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Isolation and Identification of the Principal Siderophore of the Dermatophyte *Microsporum gypseum*

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ABSTRACT: The dermatophyte *Microsporum gypseum* has been shown to produce two siderophores under conditions of low-iron stress. These compounds have been separated as Fe(III) complexes on silica gel, and the principal siderophore has been identified as ferricrocin using the methods of amino acid analysis, comparative thin-layer chromatography, partial sequencing by gas chromatography–mass spectrometry, ultraviolet spectroscopy, and proton nuclear magnetic resonance spectroscopy of the Al(III) complex.

Since the pioneering discovery by Neilands (1952) of ferrichrome in cultures of the smut fungus *Ustilago sphaerogena*, the chemistry of iron transport by fungi and bacteria has become an important and productive area of research (Neilands, 1973; Emory, 1974; Raymond & Carrano, 1979). Iron is an essential element for all forms of life, and to compensate for the extreme insolubility of Fe(III), the dominant oxidation state of that element in aerobic systems, microorganisms have evolved the capability of producing potent chelating agents, generally called siderophores, to transport iron into the cell where it is released for utilization in various life-essential processes. In fungi, various secondary hydroxamate peptides have been found most commonly as the iron-chelating ligands, while in bacteria, catecholates have proven to be important (Neilands, 1972). The ferrichrome class of iron-transporting compounds, frequently utilized by fungi, are cyclic hexapeptides consisting of three N^{δ}-acyl-N^{δ}-hydroxyl-L-ornithines linked in sequence, with the remaining three amino acids being various sequences of serine, glycine, and, occasionally, alanine.

The availability of iron is a critical factor in determining the pathogenicity of microorganisms invading living hosts, and successful microorganisms must produce iron-transport molecules which can favorably compete with iron chelators present in the host. The ability of mammalian serum to withhold iron from microorganisms is related to the binding of iron by proteins such as transferrin or lactoferrin. Upon invasion, the host response is a reduction of iron concentration in the blood plasma (Cortell & Conrad, 1967; Pekarak et al., 1972).

As early as 1923, it was recognized that serum has an inhibitory effect on dermatophytes (Jessner & Hoffman, 1923). In 1975, King et al. characterized the serum inhibition factor for dermatophyte growth as nondialyzable, fungistatic, and heat-stable at 56 °C for 4 h, and identified the factor as transferrin. They also demonstrated that addition of iron to serum lowered the inhibitory capacity of the serum and that the addition of other metals such as zinc, magnesium, manganese, or copper did not produce the same effect. The same group also showed that no differences existed between the inhibitory activity of serum from normal patients and that from patients with dermatophytosis (Carlisle et al., 1974). Recently, Kerbs et al (1979) demonstrated that deferoxamine methanesulfonate, an iron chelator, inhibited germ tube formation and growth of the dermatophyte *Trichophyton mentagrophytes* in microculture assays, and it was postulated that these results were due to iron deprivation.

Recent reports by Artis (1984) have presented evidence that the dermatophytic fungus *Trichophyton mentagrophytes* produces hydroxamate siderophores when cultured in a chemically defined, nonphysiologic low-iron medium. However, the exact chemical nature of the substances was not reported.

In spite of the probable key role that iron-transport chemistry plays in host invasion by pathogenic dermatophytes, there have been no reports in which iron-chelating agents have been identified within this important class of fungi. In this paper, we report the isolation and characterization of the principal siderophore produced by the dermatophyte *Microsporum gypseum* grown under iron-limiting conditions.

Materials and Methods

Microsporum gypseum Culture. (A) Preparation of Inoculum Medium. The inoculum medium was a modification of that of Wiebe and Winkelmann (1975) with the following composition: L-asparagine (5 g), $K_2HPO_4 \cdot 7H_2O$ (1 g), glucose (20% w/v, 100 mL), distilled, deionized water (900 mL). The pH of medium was adjusted to 6.0 prior to autoclaving at 121

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°C for 15 min. The glucose was added from a 20% w/v solution which had been separately autoclaved. Iron determination using atomic absorption spectroscopy according to usual procedures demonstrated the iron level to be less than 1 ng/mL.

(*B*) *Preparation of Iron-Limited Growth Media.* Two liters of the above-described medium was prepared at double the above concentration and was stirred 1 h with Chelex 100 (K^+ form, Bio-Rad Laboratories) and the resin removed by filtration. The resin was then washed with 2 L of distilled, deionized water, and the pH of the resulting medium was adjusted to 5. To 990 mL of this medium was added 10 mL of 10% w/v $MgSO_4$ and the resulting medium autoclaved at 121 °C for 15 min.

(*C*) *Fungal Culture.* *Microsporum gypseum*, strain TSDH-317256, was obtained from J. Steadham of the Texas State Department of Health and was maintained in stock culture on Sabouraud dextrose agar or potato dextrose agar slants at room temperature.

One hundred milliliters of Sabouraud dextrose broth (SDB) (Diffco) was inoculated from a 2–3-week-old slant culture and incubated at 25 °C for 5 days on a gyrorotary shaker at 250 rpm. The culture was then pelleted at 8000 rpm and the supernatant discarded. The pellet was resuspended in 100 mL of the modified Wiebe–Winkelmann inoculum media and incubated 2 days at 25 °C on a gyrorotary shaker. The resulting 2-day prestarved inoculum was centrifuged and the pellet washed twice with 100-mL portions of sterile physiological saline solution. The pellet was then resuspended in sterile saline solution and blended for 30 s. The blended mycelium was used to inoculate 1 L of Chelex-treated medium in 2-L flasks which had been acid-washed and rinsed with distilled, deionized water. The cultures were incubated on a gyrorotary shaker at 25 °C for 10 days.

Siderophore Isolation. *M. gypseum* iron-limited culture broth (33 L) was autoclaved and filtered, and the filtrate was concentrated to 1650 mL by vacuum rotary evaporation. To this solution was added 64 g of $FeCl_3$ and 1.2 kg of $(NH_4)_2SO_4$, and the resulting solution was stirred 1 h and extracted with five 50-mL portions of benzyl alcohol. To the combined benzyl alcohol phases was added 1.75 L of ethyl ether and the resulting solution extracted with two 65-mL portions of water. The combined aqueous extracts were evaporated to dryness by using a vacuum rotary evaporator to yield 4.7 g of a red-brown solid. Thin-layer chromatography on a 250-μm silica gel plate (Analtech) using 80% chloroform/19% ethanol/1% H_2O showed red-orange spots at R_f 0.31 and 0.15. On the same plates eluted with BuOH/methyl ethyl ketone/H_2O (2:2:1), band II exhibited an R_f of 0.24. The latter elution solvent on 250-μm Avicel plates yielded an R_f of 0.34 for band II and 0.51 for band I. When compared with an authentic sample of ferricrocin, the R_f of band II matched closely in each case. The total mixture was chromatographed on a 50 × 3 cm column packed with thin-layer chromatography (TLC) grade silica gel G (EM) and eluted with 80% $CHCl_3$/19% ethanol/1% H_2O by using the technique of vacuum liquid chromatography (Targett et al., 1979). Two well-resolved red-orange bands were obtained. Band I (178 mg) corresponded to the R_f 0.31 component while band II (1.3 g) corresponded to the R_f 0.15 component on silica gel TLC as described above.

Siderophore Characterization. (*A*) *Amino Acid Analysis.* A sample of band II in methanol was hydrogenated at 25 psi over 5% Pd/C for 8 h to reduce the complexed hydroxamate peptide to a simple peptide. Hydrolysis of the peptide was accomplished by heating in 6 N HCl for 48 h at 100 °C in a sealed vial. The aqueous HCl was removed by vacuum evaporation. A sample of the hydrolysate of the reduced material was converted to the *N*-trifluoroacetyl *n*-butyl esters according to the quantitative procedure of Roach and Gehrke (1969). Gas–liquid chromatography of the derivative was done quantitatively using a Perkin-Elmer 900 gas–liquid chromatograph fitted with a 6 ft × $^1/_8$ in. SE 30 column at a helium flow rate of 20 mL/min and oven temperature of 150 °C. Amino acid ratios were quantitated by comparison of ratios obtained with mixtures of derivatized standard samples.

(*B*) *Deferration of Iron-Complexed Peptide.* A sample of the iron-complexed band II hydroxamate was dissolved in methanol and stirred 48 h at room temperature with an excess of 8-hydroxyquinoline. The resulting solution was evaporated to dryness and partitioned between water and chloroform. After being extracted 3 times with chloroform, the aqueous phase was evaporated to dryness under vacuum.

(*C*) *NMR Spectra.* The NMR spectrum of the aluminum complex of deferrated band II, prepared as described by Llinas et al. (1972), was obtained on a Varian XL-200 NMR spectrometer at 55 °C using dimethyl-d_6 sulfoxide as solvent with tetramethylsilane as internal standard. In addition to the acetyl methyl absorption at 2.1 ppm, the crucial absorptions for characterization were those of the amide protons. These were as follows: 6.43 ppm (d, J = 8 Hz, Orn 3); 6.87 ppm (br t, Gly-2); 7.96 ppm (d, J = 8 Hz, Orn 2); 8.63 ppm (br d, Ser); 9.03 ppm (br t, Gly-1); 10.07 ppm (d, J = 6 Hz, Orn 1).

(*D*) *Gas Chromatography/Mass Spectrometry (GC/MS) Sequence Analysis.* Amino acid sequence information on band II was obtained in the following way. A sample of band II material (2 mg) was dissolved in methanol (10 mL), and 6 mg of 5% palladium on charcoal was added. The suspension was hydrogenated at 25 psi for 10 h and filtered. The filtrate was evaporated to dryness under vacuum and the residue dissolved in 2 mL of 6 N HCl. Portions of this solution (0.5 mL) were separately placed in an oven at 110 °C and removed at 15-min intervals. The combined samples were evaporated to dryness under vacuum. The hydrolyzed peptide was converted to poly(aminoalcohol) derivatives according to the procedure of Carr et al. (1981). In summary, the peptides were methylated with methanolic HCl and trifluoroacetylated with methyl trifluoroacetate/methanol (1:1). Carbonyl functions were reduced with perdeuteriodiborane in tetrahydrofuran. Following workup, the poly(amino alcohols) were trimethylsilylated with (trimethylsilyl)diethylamine and injected into the GC/MS system (Hewlett-Packard 5985 B). Gas chromatography was performed on a 30 m × 0.32 mm fused silica SE 30 capillary column. GC conditions were as follows: splitless injection at 250 °C; column oven temperature programmed from 70 to 270 °C at 8 °C/min; Pt/Ir transfer line to MS at 275 °C. Mass spectra were collected every 2 s. The electron impact source was maintained at 200 °C with an electron energy of 70 eV.

Results and Discussion

Thin-layer chromatography (TLC) of the benzyl alcohol extract of a culture of the dermatophyte *Microsporum gypseum* grown under iron-limiting conditions and then treated with Fe(III) indicated the presence of two red-orange compounds. These components were preparatively separated for characterization by using vacuum liquid chromatography on TLC-grade silica gel. Both components exhibited UV spectra typical of siderophore iron complexes. The slower moving, major component (band II), comprising 88% of the total

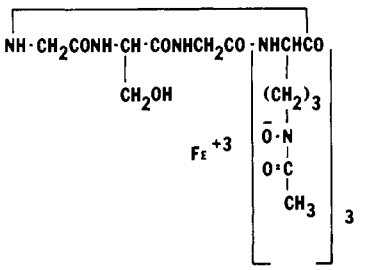

FIGURE 1: Structure of ferricrocin.

mixture, showed a λ_{max} in water at 422 nm. TLC of band II material was conducted in three solvent systems and compared to standard samples. TLC data matched well with those of ferricrocin (Figure 1) under all sets of chromatographic conditions. A sample of the band II material was subjected to hydrogenation to remove iron and reduce the *N*-hydroxy peptide to a peptide containing the corresponding amino acids. When this peptide was hydrolyzed in hydrochloric acid, ornithine (Orn), serine, and glycine were identified by TLC. The amino acids were converted to the *N*-trifluoroacetyl *n*-butyl esters, and the glycine:serine ratio was shown by gas–liquid chromatography to be 2:1. A sample of the band II peptide was subjected to partial hydrolysis, and the fragments were derivatized by an established method (Carr et al., 1981) involving conversion to the methyl esters, N-trifluoroacetylation, reduction with perdeuteriodiborane, and trimethylsilylation. Using GC/MS, we were able to establish the presence of derivatives corresponding to Orn-Orn, Orn-Gly, and Ser-Gly. We were unable to establish conditions for detection of all fragments. However, all known siderophores of this class have the Orn-Orn-Orn sequence present as apparently required for stable octahedral iron complexation of the *N*-hydroxyornithines. The presence of the Orn-Orn-Orn sequence in our peptide would require that the remaining amino acids be in the sequence Gly-Ser-Gly. Conclusive evidence for the structure of the principal siderophore of *M. gypseum* was provided by the 200-MHz proton NMR spectrum of the aluminum complex in dimethyl-d_6 sulfoxide of the band II hydroxamate peptide. The acetylated ornithine units displayed a nine-proton absorption at 2.1 ppm. As reported by Llinas et al. (1972), the amide N–H absorption patterns between 5 and 10 ppm of the aluminum complexes of the hydroxamate peptides are highly diagnostic for the structure, including the amino acid sequence of these systems. Our spectrum was identical with that reported by Llinas et al. (1972) for alumicrocin. The principal iron-transport compound produced by *M. gypseum* is thus ferricrocin (Figure 1).

This research represents the first report of the identification of a siderophore associated with a dermatophyte. Ferricrocin has been previously reported as an iron-transport compound elaborated by *Aspergillus* and *Streptomyces* species (Llinas & Neilands, 1972). Our presently reported work provides the foundation for future assessment of the hypothesis of Artis (1984) that siderophores of fungal pathogens antagonize transferrin's antifungal activity and host defense. Our work continues in identification of the minor siderophore of *M. gypseum*.

Registry No. Ferricrocin, 23086-46-6.

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