27
Tetraglycyl-ferrichrome	0.20	0.22	0.04
Ferrirubin	0.20	0.25	0.29
Ferrioxamine B	0.09	0.13	0.10
Ferrichrome A	0.02	0.09	0.24
Des (diserylglycyl)-ferrirhodin	0.04	0.11	0.16
Coprogen B	0.02	nd	0.08
Fe_2 (rhodotorulate)$_3$	0.00	nd	0.04

Solvent systems (freshly prepared): (I) chloroform/methanol/water (70:24:4); (II) chloroform/methanol/water/acetic acid (65:25:4:3); (III) *n*-butanol/acetic acid/water (80:20:20); nd = not determined

Because of its unfavourable elution behaviour, this siderophore was not incorporated in the standard mixture II used for the C_8 column. The selectivity with respect to the couples ferrichrysin/ferrichrome and neocoprogen II/tetraglycyl-ferrichrome could not be improved using the C_8 stationary phase.

However, both couples are well resolved on silica gel 60 thin layers (Table 2). The R_f values reported in this investigation differ from those reported earlier by Jalal et al. (1984a). In the present investigation we used freshly prepared solvent systems, whilst in the paper of Jalal et al. the solvent was 4 days old, which seems unsuitable for rapid identification purposes. Additionally we tested a diol phase for HPLC separation of ferrichrysin and ferrichrome as well as for neocoprogen and tetraglycyl-ferrichrome. We expected hydrogen bond formation of the two hydroxyl groups of ferrichrysin with the stationary phase. However, no retardation of this component was obtained. On the other hand, the separation of the structurally differing siderophores neocoprogen II and tetraglycyl-ferrichrome could be achieved on the diol phase (Table 1, chromatogram not shown).

The C_{18} separation was also performed on an HPLC chromatograph with a photodiode array detector. Thereby four types of ultraviolet/visible spectra of fungal siderophores could be distinguished depending on the number of anhydromevalonic acid residues (AMA) per molecule (Fig. 3). The ratio of the absorbances at λ_{max} of the ligand-to-ferric-ion charge-transfer band and at 250 nm, typical for α,β-unsaturated hydroxamates, is a characteristic value for this distinction. Absorption maxima of the charge-transfer band (c.t.) were found in a range 425–440 nm as expected. Only coprogen B showed a bathochromic shift to 494 nm at the adjusted pH 3.

Under the conditions described for the separation on a C_{18} column, siderophores could be qualitatively detected down to a concentration of about 1 µmol/l. However, reliable quantification of siderophores is only possible in the range of about 0.01–2.0 mmol/l related to a 30-µl sample volume. This corresponds to 0.3–60 nmol/sample. The exact linear range of calibration graphs depends on the particular siderophore investigated.

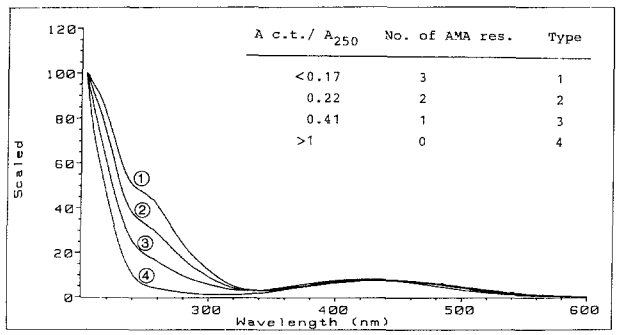

Fig. 3. Representative ultraviolet/visible spectra of siderophores obtained by photodiode array detection of the HPLC eluate: coprogen (type 1), neocoprogen I (type 2), neocoprogen II (type 3) and ferrichrome (type 4). *c.t.*, charge-transfer; *AMA*, anhydromevalonic acid

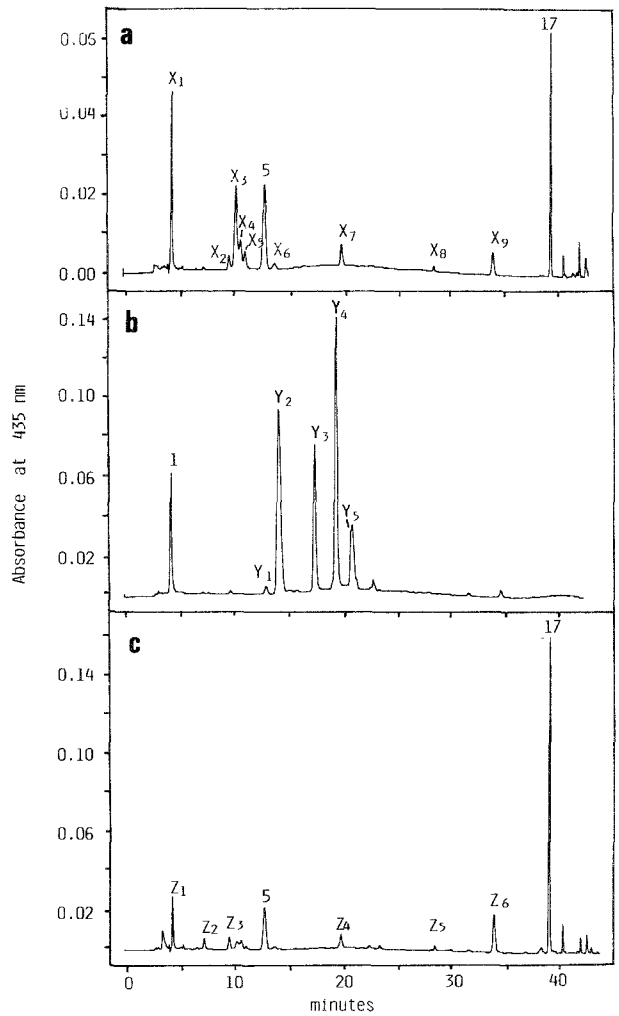

Fig. 4a–c. Separation of siderophores produced by *Penicillium resticulosum* **(a)**, *Fusarium dimerum* **(b)** and *Aspergillus fumigatus* **(c)**. Siderophores were isolated after 5 days of cultivation of the fungi in low-iron medium, adsorbed on a XAD-2 column and desorbed with methanol. This eluate was directly applied to the HPLC column C_{18} and separated as described for the standard solution in Fig. 2a. Numbered peaks correspond to assigned siderophores (see Table 3), whereas peaks X_1–X_6, Y_1–Y_4, Z_1–Z_5 are still unknown components

Using a detector wavelength of 215 nm, sensitivity can be enhanced by a factor of about 6–10, depending on the number of anhydromevalonic acid residues per molecule.

HPLC separation of culture samples

Figure 4 shows a comparison of siderophores produced by fungal strains of three different genera: *Penicillium resticulosum, Fusarium dimerum*, and *Aspergillus fumigatus*. From the HPLC chromatograms it is obvious that the majority of the peaks is still unknown. Photodiode array detection allowed the distinction between siderophores of the type 1–4 and non-siderophores according to their ultraviolet/visible spectra (Table 3). The quantitative determination of siderophore production of the three species after 5 days of growth in low-iron medium is also shown in Table 3. These values greatly depend on the strain used, the size of the inoculum (e.g. the amount of germinated conidiospores), as well as on the medium and cultivation conditions. Time-dependent siderophore production by *Neurospora crassa* was quantitatively followed between the 3rd and 5th day of cultivation. Results are presented in Fig. 5 and Table 4.

Discussion

Analysis of siderophore production

According to our assignment (Table 3) the main siderophores of *P. resticulosum* after 5 days of growth under iron limitation are triacetylfusarinine C (peak 17) and ferricrocin (peak 5). In addition, we found a peak X_3 at a retention time of 10.38 min (Fig. 4a) which seemed to correspond to neocoprogen II in both the C_{18} and the diol HPLC systems (data not shown). But diode array detection revealed the non-siderophore character

Table 3. Assignment and quantification of siderophore production of fungi after 5 days of cultivation in low-iron medium as illustrated in Fig. 3

Fungus	Peak	Retention time	Assignment of siderophore[a]	Concentration
		(min)		(mg/l)
a) *P. resticulosum*	X_1	4.46	No siderophore	
	X_2	9.63	No UV/VIS spectrum	
	X_3	10.38	No siderophore	
	X_4	10.84	No siderophore	
	X_5	11.26	No UV/VIS spectrum	
	5	13.02	Ferricrocin	33
	X_6	13.83	No siderophore	
	X_7	20.09	Siderophore (type 1)	
	X_8	28.60	Siderophore (type 1)	
	X_9	34.09	Siderophore (type 1)	
	17	39.48	Triacetylfusarinine C	30
b) *F. dimerum*	1	4.30	Coprogen B (type 1)	4
	Y_1	13.06	No UV/VIS spectrum	
	Y_2	14.19	Siderophore (type 1)	71[c]
	Y_3	17.53	Siderophore (type 1)	36[c]
	Y_4	19.47	(Dimethylcoprogen[b]) (type 1)	46[c]
	Y_5	21.05	No UV/VIS spectrum	
c) *A. fumigatus*	Z_1	4.46	No Siderophore	
	Z_2	7.37	No UV/VIS spectrum	
	Z_3	9.70	No UV/VIS spectrum	
	5	12.94	Ferricrocin	14
	Z_4	19.94	Siderophore (type 1)	
	Z_5	28.63	Siderophore (type 1)	
	Z_6	34.14	Siderophore	
	17	39.26	Triacetylfusarinin C	27

[a] Assignment based on retention times, spectrum and the ultraviolet/visible (UV/VIS) coinjection procedure
[b] Structure determined by D. van der Helm (personal communication)
[c] Estimated values using coprogen characteristics (absorption coefficient, calibration graph, molecular mass)

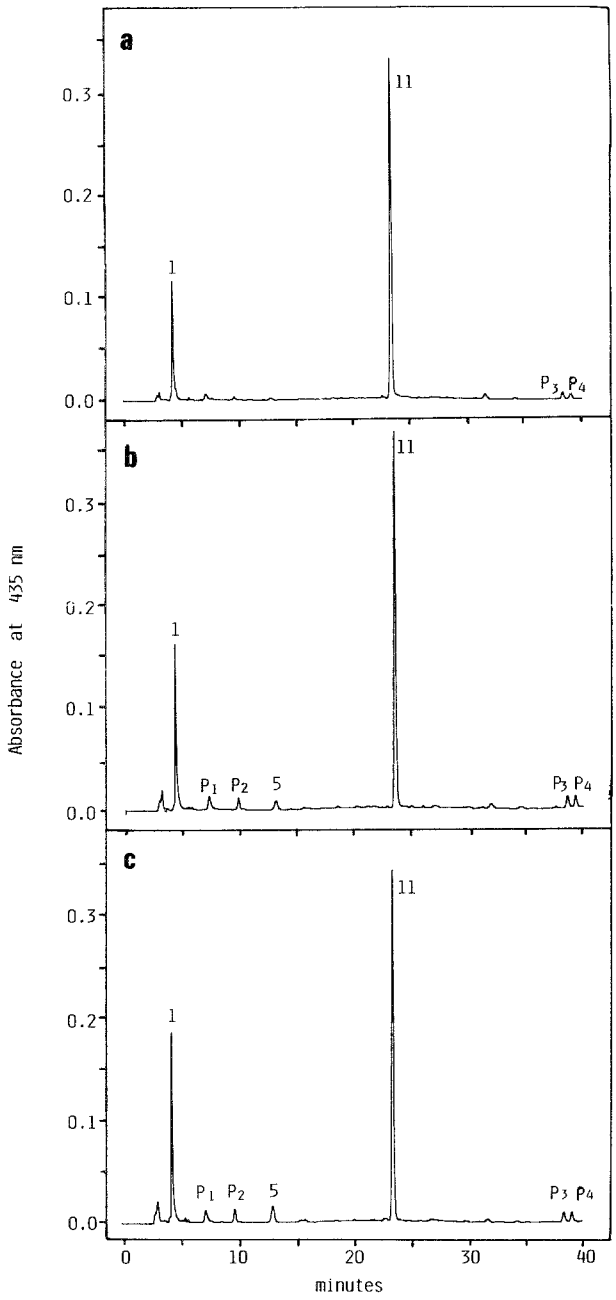

Fig. 5a–c. Time-dependent siderophore production in low-iron culture of *Neurospora crassa*. Low-iron medium (200 ml) was inoculated with conidiospores of *N. crassa* 74A (wild type). After 3 days **(a)**, 4 days **(b)** and 5 days **(c)**, aliquots of the culture filtrate were adsorbed on XAD-2, and the methanol eluate was analysed on Nucleosil C_{18} using the procedure as described in Fig. 2a

of this component. Also the dominant peak X_1, having a slightly different retention time compared to coprogen B, belonged to a non-siderophore compound. Typical siderophore spectra of

Table 4. Production of siderophores of *Neurospora crassa* determined after 3, 4, and 5 days of cultivation in low-iron medium

Peak	t_R (min)	Assignment[a] of siderophore	Concentration after		
			3 d (mg/l)	4 d	5 d
1	4.36	Coprogen B	9.1	13.5	13.7
P_1	7.29	No siderophore			
P_2	9.86	No siderophore			
5	13.10	Ferricrocin	0.6	2.3	3.0
11	23.53	Coprogen	32.4	34.3	28.5
P_3	38.60	No UV/VIS spectrum			
17	39.30	No siderophore			

[a] See Table 3

type 1 were produced by the components X_7, X_8 and X_9.

The pattern of siderophore production of low-iron cultures of *F. dimerum* is shown in Fig. 4b. From these siderophores only coprogen B (peak 1) is already known to be produced by this strain (Diekmann 1970). However, three main peaks (Y_{2-4}) are still unknown. Out of these the major peak (Y_4) is identical with N^{α}-dimethylcoprogen isolated from cultures of *Alternaria alternata* (Jalal et al. 1988, this journal). The isolation and structural elucidation of components Y_2 and Y_3 are being carried out presently in our laboratories.

The siderophores produced by low-iron cultures of *A. fumigatus* (Fig. 4c) resemble those found in *P. resticulosum*. Again triacetylfusarinine C (peak 17) is the major product followed by ferricrocin (peak 5). The peaks Z_1, Z_4, Z_5 and Z_6 correspond to X_1, X_7, X_8 and X_9. Identical ultraviolet/visible spectra support the presence of the same siderophores in both genera. Moreover, by comparison with the standard mixture, it could be excluded that these siderophores are identical with any known fungal trihydroxamates. We therefore assume that they represent new structures or derivatives of known siderophores. As shown in the present investigation, triacetylfusarinine C is also found in the culture filtrate of *P. resticulosum* confirming the earlier findings of Moore and Emery (1976), although the *Penicillium* strain described in that paper was later identified as *Mycelia sterilia* (Adjimani and Emery 1987).

HPLC analysis provides a highly valuable tool for the control of siderophore production from samples of low-iron culture filtrates. To demonstrate the efficiency of this method we followed

the siderophore production in *N. crassa*. This fungus is known to produce coprogen B (peak 1) and coprogen (peak 11). These main products are clearly detectable even after only three days of low-iron cultivation (Fig. 5, Table 4). Prolonged cultivation (4 and 5 days) indicated the presence of additional minor peaks among which ferricrocin (peak 5) has been reported to occur. Ferricrocin is believed to be a predominantly intracellular siderophore in *N. crassa* (Horowitz et al. 1976). Peaks P_1 and P_2 are not siderophores.

Chromatographic behaviour and structural properties

The chromatographic behaviour of the ferrisiderophores largely obeys common rules for reversed-phase systems. Highest mobility was found for the components coprogen B and ferrioxamine B which can be protonated. Within the series coprogen, neocoprogen I, and neocoprogen II the interaction with the C_{18} phase decreased on stepwise exchange of the anhydromevalonic acid end groups by acetyl residues. The order of elution of the components
ferrichrome (—Gly—Gly—Gly—),
ferricrocin (—Gly—Ser—Gly—) and
ferrichrysin (—Ser—Ser—Gly—),
differing in the exchange of one or two glycine residues against serine in their peptide backbone, was surprising. An increasing number of serine hydroxyl functions was expected to enhance the polarity of the three siderophores in the order listed above. Instead of exhibiting the expected decrease in retention time, ferrichrome and ferrichrysin were eluted together after ferricrocin. Obviously the introduction of only one Ser residue enhances the polarity somewhat, whereas two serines again alleviate this difference. No plausible explanation was found. On the other hand, the exchange of glycine against the more lipophilic alanine in ferrichrome C resulted in the expected higher retention time in comparison to ferrichrome, whereas the increase in peptide ring size in tetraglycyl-ferrichrome lowered the elution time due to higher polarity.

Within the series ferrichrysin, asperchrome D1 and B1, ferrirubin and ferrirhodin all compounds possess the same tripeptide segment —Ser—Ser—Gly—, whereas the three N^5-acetyl groups surrounding the iron center are successively replaced by the longer anhydromevalonic acid residues. The resulting differences in lipophilicity and molecular size agree with the order of elution of these components. The mobility of these siderophores also depends on the configuration of the anhydromevalonic acid residues, the 3-*trans*-ferrirubin being eluted before the 3-*cis*-ferrirhodin. From x-ray structures it is known that the 0=C—C=C torsional angles of the three anhydromevalonic acid residues are close to 0°, leading to a preferred spatial orientation of the methyl and hydroxyethyl groups situated at the double bond with respect to the chelated iron center (Huschka et al. 1986; van der Helm et al. 1987). In ferrirubin the methyl groups point towards the central atom and the hydroxyethyl groups point outward and interact with the stationary HPLC phase. This stereochemistry is reversed for ferrirhodin showing higher lipophilicity compared to ferrirubin due to its outward oriented methyl groups. The shorter retention time of des(diserylglycyl)ferrirhodin in comparison to ferrirhodin is explained by the missing backbone. The influence of the three *trans*-(4-carboxy-3-methylpent-2-enoyl) residues in ferrichrome A is obviously very similar to that of the anhydromevalonic acid residues. The smallest mobility in the C_{18} system was exhibited by triacetylfusarinine C (triacetylfusigen). Probably its disc-like shape with three acetyl groups on the outside allows strong interaction with the stationary phase. The order of elution remained the same for C_{18} and C_8 columns, with the exception of des(diserylglycyl)-ferrirhodin showing the highest retardation of all components on the C_8 phase.

Taking into account our experiences, the screening for siderophores is recommended on the basis of our optimized C_{18} separation, despite the fact that the time required is twice as long as for the C_8 column. A sharp separation and reproducibility as obtained with the C_{18} column is essential for screening purposes in order to separate the various naturally occurring siderophores. At an equilibration time of 20 min the deviation in retention times was about ± 0.05 min within a series of 20 consecutive separations. At a wavelength of 435 nm the detection and quantification of siderophores in samples of culture filtrates is reliable and seldomly disturbed by other chromophores. The identification of siderophores in culture media can be achieved by comparison of the retention times with those recorded for the standard mixture I, and should additionally be confirmed by coinjection and, if possible, by photodiode array detection. Furthermore the application of the recently developed HPLC/mass spectrometry on-line detection technique would improve and accelerate the identification of separated siderophore components.

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