Siderophores Produced by *Magnaporthe grisea* in the Presence and Absence of Iron

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An analysis of siderophores produced by *Magnaporthe grisea* revealed the presence of one intracellular storage siderophore, ferricrocin, and four coprogen derivatives secreted into the medium under iron depletion. Structural analysis showed that the compounds are coprogen, coprogen B, 2-*N*-methylcoprogen and 2-*N*-methylcoprogen B. Siderophore production under low and high iron conditions was quantified.

Key words: Magnaporthe grisea, 2-*N*-Methylcoprogen, 2-*N*-Methylcoprogen B

Introduction

Magnaporthe grisea, a highly virulent plant-pathogenic fungus, infects rice plants and other agriculturally important cereals including wheat, rye and barley causing blast disease which results in economically significant crop losses (Leung *et al.*, 1988; Ou, 1985).

Iron is an essential growth factor for nearly all organisms. Accessibility of iron is highly limited in natural environments due to its very low solubility at neutral pH. Fungi use siderophores as iron-complexing compounds to sequester iron (Haas, 2003; Leong and Winkelmann, 1998; Winkelmann, 2001). Siderophore biosynthesis is essential for virulence in host-fungus interactions, *e.g.* in the human pathogen *Aspergillus fumigatus* (Schrettl *et al.*, 2004), but not in others, like the plant pathogen *Ustilago maydis* (Mei *et al.*, 1993). Furthermore, siderophores function intracellularly as storage compounds for iron, thereby preventing the formation of free radicals which are formed by unbound iron (Eisendle *et al.*, 2003).

In the present work, we have isolated and quantified the intracellular storage siderophore, ferricrocin, and four coprogen derivatives which are secreted by *M. grisea* under iron depleted growth conditions: coprogen, coprogen B, 2-*N*-methylcoprogen and 2-*N*-methylcoprogen B. The structure elucidation of the novel methylated coprogens is also presented.

Materials and Methods

Culture conditions

M. grisea was grown in complete medium (CM) (10 g/l glucose, 1 g/l bacto yeast extract, 1 g/l casein hydrolysate, 2 g/l soy peptone, 6 g/l $NaNO_3$, 0.5 g/l KCl, 0.5 g/l $MgSO_4$, 1.5 g/l KH_2PO_4, 1.7 mg/l $CoCl_2 \cdot 6H_2O$, 1.6 g/l $CuSO_4 \cdot 5H_2O$, 50 mg/ml EDTA, 5 mg/l $FeSO_4 \cdot 7H_2O$, 11 mg/l H_3BO_3, 5 mg/ml $MnCl_2 \cdot 4H_2O$, 1.5 mg/ml $Na_2MoO_4 \cdot 2H_2O$, 22 mg/l $ZnSO_4 \cdot 7H_2O$, pH 6.5). For cultures on solid media 1.5% of agar were added.

For iron depletion studies mycelium of *M. grisea* was washed four times with Sundström minimal medium (Sundström, 1964) (20 g/l glucose, 1.4 g/l L-asparagine, 0.35 g/l KH_2PO_4, 0.15 g/l $K_2HPO_4 \cdot 3H_2O$, 0.5 g/l $Na_2SO_4 \cdot 10H_2O$, 0.1 mg/l thiamine, 5 mg/l $CaCl_2$, 20 mg/l $MgCl_2 \cdot 6H_2O$, 0.27 g/l sodium citrate, 0.26 g/l citric acid, 0.22 g/l $MnSO_4 \cdot 4H_2O$, 0.20 g/l $ZnSO_4 \cdot 7H_2O$), transferred to Erlenmeyer flasks with Sundström minimal medium and incubated with agitation (120 rpm) at 28 °C. Iron-augmented medium contained 10 μM $FeCl_3$.

Isolation and characterization of siderophores

Mycelia were separated from the culture broth by filtration from a 7-d-old culture of *M. grisea*, grown in iron-depleted medium. Mycelia were extracted with methanol or acetone (1 h) and filtered, followed by concentration of the organic solution. The culture filtrate was loaded onto an XAD-16 column and washed with three column volumes of water. Siderophores were eluted with methanol or ethanol. After evaporation of the solvent *in vacuo*, the extract was dissolved in methanol and subjected to gel filtration on Sephadex LH20 in methanol. Fractions containing siderophores (*i.e.* showing red color after addition of $FeCl_3$) were pooled, concentrated and purified by

preparative HPLC [Zorbax Eclipse XDB-C8, 9.4 × 250 mm, 5 μm (Agilent), elution with 14% acetonitrile, 86% 0.1% formic acid, isocratic] using a Jasco modular HPLC system (Gross-Umstadt, Germany) consisting of a pump PU-980 and DAD MD-910.

For MS-MS analysis, samples and standards were dissolved in 50% methanol with 0.1% formic acid to a final concentration of 0.1 μg/μl. Experiments were performed on a Q-TOF2 (Micromass, Manchester, UK) with direct application of the sample in a nano-ESI capillary, 1 kV cone voltage, 80 °C source temperature and collision energies from 10 to 80 eV. Reference substances derived from the substance collection of the IBWF, Kaiserslautern, Germany.

Quantification of siderophores

M. grisea was grown in 300 ml Sundström medium with or without 10 μM $FeCl_3$ in duplicates. After 8 d, the mycelia were separated from the broth. The mycelial dry weight was determined from one half of the culture by washing and lyophilisation. Mycelia from the second half were extracted with methanol (2 × 75 ml). Extracellular siderophores were extracted by adsorption on an XAD-16 column as described above (2 × 100 ml from each culture). Siderophores in the extracts from mycelia and culture filtrate were analysed by HPLC-MS. The values given are the mean of two measurements of the duplicates.

Structure elucidation

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $(CD_3)_2SO$, and the solvent signals (2.50 and 39.51 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra (HRESI) were recorded with a Micromass Q-TOF MICRO instrument (Micromass).

2-N-Methylcoprogen: Colourless oil. – ^{1}H NMR [500 MHz, $(CD_3)_2SO$] δ [integral, mult., J (Hz)] = 9.68 (3 H, brs, 5/5′/5″-N-OH), 8.12 (2 H, s, 2′/2″-NH), 6.21 (3 H, brs, 7/7′/7″-H), 4.89 (1 H, dd, J = 4.8 and 10.6, 2-H), 4.19 (1 H, m, 10′-Ha), 4.15 (1 H, m, 10′-Hb), 3.81 (2 H, m, 2′/2″-H), 3.53 (4 H, m, 10/10″-H_2), 3.50 (6 H, m, 5/5′/5″-H_2), 2.84 (3 H, s, 2-N-CH_3), 2.38 (2 H, m, 9′-H_2), 2.23 (4 H, m, 9/9″-H_2), 2.01 (9 H, s, 11/11′/11″-H_3), 2.00 (3 H, s, 2-N-Ac), 1.80 (1 H, m, 3-Ha), 1.68 (2 H, m, 3′/3″-Ha), 1.67 (1 H, m, 4-Ha), 1.66 (1 H, m, 3-Hb), 1.60 (2 H, m, 3′/3″-Hb), 1.59 (4 H, m, 4′/4″-H_2), 1.45 (1 H, m, 4-Hb). – ^{13}C NMR [125 MHz, $(CD_3)_2SO$]: δ = 170.9 (C-1), 170.7 (2-N-Ac), 169.9 (C-1′/1″), 166.6 (C-6/6″), 166.3 (C-6′), 150.9 (C-8/8″), 148.7 (C-8′), 117.2 (C7′), 116.2 (C-7/7″), 62.3 (C-10′), 59.2 (C-10/10″), 55.8 (C-2), 53.8 (C-2′/2″), 46.8 (C-5/5′/5″), 43.8 (C-9/9″), 38.8 (C-9′), 32.5 (2-N-CH_3), 30.4 (C-3′/3″), 25.0 (C-3), 23.1 (C-4), 22.2 (C-4′/4″), 21.5 (2-N-Ac), 18.2 (C-11/11″), 17.9 (C-11′). – HRESI-MS: m/z = 783.4133 [M+H$^+$] ($C_{36}H_{59}N_6O_{13}$ requires 783.4140).

Results and Discussion

Magnaporthe grisea produces siderophores of the hydroxamate type

Ferricrocin was identified in mycelial extracts of *M. grisea* as the sole intracellular siderophore by means of MS-MS and has already been reported for iron-depleted cultures (Schwecke *et al.*, 2006). In *Neurospora crassa* intracellular ferricrocin functions as iron-storage compound and is translocated to the conidia (Matzanke *et al.*, 1988). The production of this siderophore was increased in the presence of iron. When grown in iron-depleted medium, four additional siderophores were excreted into the culture filtrate: coprogen, 2-*N*-methylcoprogen, coprogen B and 2-*N*-methylcoprogen B (Fig. 1). In order to exclude artificial methylation

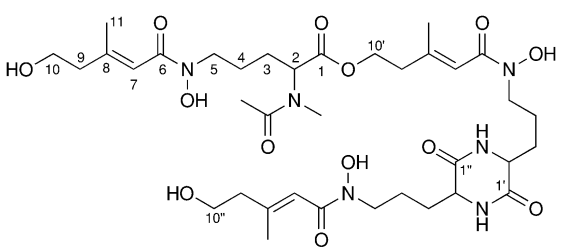

Fig. 1. Structure of 2-*N*-methylcoprogen.

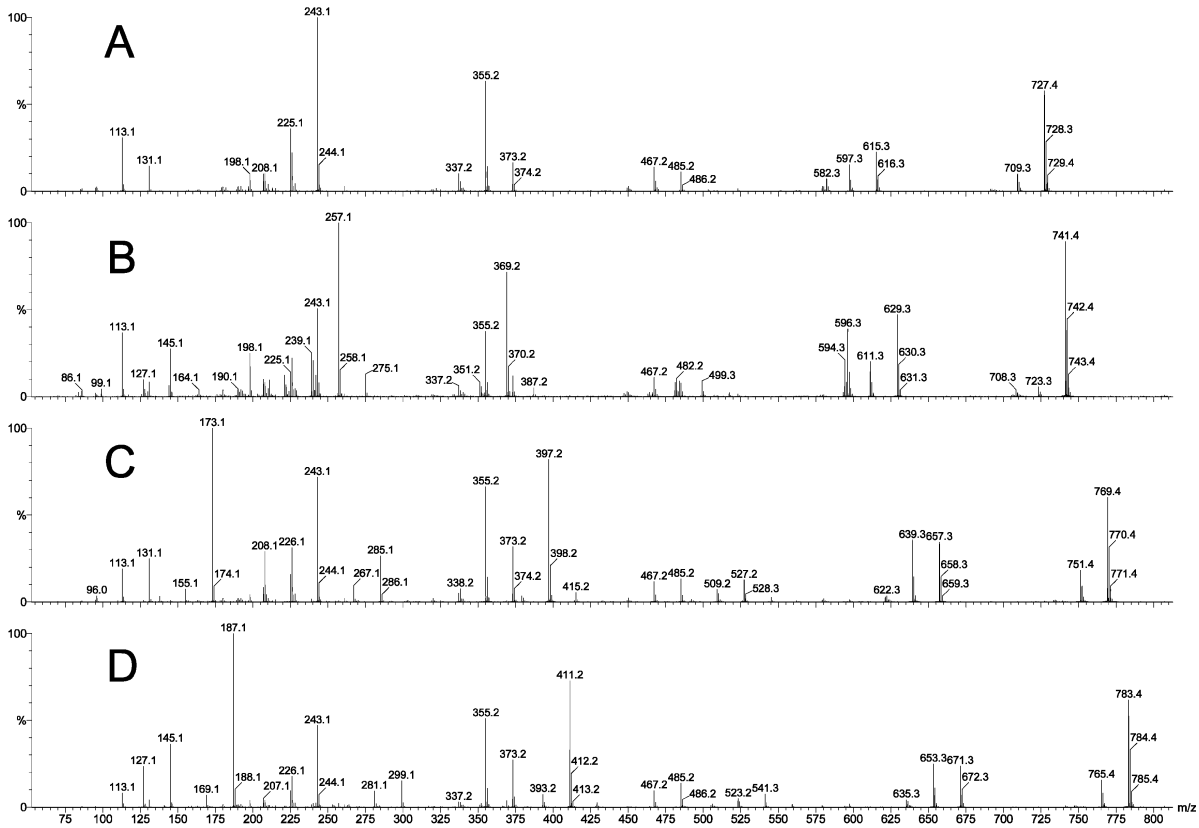

Fig. 2. Fragmentation spectra of the desferri-forms of coprogen B (A), 2-*N*-methylcoprogen B (B), coprogen (C) and 2-*N*-methylcoprogen (D).

during the isolation procedure, extraction and purification were also carried out using ethanol and acetone, respectively, and the same results were obtained. A methylated coprogen B has been previously isolated from *Histoplasma capsulatum* (Howard *et al.*, 2000) but no structural elucidation has been performed. Coprogen and coprogen B were identified by comparison of fragmentation spectra obtained by MS-MS and comparison of 1D NMR data with reference substances. The structure of 2-*N*-methylcoprogen was determined by HRMS spectrometry and 2D NMR spectroscopy, while that of 2-*N*-methylcoprogen B was suggested by the MS-MS data obtained with the couple coprogen B/2-*N*-methylcoprogen B and compared with coprogen/2-*N*-methylcoprogen (Fig. 2). The novel methylated coprogens bring the number of coprogen derivatives to a total of fourteen (Renshaw *et al.*, 2002).

Structure determination of the new coprogen derivatives

A complete structure elucidation with assignment of all NMR data was carried out with 2-*N*-methylcoprogen (see Fig. 1 for structure and numbering). The spectroscopic data showed similarities to those of coprogen itself, and high resolution MS data clearly indicated that the difference was due solely to the presence of an additional methyl group. The position of this was determined by correlations observed in the HMBC spectrum: 2-N-CH_3 correlates to both C-2 and the carbonyl carbon atom of the acetyl group, and 2-H correlates with 2-H–CH_3.

Quantification of the siderophores

For quantification of the siderophores produced by *M. grisea*, cultures were incubated in Sundström medium with or without supplementation of

10 μM $FeCl_3$. After 8 days of incubation, siderophores were quantified in mycelial extracts and in the culture broth by HPLC-MS. When grown in medium with iron, amounts of coprogen were below the limit of detection (LOD 0.07 μg/ml extract). When iron was absent, *M. grisea* produced a total of (130.5 ± 78.4) μg coprogens per ml culture filtrate. The predominant siderophores were 2-*N*-methylcoprogen [(48.1 ± 31.5) μg/ml] and coprogen [(41.7 ± 23.3) μg/ml], followed by 2-*N*-methylcoprogen B [(22.4 ± 14.7) μg/ml] and coprogen B [(18.4 ± 9.7) μg/ml].

Ferricrocin, the intracellular storage siderophore of *M. grisea*, was detected both in mycelial extracts of iron-depleted and iron-augmented cultures. The ferricrocin content of cultures grown in the presence of iron was about 9-fold higher [(242.9 ± 48.6) μg/g dry weight mycelium] as compared to growth in the absence of iron [(27.6 ± 6.0) μg/g dry weight].

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