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Synthesis and properties of different metal complexes of the siderophore desferriferricrocin

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Abstract

Desferriferricrocin is a cyclic hexa-peptide siderophore with three hydroxamates as primary coordination groups. It forms metal complexes with Fe(III), Cr(III), Al(III), Ga(III), Cu(II), and Zn(II). These complexes were prepared and characterized using UV–vis, circular dichroism spectroscopy (CD), nuclear magnetic resonance spectroscopy (NMR), and electrospray ionization mass spectroscopy (ESI-MS). The mononuclear trivalent metal complexes of desferriferricrocin were stable in aqueous solutions, and their coordination centers primarily adopted the Λ configuration. The formation of multinuclear complexes of desferriferricrocin was determined by ESI-MS. Desferriferricrocin was able to bind up to three Cu(II) and two Zn(II) respectively. Heteronuclear complexes containing one trivalent and one divalent were also determined. In these complexes, amide nitrogens were utilized as alternative binding groups of desferriferricrocin in addition to the primary binding groups, the hydroxamates.

Introduction

Siderophores are low molecular weight organic molecules produced by many microorganisms under iron-deficient conditions. They serve to chelate and solubilize available ferric iron (Drechsel & Winkelmann 1997). The chelated iron(III) is transported into the cells through high-affinity iron transport systems which may include a shuttle (Emery 1971), reductive taxi (Ecker & Emery 1983), ligand exchange (Müller *et al.* 1985), or endocytosis transport mechanism (Yun *et al.* 2000).

Desferriferricrocin is a common ferrichrome-type siderophore with a cyclic hexapeptide backbone. Desferriferricrocin is produced under iron-limiting conditions by a number of *Aspergillus* and *Neurospora* species (Winkelmann & Huschka 1987) to facilitate iron uptake in these species (Wiebe & Winkelmann 1975; Huschka *et al.* 1985). Desferriferricrocin was also produced by a number of mycorrhizal fungi including the ericoid mycorrhizal fungi *Hymenoscyphus ericae* and *Oidiodendron griseum* (Haselwandter *et al.* 1992), the ectendo-mycorrhizal fungi *Wilcoxina* spp. (Prabhu *et al.* 1996), and the ecto-mycorrhizal fungus *Cenococcum geophilum* (Haselwandter & Winkelmann 2002). In these mycorrhizal associations, desferriferricrocin may also enhance iron uptake of their associated host plants (Shaw *et al.* 1990; Haselwandter 1995).

Desferriferricrocin uses three hydroxamates as its binding groups. It strongly binds ferric iron (Figure 1) with stability constant as high as $10^{30.4}$ (Wong *et al.* 1983). Desferriferricrocin may also bind metals other than iron. The complex of desferriferricrocin with Al(III), as well as the complexes of the closely related desferriferrichrome, with Al(III), Ga(III) (Llinás *et al.* 1972), and Cr(III) (Leong & Raymond 1974b), have been characterized by NMR and circular dichroism spectrometry (CD). These metal complexes may be

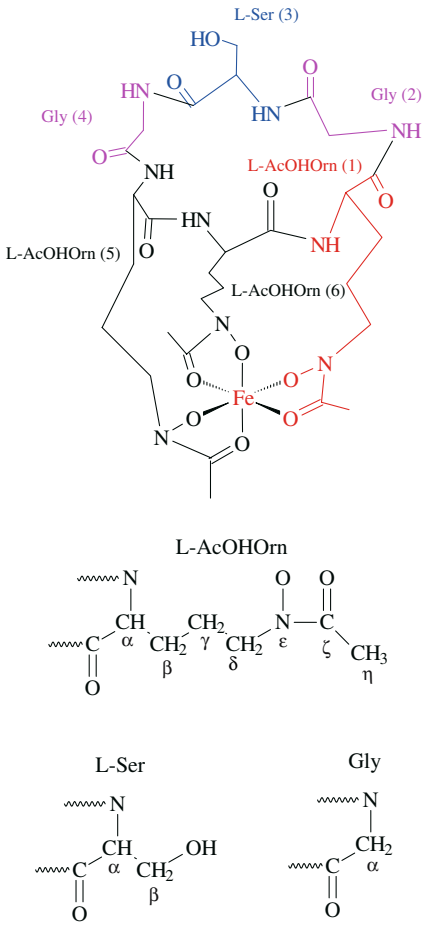


Figure 1. The primary structure of ferricrocin. Its six constituent amino acids are numbered and labeling of the individual amino acid side chain is indicated.

taken up or excluded by the cell depending if they are recognized by their corresponding transport systems. For example, the siderophores schizokinen and *N*-deoxyschizokinen produced by *Bacillus megaterium* formed stable complexes with trivalent aluminum. These complexes were recognized and taken up by a high-affinity transport system of *B. megaterium* leading to an enhanced aluminum uptake at low levels of aluminum for iron-deficient *B. megaterium* (Hu & Boyer 1996). In contrast, the schizokinen-Cu(II) complex was not recognized by its uptake receptor and was excluded by a cyanobacterium, *Anabaena* sp. In this case, the addition of the siderophore to the culture media lowered the intracellular copper content (Clarke *et al.* 1987).

Similar work has not been done with the siderophores produced by mycorrhizal fungi. To understand the importance of siderophores in the transport of metal ions in these species, it is necessary to characterize the structure of these siderophore complexes. Here we prepared the complexes of desferriferricrocin with Fe(III), Al(III), Ga(III), Cr(III), Cu(II), and Zn(II) and characterized them by UV–vis, CD, NMR, and ESI-MS spectroscopy.

Materials and methods

Preparation of desferriferricrocin and its mononuclear trivalent metal complexes

Ferricrocin, the Fe(III) complex of desferriferricrocin, was obtained from an iron-deficient culture of *Aspergillus fumagatus* and extracted as described by Garibaldi & Neilands (1955). Ferrous sulfate was added to the cell-free media at levels sufficient to form the deep red color of the iron complex and $(NH_4)_2SO_4$ was added at 70.6 g per 100 ml. Ferricrocin was extracted into benzyl alcohol and back extracted into water following addition of three volumes of diethyl ether. The aqueous extract was applied to a Biogel P2 column (2.5 × 80 cm; Bio-Rad, Hercules, CA) and eluted using 10% methanol as the mobile phase. Fractions absorbing at 425 nm were pooled and further purified by preparative HPLC using a Hamilton PRP-1 column (10 μm, 7 × 305 mm) eluted with a 30 min linear gradient of 5–50% acetonitrile containing 1% acetic acid at 2.0 ml min^{-1}. Desferriferricrocin was prepared by removing the iron from the ferricrocin with excess sublimed 8-hydroxyquinoline (Meyer 1978). The 8-hydroxyquinoline–iron complex was precipitated by centrifugation, the remaining 8-hydroxyquinoline–iron complex was extracted using chloroform, and the remaining desferriferricrocin was purified by preparative HPLC as described above.

The aluminum(III) complex of desferriferricrocin, alumicrocin, was prepared by mixing 1 ml of 10 mM desferriferricrocin in methanol with 1 ml of 40 mM $Al(NO_3)_3{\cdot}9H_2O$ in methanol. The reaction mixture was stirred at room temperature for 12 h. An equal volume of water was added and alumicrocin was purified by preparative HPLC as described above. The gallium(III) and chromium(III) complexes, gallicrocin and chromicrocin, were prepared similar to the aluminum

complex using 40 mM gallium nitrate, $Ga(NO_3)_3 \cdot H_2O$, or 40 mM chromium nitrate, $Cr(NO_3)_3 \cdot 9H_2O$, in place of aluminum nitrate and stirred for 24 h at room temperature before the purification step.

Characterization of the complexes

Desferriferricrocin and its metal complexes were characterized by electrospray mass spectroscopy (ESI-MS) on a Hewlett Packard 1100 series LC-MSD using a 4.6×150 mm, 5 μm, Hamilton PRP-1 column (Hamilton, Reno, NV) eluted with an 18 min linear gradient of 5–50% acetonitrile containing 1% acetic acid at a flow rate of 0.8 ml min^{-1}. The ESI-MS conditions used an ion mass range scan of 300–1800 m/z, drying gas flow rate of 12.0 l min^{-1}, nebulizer pressure of 50 PSI, drying gas temperature of 300 °C, capillary voltage of 3000 V, and fragmentation voltage ranged between 40 and 180 V. UV–vis and CD spectra were recorded on an Aminco MR 3000 photodiode array spectrometer and an AVIV 202 circular dichroism spectrometer. The ^1H and ^{13}C NMR spectra in H_2O containing 5% D_2O were obtained using a Bruker AVANCE 600 spectrometer. The water resonance peak was suppressed using the WATERGATE sequence during the acquisition (Liu *et al*. 1998) and the assignment was confirmed using the 2D COSY and HECTOR spectra (Zou 2003).

For preparation of the multinuclear complexes, a 10-fold molar excess of $Cu(NO_3)_2 \cdot 3H_2O$ or $ZnSO_4 \cdot 7H_2O$ was added to 0.5 mM desferriferricrocin, ferricrocin, alumicrocin, gallicrocin, or chromicrocin in water. These solutions were measured by flow injection analysis (FIA) using ESI-MS with the same operating parameters as used for LCMS as described above.

Results

Characterization of desferriferricrocin and its Al(III), Ga(III), Fe(III), and Cr(III) complexes

Desferriferricrocin, alumicrocin, and gallicrocin were synthesized and their UV absorption and CD spectra are shown in Figure 2. Alumicrocin and gallicrocin showed a negative CD band near 202 nm and a positive CD band near 230 nm. Ferricrocin and chromicrocin showed a similar positive CD band near 239 nm and a shoulder peak with the maximum at 233 nm respectively (Figure 3). Both displayed a negative CD band near 218 nm. The CD maximum at 230 nm for gallicrocin had the largest magnitude ($\Delta\varepsilon = 49.8$ M^{-1} cm^{-1}), followed by alumicrocin, ferricrocin, and chromicrocin with intermediate magnitudes ($\Delta\varepsilon \approx 5$–$11$ M^{-1} cm^{-1}), and then desferriferricrocin with the smallest magnitude ($\Delta\varepsilon = 0.89$ $M^{-1}cm^{-1}$). In addition to the strong absorption in the UV region, both ferricrocin and chromicrocin absorbed at visible wavelengths. Ferricrocin showed its characteristic absorbance at 425 nm, and chromicrocin absorbed at 257, 431, and 579 nm. Neither alumicrocin nor gallicrocin absorbed strongly in the visible region. The absorbance maxima, absorption coefficients (ε), CD maxima or minima, and $\Delta\varepsilon$ for all complexes are summarized in Table 1.

The HPLC retention times (RT) and singularly protonated molecular ions (MH^+) for desferriferricrocin, and its Al(III), Fe(III), Cr(III), and Ga(III) complexes are given in Table 2. Desferriferricrocin had a shorter retention time than its metal complexes on reverse-phase HPLC. Electrospray ionization of the trivalent metal complexes gave the singly protonated molecular ion. The molecular weight increase for the metal complexes over desferriferricrocin (m/z 718) were 53 amu ($+^{56}$Fe-3H) for the Fe(III) complex, 24 amu ($+^{27}$Al-3H) for the Al(III) complex, and 66 + 68 amu ($+^{69}$Ga-3H, $+^{71}$Ga-3H) for the Ga(III) complex.

The ^1H and ^{13}C-NMR spectra for desferriferricrocin, alumicrocin, and gallicrocin were obtained (Table 3). Spectra could not be obtained for ferricrocin and chromicrocin due to line broadening from the paramagnetic nature of trivalent iron ($t_{2g}^3 e_g^2$) and chromium (t_{2g}^3) (data not shown). The ^1H spectra of desferriferricrocin differed significantly from its Al(III) and Ga(III) complexes, whose amide proton resonances spanned a broader range, and whose methylene protons were clearly distinct.

Characterization of the Cu(II) and Zn(II) complexes with desferriferricrocin

In contrast to the four previously described trivalent metal complexes, no change was observed in the UV–vis or ^1H-NMR spectrum of

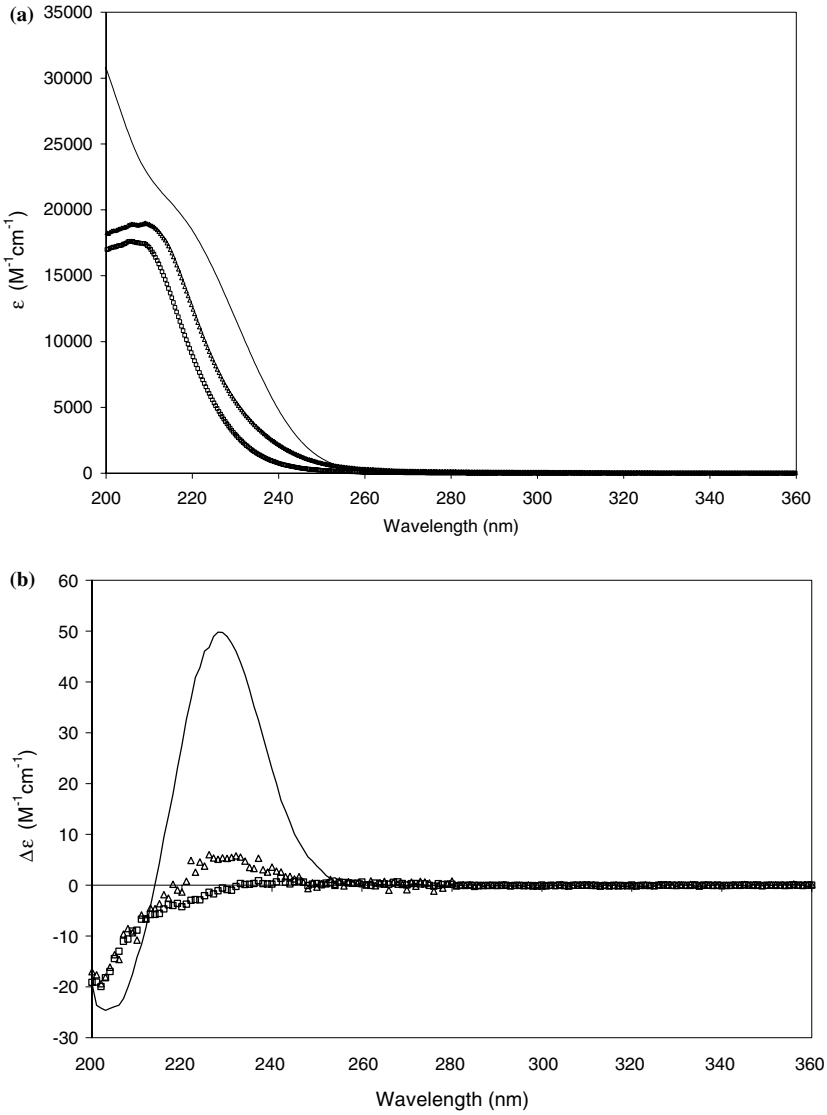


Figure 2. (a) UV absorption spectra and (b) circular dichroism spectra of desferriferricrocin (open squares), alumicrocin (open triangles), and gallicrocin (solid line) in aqueous solutions.

desferriferricrocin after the addition of Zn^{2+} at neutral pH. Increasing the pH to 13 resulted in the formation of a Zn(II)-desferriferricrocin complex(s) as indicated by the appearance of many new sharp peaks in the ^{1}H-NMR spectrum (Zou 2003). In a similar experiment with Cu^{2+}, line broadening of the ^{1}H-NMR due to the paramagnetic property of Cu^{2+} (d^{9}) made the spectrum un-interpretable, but the UV–vis absorbance spectra of the copper complex showed a weak absorbance maximum near 780 nm ($\varepsilon \approx 50\ M^{-1}\ cm^{-1}$). This absorbance shifted from 780 to 554 nm, and an intense absorbance near 250 nm ($\varepsilon \approx 1600\ M^{-1}cm^{-1}$) emerged when the pH was increased to 13.

The Cu(II) and Zn(II) complexes of desferriferricrocin were not stable to HPLC, so FIA analysis electrospray ionnization mass spectroscopy (FIA-ESI-MS) was used to directly identify the metal species in solution (Figure 4). Deferriferricrocin lost up to six protons while binding up to three coppers. The addition of zinc sulfate to desferriferricrocin gave similar multi-metal complexes

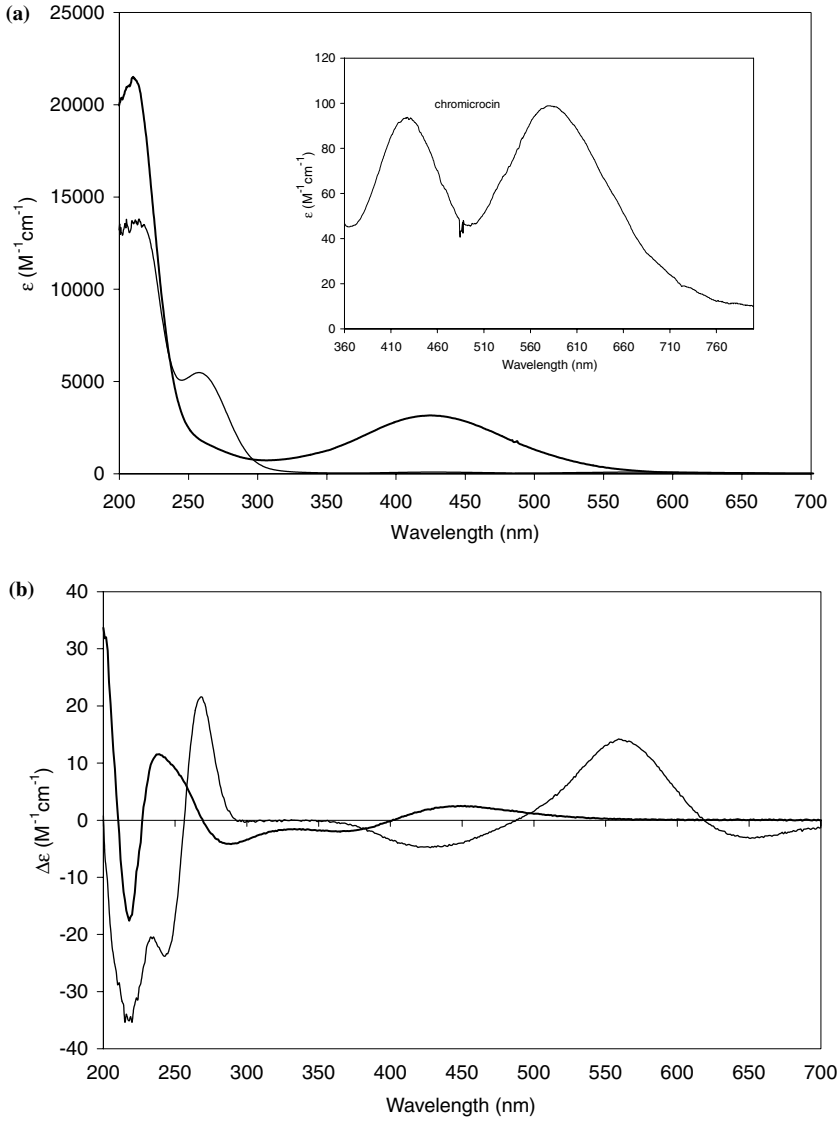


Figure 3. (a) UV–vis absorption and (b) circular dichroism spectra of ferricrocin (thick line) and chromicrocin (thin line) in aqueous solutions.

although binding of only one or two Zn(II) was observed (Figure 5).

Heteronuclear complexes of desferriferricrocin

When copper nitrate was added to the iron-containing complex ferricrocin, heteronuclear complexes were detected by FIA-ESI-MS (Figure 6). Divalent copper was either bound with one ferricrocin, two ferricrocins, one ferricrocin and one nitrate ion, or one ferricrocin after replacing two protons from ferricrocin. Addition of zinc sulfate to ferricrocin gave divalent zinc bound in one of four ways: with one ferricrocin, two ferricrocins, one ferricrocin and one SO_4^{2-}, or one ferricrocin after replacing two protons from the ferricrocin (Figure 7).

Cu(II) and Zn(II) also formed complexes with alumicrocin, gallicrocin, and chromicrocin, similar to ferricrocin. Cu(II) either bound to one desferriferricrocin–metal(III) complex after extracting two protons, or bound to one desferriferricrocin–metal(III) complex and one nitrate anion without extracting a proton (Table 4). For Zn(II), only

Table 1. Summary of the electronic and CD spectra of desferriferricrocin and its Al(III), Ga(III), Fe(III), and Cr(III) complexes.

Compound	Absorbance maximum in nm (ε)	CD maximum in nm ($\Delta\varepsilon$)
Desferriferricrocin		202 (−19.9)
		237 (0.89)
Alumicrocin		202 (−19.4)
		232 (5.77)
Gallicrocin		203 (−24.6)
		228 (49.8)
Ferricrocin	425 (3166)	218 (−17.6)
		239 (11.6)
		289 (−4.18)
		365 (−2.00)
		452 (2.51)
Chromicrocin	257 (5488)	217 (−34.8)
	431 (93.6)	233 (−20.4)*
	579 (98.9)	243 (−23.8)*
		268 (21.6)
		430 (−4.72)
		559 (14.2)
		651 (−3.17)

*Shoulder peak.

Table 2. Molecular weight and HPLC retention time of desferriferricrocin and its Al(III), Ga(III), Fe(III), and Cr(III) complexes as detected by ESI-MS.

Compound	RT (min)*	MH^+ (m/z)
Desferriferricrocin	5.71	718.3
Ferricrocin	7.01	771.3
Alumicrocin	6.30	742.3
Gallicrocin	6.60	784.2, 786.2
Chromicrocin	6.54	767.3

*HPLC conditions utilized a 4.6 × 150 mm, 5 μm, Hamilton PRP-1 column with an 18-minute linear gradient of 5–50% acetonitrile containing 1% aqueous acetic acid at a flow rate of 0.8 ml/min.

one type of complex was detected, namely one Zn(II) was bound with the desferriferricrocin–metal(III) complex after extracting two protons. The observed isotope pattern in the mass spectra showed excellent agreement (2%) with the calculated isotope abundance for the different metal complexes.

Discussion

The UV–vis absorption and CD spectra of ferricrocin measured here agreed well with those previously reported (Wong *et al*. 1983; Prabhu *et al*. 1996). Ferricrocin showed an absorbance maximum near 425 nm corresponding to a ligand metal charge transition (LMCT), and displayed a positive CD band near 452 nm and two negative bands near 365 and 289 nm respectively due to charge transfer transitions. A comparison with the CD spectrum of crystalline ferrichrome (van der Helm *et al*. 1980) indicated that ferricrocin formed the Λ configuration about its coordination center in aqueous solution. The weak absorption of chromicrocin near 579 and 431 nm are assigned to two spin-allowed d–d transitions, i.e. the $^4A_{2g} \rightarrow {}^4T_{2g}$ and $^4A_{2g} \rightarrow {}^4T_{1g}$ octahedral transitions, respectively (Leong & Raymond 1974a). The strong absorption near 257 nm was due to a charge transfer transition from the t_{1u} symmetry σ-bonding orbitals of the oxygen donors to the e^*_g σ-antibonding metal orbitals (Kuroda & Saito 1994). The CD spectrum of chromicrocin was more complicated, but can be explained assuming D_3 crystal field symmetry. There, the ground state $^4A_{2g}$ became $^4A_2$, the excited state $^4T_{2g}$ split into $^4A_1$ and ^4E, and the excited state $^4T_{1g}$ split into $^4A_2$ and ^4E. Correspondingly, the $^4A_{2g} \rightarrow {}^4T_{2g}$ and $^4A_{2g} \rightarrow {}^4T_{1g}$ octahedral transitions decomposed into $^4A_2 \rightarrow {}^4A_1 + {}^4E_a$ and $^4A_2 \rightarrow {}^4A_2 + {}^4E_b$ respectively. The $^4A_2 \rightarrow {}^4A_2$ was forbidden. The remaining three transitions were in sequence: $^4A_2 \rightarrow {}^4A_1$ (651 nm) < $^4A_2 \rightarrow {}^4E_a$ (559 nm) < $^4A_2 \rightarrow {}^4E_b$ (430 nm) according to increasing energy levels, and the CD band due to $^4A_2 \rightarrow {}^4E_a$ transition had the opposite sign with respect to the $^4A_2 \rightarrow {}^4A_1$ and $^4A_2 \rightarrow {}^4E_b$ transitions. Similarly, the high energy charge transition (CT) factored into two transitions due to the splitting of $^1T_{1u}$ octahedral state into ^1E and $^1A_2$ state in D_3 symmetry. These two transitions (243 nm and 268 nm) had opposite signs and similar magnitude as observed in the CD spectrum of chromicrocin. Furthermore, positive sign of the $^4A_2 \rightarrow {}^4E_a$ CD band meant that chromicrocin had a Λ configuration at the coordination center, similar to the Cr(III) complex of desferriferrichrome (Leong & Raymond 1974b). Alumicrocin and gallicrocin absorbed in UV region only (210 nm, $\varepsilon > 10000\ M^{-1}cm^{-1}$), which was assigned to the $\pi \rightarrow \pi^*$ transition of hydroxamate ligand. The n →

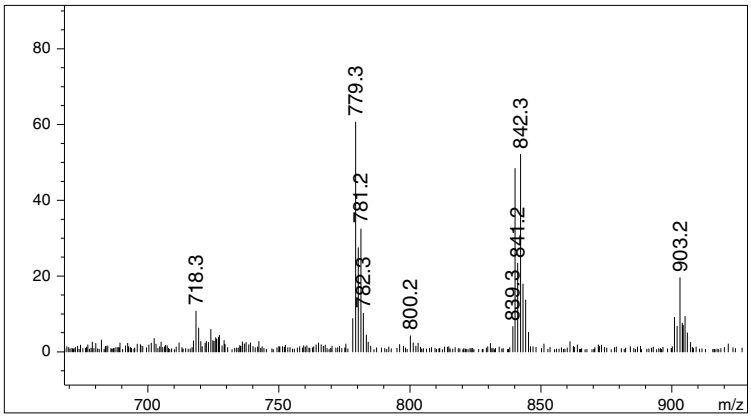
Table 3. The assignment of the ^{1}H and ^{13}C resonances of desferriferricrocin (deFCR), alumicrocin (AlCR), and gallicrocin (GaCR) at 273 K in H_2O containing 5% D_2O.

	HN	H_α	H_β	H_γ	H_δ	H_η	$C_{C=O}$	C_α	C_β	C_γ	C_δ	C_ζ	C_η
AcOHOrn1													
deFCR	8.03	4.18	1.65	1.50	3.57	2.03	173.91	54.33	27.79	22.53	51.31	171.79	19.99
AlCR	6.47	4.30	1.12, 1.98	1.61	3.48, 3.72	2.02							
GaCR	6.46	4.38	1.10, 2.01	1.65	3.53, 3.82	2.12	170.69	52.83	26.31	25.41	50.08	163.68	15.81
Gly2													
deFCR	8.38	3.69, 3.98					172.07	43.20					
AlCR	7.12	3.82, 3.92											
GaCR	7.21	3.83, 3.99					170.06	41.43					
Ser3													
deFCR	8.40	4.36	3.79				174.42	55.76	60.91				
AlCR	8.44	4.04	3.68										
GaCR	8.48	4.10	3.72				174.53	57.78	60.36				
Gly4													
deFCR	8.44	3.86, 4.35					172.45	43.40					
AlCR	9.02	3.66, 3.98											
GaCR	9.06	3.70, 4.01					171.74	43.39					
AcOHOrn5													
deFCR	8.19	4.20	1.80	1.50	3.53	2.03	174.12	54.51	28.96	23.34	47.62	169.60	19.70
AlCR	8.16	4.80	1.75	1.15, 1.34	3.33, 3.65	2.04							
GaCR	8.18	4.85	1.77	1.20, 1.40	3.40, 3.77	2.13	172.21	52.81	21.07	20.42	49.94	164.33	16.80
AcOHOrn6													
deFCR	8.07	4.22	1.70	1.60	3.53	2.03	174.26	54.34	28.69	22.77	47.62	169.60	19.70
AlCR	10.07	4.27	1.84, 2.33	1.66, 1.98	3.58, 4.02	2.03							
GaCR	10.05	4.31	1.92, 2.29	1.71, 2.03	3.68, 4.11	2.11	177.16	58.40	25.44	25.53	49.05	163.82	16.44

π^* transition of the peptide backbone at 210–230 nm was very weak ($\varepsilon < 100$ $M^{-1}cm^{-1}$) and a $\pi \rightarrow \pi^*$ ($\varepsilon > 5000$ $M^{-1}cm^{-1}$) transition of the peptide backbone would be at 185–190 nm (Nielsen & Schellman 1967). Neither of these transitions can be assigned to the absorbance at 210 nm observed in alumicrocin and gallicrocin. In the CD spectra, the $\pi \rightarrow \pi^*$ transition of the hydroxamate ligands gave rise to a exciton couplet, and the positive low energy CD band at 228–232 nm suggested that these complexes also adopted a Λ configuration at the coordination center (Ziegler & von Zelewsky 1998; Terpin *et al*. 2001). The magnitude of this CD band increased from alumicrocin to gallicrocin. This is similar to the Al(III) and Ga(III) complexes with 1,3-butanedione, where the magnitude also increased with the ionic radii, Ga^{3+} (0.76 Å) > Al^{3+} (0.68 Å), of the metal centers (Okawa *et al*. 1988). A similar CD band was observed in ferricrocin and chromicrocin although it overlapped with other absorptions.

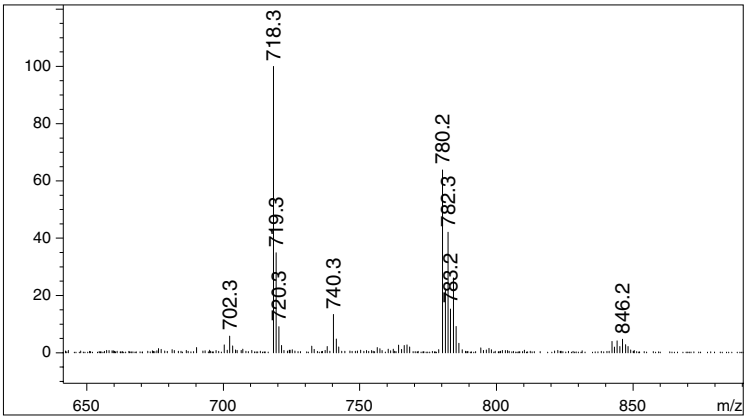
The ^{1}H and ^{13}C-NMR spectra were used to fully assign the NMR chemical shifts in desferriferricrocin, alumicrocin, and gallicrocin. The spectrum of alumicrocin expanded that initially reported by Llinàs *et al*. (1972). They have been recently used to calculate the solution structure of both alumicrocin and gallicrocin (Zou 2003). Unlike the Al(III) and Ga(III) desferriferricrocin complexes, a stable Zn(II) desferriferricrocin complex was not formed when Zn^{2+} was mixed with desferriferricrocin in aqueous solution. This was expected on thermodynamic grounds since hydroxamic acids are very weak acids ($pK_a \approx 9$) and the formation constant for the complexes of the dihydroxamate ligands with Zn(II) is low (Evers *et al*. 1989). This can be shown as the following:

$$Zn^{2+} + L^{2-} \rightarrow ZnL \quad K = 10^{7.76}\text{–}10^{8.80}$$



Molecular ions	*m/z*	Intensity (%)
[desferriferricrocin]H^+	718.3	11
[Cu(II)(desferriferricrocin-2H)]H^+	779.3	60
[$Cu(II)_2$(deferriferricrocin-4H)]H^+	840.2	45
[$Cu(II)_3$(desferriferricrocin-6H)]H^+	901.0	8

Figure 4. The flow injection analysis electrospray ionization mass spectrum (FIA-ESI-MS) and identification of Cu(II) desferriferricrocin complexes.



Ion	*m/z*	Intensity (%)
[desferriferricrocin]H^+	718.3	95
[Zn(II)(desferriferricrocin-2H)]H^+	780.3	65
[$Zn(II)_2$(desferriferricrocin-4H)]H^+	842.3	5

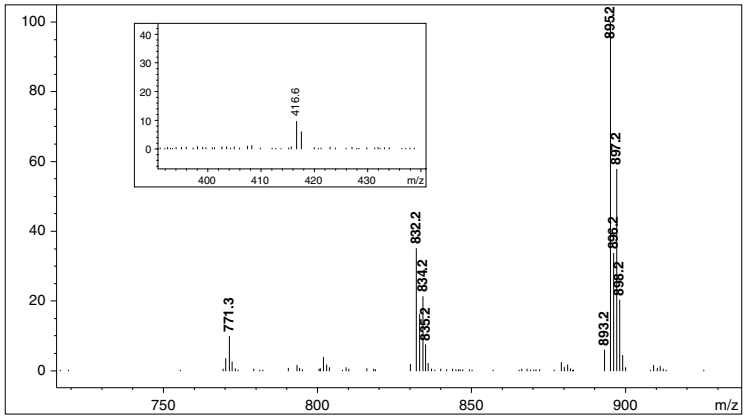
Figure 5. The flow injection analysis electrospray ionization mass spectrum (FIA-ESI-MS) and identification of Zn(II) desferriferricrocin complexes.

Accordingly, the equilibrium constant for the reaction:

$$Zn^{2+} + H_2L \rightarrow ZnL + 2H^+$$

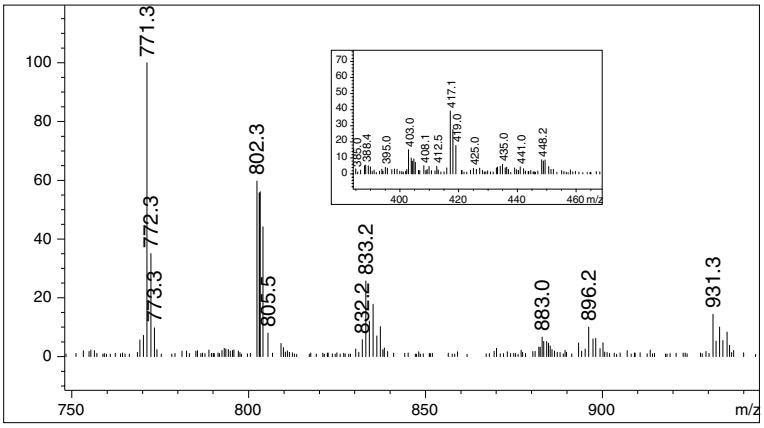
is about 10^{-10}. Considering the hydrolysis of Zn^{2+}:

$$Zn^{2+} + H_2O \rightarrow Zn(OH)^+ + H^+ \quad K = 10^{-9}$$



Molecular ions	*m/z*	Intensity (%)
$[Cu(II)(ferricrocin)]^{2+}$	416.6	10
$[Ferricrocin]H^{+}$	771.3	10
$[Cu(II)(ferricrocin)_2]^{2+}$	801.8	5
$[Cu(II)(ferricrocin\text{-}2H)]H^{+}$	832.2	35
$[Cu(II)(ferricrocin)(NO_3^{-})]^{+}$	895.2	100

Figure 6. The flow injection analysis electrospray ionization mass spectrum (FIA-ESI-MS) and identification of mono-nuclear ferricrocin and its Cu(II) complexes.



Molecular ions	*m/z*	Intensity (%)
$[Zn(II)(ferricrocin)]^{2+}$	417.1	39
$[Ferricrocin]H^{+}$	771.3	100
$[Zn(II)(ferricrocin)_2]^{2+}$	802.3	60
$[Zn(II)(ferricrocin\text{-}2H)]H^{+}$	833.2	26
$[Zn(II)(ferricrocin)(SO_4^{2-})]H^{+}$	931.3	14

Figure 7. The flow injection analysis electrospray ionization mass spectrum (FIA-ESI-MS) and identification of the heteronuclear Zn(II) complexes of ferricrocin.

which decreases the pH of the solution, the equilibrium concentration of ZnL would be about 10^{-7} M if 10^{-3} M of Zn^{2+} and an equal amount of a dihydroxamate ligand was mixed together in pure water. The formation of ZnL complex under these conditions would be too low to be observed in

Table 4. Mass to charge *(m/z)* ratios for the Cu(II) and Zn(II) complexes derived from alumicrocin, gallicrocin, and chromicrocin.

Molecular ions	*m/z*
$[Cu(II)(alumicrocin\text{-}2H)]H^+$	803.2
$[Cu(II)(gallicrocin\text{-}2H]H^+$	845.2
$[Cu(II)(chromicrocin\text{-}2H)]H^+$	828.2
$[Cu(II)(alumicrocin)NO_3^-]^+$	866.3
$[Cu(II)(gallicrocin)NO_3^-]^+$	908.2
$[Cu(II)(chromicrocin)NO_3^-]^+$	891.3
$[Zn(II)(alumicrocin\text{-}2H)]H^+$	804.2
$[Zn(II)(gallicrocin\text{-}2H)]H^+$	846.3
$[Zn(II)(chromicrocin\text{-}2H]H^+$	829.3

The complexes were formed by addition of copper nitrate or zinc sulfate to the preformed Al(III), Ga(III), and Cr(III) desferriferricrocin complexes.

NMR spectra. As the pH was increased to 13, the formation of Zn(II) complexes with desferriferricrocin was observed in the ^{1}H-NMR spectrum. A similar phenomena has been reported for Zn(II) and mugineic acid. Mugineic acid is a stronger acid ($pK_a \approx 3$) than hydroxamate acids and thus will favor the formation of Zn(II) mugineic acid complexes at lower pH (Nomoto *et al*. 1987). The formation of the Cu(II) desferriferricrocin complex was visible in UV–vis spectra as has been reported for Cu(II) desferrioxamine B complexes (Biruš *et al*. 1999). The ligating groups changed from hydroxamates to peptide nitrogens as the pH increased to 13 as indicated by the blue shift of the absorption from 780 to 550 nm along with the appearance of a strong charge transfer transition at 250 nm (Tsangaris *et al*. 1969; Sigel & Martin 1982;).

Flow injection analysis using electrospray ionization mass spectrometry (ESI-MS) gave additional structural information of the complexes of desferriferricrocin with Cu(II) and Zn(II). ESI is a ''soft ionization'' method and transfers pre-existing ions in solution to the gas phase where these ions can be separated and characterized by MS (Stewart 1999). These results must be viewed with caution as the ions may also be formed in the gas phase as an artifact of the electrospray ionization process (see below). The complexes, Cu(II) and Zn(II) desferriferricrocin complexes, if formed at all in solution of neutral pH, were very weak and could not be isolated by liquid chromatography even using a polymeric (non-silica) column. Both Cu^{2+} and Zn^{2+} replaced a varied number of protons from desferriferricrocin. Divalent copper replaced up to six protons. These were most likely the three protons on the hydroxamic acids and three readily exchangeable protons from amide groups of the peptide backbone. This suggests that desferriferricrocin has two metal-binding sites: the primary site being the hydroxamic acids and the secondary site being the amide linkages.

The complexation between an amide group of a peptide and metal ions is not new. The replacement of an amide proton by a metal ion makes a strong coordination bond (Sigel & Martin 1982). However, such proton displacement usually requires highly basic conditions (pH > 13) (Brenk *et al*. 1994; Comba *et al*. 1998), similar to what was observed with the Cu(II) and Zn(II) desferriferricrocin complexes. As the solution becomes more basic, hydrolysis of the metal ion occurs before complexation with the amide nitrogens. The presence of a strong anchor that can coordinate with the metal at an intermediate pH will prevent this hydrolysis. In peptides, such anchors can be terminal amino groups, amino groups on the side chain, imidazole nitrogens, and hydroxyl or thioether groups. If there is no such anchor, the metal ion will react with water at an intermediate pH range before it can replace the amide protons.

ESI can efficiently produce proton-displaced, metal–peptide complexes in the gas phase at a neutral pH (Gatlin *et al*. 1995). Deprotonation occurs at the amide NH group without the need for high pH. Extraction of amide protons in the ESI-MS gas phase is assisted by a unique charge reduction reaction (Mollah *et al*. 2000). This results from the instability of the highly charged ion cluster. During the evaporation process, the ion clusters become smaller and smaller until the existing ion solvation sphere can no longer delocalize the highly positive charge. The metal ion then reacts with solvated ligands. Normally, the ligand in the inner solvation sphere with the lowest ionization potential, and the ability to form strong covalent bonds with the metal ion, will undergo charge reduction in preference to other ligands, in parallel, the metal ion with the highest ionization potential and greatest tendency to form stable covalent bonds with ligands are preferred over other metal ions to undergo charge reduction. In agreement with our results, copper(II) has a higher

tendency than zinc(II) to displace amide protons (Sigel & Martin 1982). This process involves transfer of a proton from one ligand to another and the formation of a coordination bond as illustrated below:

$$ML_1L_2^{n+} \rightleftharpoons M(L_1 - H)^{(n-1)^+} + (L_2 + H)^+$$

where L_1 represents desferriferricrocin or desferriferricrocin–metal complexes, and L_2 represents another ligand such as a water molecule, sulfate, or nitrate.

Complexation at the amide nitrogen or oxygen makes it possible to form multinuclear complexes with a siderophore. Previously, dinuclear species of dihydroxamate siderophore were reported, where no amide nitrogen was involved in complexation (Spasojevic *et al*. 2001). Another example of a multinuclear complex is the plutonium(IV) siderophore complex. A hydrated aluminum(III) was bound with a plutonium(IV) siderophore (desferrioxamine E) by hydrogen bonds between the three oximate oxygens of the siderophore and its coordinated water molecules (Neu *et al*. 2000). Here, coordinated water molecules were not detected, possibly due to the weak binding between the water molecules and metal ions. Divalent copper or zinc was found to directly bind to one or two ferricrocins possibly through the amide oxygen. Coordinated nitrate or sulfate ion was also observed. The coordinated amide oxygens, nitrate, or sulfate was replaced by amide nitrogens at high fragmentation voltages. This implied that coordination through the amide nitrogen was more stable, but needed more energy to overcome the transition energy barrier.

The complexation of metal ions by siderophores is a promising mechanism for heavy metal bioremediation. Such complexes would reduce the free metal concentration and therefore the toxicity of heavy metals. However if the metal ions are easily dissociated and absorbed by living organisms, it may increase the solubility of the metal ion, and consequently their toxicity. Desferriferricrocin has two binding groups: hydroxamates and amides. Binding of metal ions with hydroxamate oxygen and binding of metal ions with amide nitrogen both form strong coordination bonds, but binding between metal ions and amide oxygen forms weak coordination bond (Sigel & Martin 1982). Formation of a strong coordination complex between the siderophore and the metal ion would decrease metal toxicity whereas the formation of a weak coordination complex between the siderophore and a metal ion might increase metal toxicity. Biological effects may also be important. For instance, the siderophore schizokinen reduced the toxicity of copper at a ratio of schizokinen to Cu^{2+} of 1.5 to 1 in the cyanobacterium *Anabaena* sp. (Clarke *et al*. 1987). However, it increased the toxicity of copper at a ratio of schizokinen to Cu^{2+} dropped of 1 to 1 in *Bacillus megaterium* (Arceneaux *et al*. 1984). This illustrated that any application of siderophores for heavy metal remediation must be done cautiously.

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