

Chiu-Fou Tseng · Alain Burger · Gaëtan L. A. Mislin  
Isabelle J. Schalk · Steve S.-F. Yu · Sunney I. Chan  
Mohamed A. Abdallah

## Bacterial siderophores: the solution stoichiometry and coordination of the Fe(III) complexes of pyochelin and related compounds

Received: 4 August 2005 / Accepted: 1 February 2006 / Published online: 21 March 2006  
© SBIC 2006

**Abstract** Pyochelin, its analog 3''-nor-NH-pyochelin, and the related methyl hydroxamate, 2-(2'-hydroxyphenyl)-4,5-dihydrothiazol-4-carboxylic acid methoxymethyl amide, have been prepared together with their Fe(III) complexes. The solution stoichiometry and the coordination of the three Fe(III) complexes in methanol or buffered (pH~2) 50:50 (v/v) methanol–water mixtures were determined using various spectroscopic methods: UV–vis absorption, X-ray absorption, extended X-ray absorption fine structure and electron paramagnetic resonance. All three systems showed both a 1:1 and 2:1 ligand–Fe(III) stoichiometry, but presented different coordination properties. Conditional formation constants (pH~2) were determined for both the 1:1 and 2:1 complexes in all three systems. Computation of the coordination-conformational energies by semiempirical methods indicated that the coordination in the case of the 2:1 complexes of pyochelin–Fe(III) and 3''-nor-NH-pyochelin–Fe(III) was asymmetrical, with one molecule of pyochelin (or 3''-nor-NH-pyochelin) tetradentately coordinated (O1, N1, N2 and O3) to the Fe(III), and the second molecule bound bidentately (O1, N1 or N2, O3), to complete the octahedral geometry. In contrast, two molecules of the methyl hydroxamate each provided a set of tridentate ligand atoms in the formation of the 2:1

ligand–Fe(III) complex. These results are consistent with the role of pyochelin in the uptake of iron by the FptA receptor in the outer membrane of *Pseudomonas aeruginosa* and in several gram-negative bacteria.

**Keywords** Coordination complexes of ferric ion · Iron uptake · Pyochelin · *Pseudomonas aeruginosa* · Siderophores

### Introduction

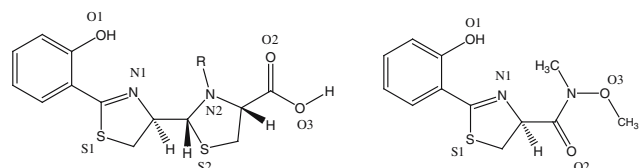
Pyochelin **1a** (Scheme 1) is a hydroxyphenylthiazoliny-thiazolidine type of siderophore, which was isolated for the first time from the iron-deficient cultures of *Pseudomonas aeruginosa* ATCC 15692 by Liu and Shokrani [1]. This siderophore was shown later to be produced not only by a great number of strains of *P. aeruginosa* but also by many strains of *Burkholderia* (previously *Pseudomonas*) *cepacia* [2, 3]. Both species are involved in severe lung infections occurring in cystic fibrosis patients.

The structure of pyochelin was established later by Cox et al. [4]: it possesses a hydroxyphenylthiazoline group as well as a carboxylic acid group, both probably involved in the chelation of Fe(III) to form an octahedral complex with a 2:1 stoichiometry. Although its association constant with Fe(III) is very weak (estimated to be  $5 \times 10^5 \text{ M}^{-1}$  in methanol) [5], the pyochelin-mediated iron transport through membranes is very efficient [6].

C.-F. Tseng · S. S.-F. Yu (✉) · S. I. Chan  
Institute of Chemistry, Academia Sinica,  
128 Academia Road Section 2, Nankang,  
Taipei 115, Taiwan, Republic of China  
E-mail: sfyu@chem.sinica.edu.tw

C.-F. Tseng · A. Burger · G. L. A. Mislin · I. J. Schalk  
M. A. Abdallah  
Métaux et Microorganismes: Chimie,  
Biologie et Applications UMR 7175-LC1 Institut Gilbert Laustriat,  
CNRS-Université Louis Pasteur (Strasbourg I) ESBS,  
Boulevard Sébastien Brant, Illkirch 67400, France  
E-mail: mislin@esbs.u-strasbg.fr

*Present address:* A. Burger  
Laboratoire de Chimie des Molécules Bioactives et des Arômes,  
UMR 6001 CNRS, Université de Nice Sophia Antipolis,  
Parc Valrose, 06108, Nice Cedex 2, France



**1a** pyochelin, R = CH<sub>3</sub>      **2** methyl hydroxamate  
**1b** 3''-nor-NH-pyochelin, R = H

Scheme 1

Earlier reports essentially based on mass spectrometry confirmed the 2:1 stoichiometry of the pyochelin metal complexes; however, the coordination between pyochelin and the metal remained uncertain. The precise role of each of the four potential coordinating atoms in the two pyochelins [the phenolic oxygen (O1), the thiazoline nitrogen (N1), the thiazolidine nitrogen (N2), and the carboxylate oxygen (O3)] remained unclear [7, 8], not to mention the participation of the sulfur atoms. More recently a mixed 1:1:1 complex between a tetradentate pyochelin, a bidentate cepabactin, and Fe(III) was isolated, purified, and characterized [9]. Simultaneously, the specific outer-membrane receptor FptA loaded with ferripyochelin with a similar 1:1:1 mixed complex between pyochelin (tetradentate), ethylene glycol (bidentate), and Fe(III) was crystallized and its three-dimensional structure reported [10].

We present here a physicochemical study of the Fe(III) complexes of pyochelin using various spectroscopic methods: UV–vis absorption, X-ray absorption (XAS), extended X-ray absorption fine structure (EXAFS) and electron paramagnetic resonance (EPR), in order to fully characterize the solution stoichiometry and the coordination of the Fe(III) complexes of pyochelin, and two related compounds, namely 3''-nor-NH-pyochelin **1b**, and the methyl hydroxamate, 2-(2'-hydroxyphenyl)-4,5-dihydrothiazol-4-carboxylic acid methoxymethyl amide **2**. The latter is an intermediate in the synthesis of pyochelin and analogs [11–13]. We show that all three systems exhibit ligand–Fe(III) complexes of 1:1 and 2:1 stoichiometry in methanol or buffered (pH~2) 50:50 (v/v) methanol–water mixtures, but with different coordination properties. Computation of coordination–conformational energies by semiempirical methods indicate that in the 2:1 complexes, one molecule of pyochelin (and 3''-nor-NH-pyochelin) acts as a tetradentate (O1, N1, N2 and O3) ligand to coordinate with Fe(III), and the second molecule acts as a bidentate (O1, N1 or N2, O3) ligand to form asymmetrical octahedral ferric complexes. In contrast, the methyl hydroxamate essentially serves as a tridentate ligand.

In principle, X-ray crystallographic analysis is the method of choice to address the structural issues of concern here. Although the X-ray structure of an Fe(III) complex of pyochelin was alluded to in a recent publication [14], unfortunately the description of this structure was unclear, and no structural data appeared till the publication of the three-dimensional structure of FptA, the specific outer-membrane receptor, loaded with ferric pyochelin [10].

## Materials and methods

### Preparation of the ligands

Pyochelin, 3''-nor-NH-pyochelin, and the methyl hydroxamate were synthesized according to published procedures [11–13].

### Buffer preparation

A 0.106 M formic acid solution was mixed with varying volumes of triethylamine, and then diluted with methanol to obtain buffered (0.053 M formic acid, pH~2) 50:50 (v/v) methanol–water mixtures. The latter were used throughout the study, except where noted otherwise.

### UV–vis measurements

Spectral titrations were carried out either at a fixed Fe(III) concentration and varying the ligand (pyochelin, 3''-nor-NH-pyochelin, or the methyl hydroxamate) concentration, or at a fixed ligand concentration and varying the metal-to-ligand concentration ratio. In the case of 3''-nor-NH-pyochelin, the samples were prepared by adding a fixed volume of FeCl<sub>3</sub> in a buffered (pH 2.72) 50:50 (v/v) methanol–water mixture to a fixed volume of the ligand at varying concentrations in the same buffer so that the final concentration of Fe(III) (0.250 mM, pH 2.72) remained constant throughout the first series of UV–vis measurements. The same method was used to prepare the samples for the second series of experiments, where the concentration of 3''-nor-NH-pyochelin was held constant at the final value of 0.107 mM in the same buffered methanol–water mixture and the Fe(III) concentration was varied. The two series of titration experiments will henceforth be referred to as forward and reverse titrations, respectively.

The same procedure was used in the preparation of the samples for the spectral titration of Fe(III) with varying concentrations of the methyl hydroxamate. Here, the Fe(III) concentration was fixed at 0.25 mM in the final buffered (pH 2.89) 50:50 (v/v) methanol–water solutions. For the spectral titration of the methyl hydroxamate with varying concentrations of Fe(III), the methyl hydroxamate concentration was fixed at 0.125 mM in the same buffered methanol–water solutions.

The spectral titration of pyochelin was carried out using samples that were prepared by adding a fixed volume of pyochelin solution at varying concentrations in methanol to a fixed volume of the FeCl<sub>3</sub>–methanol solution so that the final concentration of Fe(III) (0.33 mM) remained constant throughout the series of measurements (forward titration). In the reverse titration, increasing aliquots of 0.33 mM FeCl<sub>3</sub> in methanol were added to solutions of pyochelin in methanol so that the final concentration of the pyochelin (0.232 mM) remained constant throughout the series of measurements. The pH of the 0.33 mM FeCl<sub>3</sub> solution in methanol was determined with a pH meter to be approximately 2. All UV–vis spectra were recorded using an HP 8543 spectrophotometer.

### EPR of pyochelin–Fe(III) complex

EPR spectra were obtained at the X-band using a Bruker E580 spectrometer equipped with a Bruker TE102

cavity. The samples were aliquots taken from the corresponding samples prepared for the UV-vis spectral titrations mentioned before. These aliquots were loaded onto 4-mm outer diameter EPR tubes and plunged into a liquid nitrogen chilled isopentane solution for freezing. The EPR spectra were recorded at 4 K, and this temperature was maintained using an Oxford Instruments continuous liquid-helium cryostat equipped with a helium pump to lower the vapor pressure of the liquid helium.

#### XAS of 2:1 pyochelin-Fe(III), 3''-nor-NH-pyochelin-Fe(III), and methyl hydroxamate-Fe(III) complexes

For XAS measurements, the complexes were prepared in excess ligand and purified by a Sephadex LH-20 column. Methanol was used as the elution buffer. The samples eluted were dried and rid of solvent by lyophilization, and the complexes were extracted by ether. After lyophilization again to remove solvent, the solids were loaded onto sample holders (1.4 cm×1.4 cm×0.2 cm) covered with sheets of kapton, and cooled to 10 K using an Oxford Instruments continuous liquid-helium cryostat. XAS data were collected at the National Synchrotron Radiation Research Center (NSRRC) in Hsinchu, Taiwan (Beamline Wiggler 17C) using a Si(111) double-crystal monochromator in the region of the K-edge for Fe (7.112 keV). The absorption edges of the Fe in FeO, Fe<sub>2</sub>O<sub>3</sub>, and Fe<sub>3</sub>O<sub>4</sub> were used as internal standards for calibration of the energy of the X-ray photons.

#### Computation of coordination-conformational energies

Conformation energies and equilibrium geometries for the various conformers of the 2:1 pyochelin-Fe(III), 3''-nor-NH-pyochelin-Fe(III) and the methyl hydroxamate-Fe(III) complexes were calculated by the program Spartan'02. This semiempirical module also provides for the calculation of heats of formation. Various models of the complexes were simulated by the semiempirical PM3 method. It is reasonable to assume that the phenol moiety is neutral at pH~2, and is a phenolate in the Fe(III) complex. On the other hand, at pH~2, the carboxylic acid moiety in pyochelin might not be ionized in methanol or in the 3''-nor-NH-pyochelin in the buffered methanol-water mixtures. However, it is certainly ionized upon the formation of the Fe(III) complex. In any case, we have compared the conformational energies of the complexes with and without this ionization of the carboxylic acid in our calculations.

## Results

We carried out spectroscopic studies and theoretical calculations to elucidate the ferric coordination chemistry of pyochelin and its analogs, 3''-nor-NH-pyochelin

and methyl hydroxamate. It has been reported that pyochelin can chelate with Fe(III) in a 2:1 stoichiometry [5]. The resultant complexes presumably involve two symmetrical tridentate ligands, or two ligands asymmetrically coordinated to the iron, one tetradentate and the other bidentate, to form the hexa-coordinated octahedral geometry.

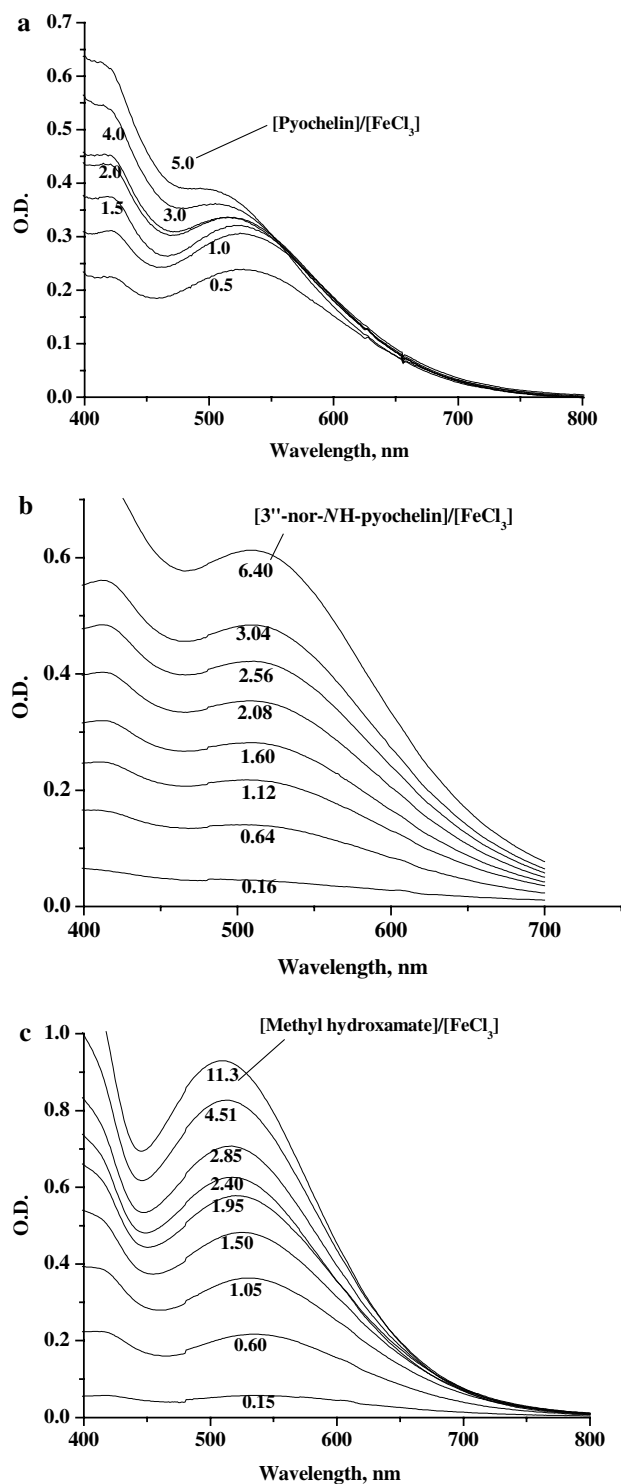
#### UV-vis spectra

To verify the stoichiometry of the limiting metal-ligand complex formed and to assist in the identification of the potential Fe(III) ligand atoms of pyochelin, we compared the titration of FeCl<sub>3</sub> solutions in methanol with pyochelin versus the titration of FeCl<sub>3</sub> with 3''-nor-NH-pyochelin and methyl hydroxamate in 50:50 methanol-water mixtures. The titration with pyochelin was undertaken in methanol, as this siderophore is sparingly soluble even in 50:50 (v/v) methanol-water mixtures. In order to optimize the solution conditions for the formation of the three iron complexes, the titrations were carried out at pH~2. The pH of a 0.33 mM FeCl<sub>3</sub> solution in methanol was determined to be approximately 2; and the methanol-water mixtures were buffered at pH 2-3 with 0.053 M formic acid.

Changes in the UV-vis absorption of the solutions upon the formation of the iron siderophore complexes were monitored. In the first series of experiments, a fixed concentration of Fe(III) was titrated against increasing concentrations of the ligands (forward titration). In a separate series of experiments, solutions of the ligands at a fixed concentration were treated with increasing concentrations of FeCl<sub>3</sub>, again in methanol or buffered methanol-water mixtures (reverse titration).

#### Pyochelin

The titration of an FeCl<sub>3</sub> solution in methanol with increasing concentration increments of pyochelin has previously been reported. These data were interpreted in terms of the formation of a 2:1 pyochelin-Fe(III) complex [5]. We repeated this experiment. In Fig. 1a, we summarize the UV-vis spectra obtained for a 0.33 mM FeCl<sub>3</sub> solution in methanol containing increasing increments of pyochelin. The various samples were prepared by adding a fixed volume of pyochelin solution at varying concentrations in methanol to a fixed volume of the FeCl<sub>3</sub>-methanol solution so that the final concentration of Fe(III) remained constant throughout the series of measurements. It is clear from these data that a complex with  $\lambda_{\max} \sim 525$  nm was formed at pyochelin-to-Fe(III) concentration ratio of approximately 0.5. As the pyochelin-to-Fe(III) concentration ratio was increased to 1:1 and above, another complex was formed with  $\lambda_{\max} \sim 500$  nm, presumably with a higher ligand-metal stoichiometry (vide infra).



**Fig. 1** UV-vis spectra of Fe(III) complexes obtained with increasing concentration increments of pyochelin and structurally related compounds. **a** Pyochelin was added to a 0.33 mM FeCl<sub>3</sub> solution in methanol. **b** 3''-nor-NH-Pyochelin was added to a 0.25 mM FeCl<sub>3</sub> solution in buffered (pH 2.72) 50:50 (v/v) methanol-water mixtures. **c** Methyl hydroxamate was added to a 0.25 mM FeCl<sub>3</sub> solution in buffered (pH 2.89) 50:50 (v/v) methanol-water mixtures. All spectra were baseline-corrected, i.e., by subtracting the baseline from the raw data. There were no apparent spectral contributions to the baseline from Fe(III) species in the vicinity of 500 nm in the absence of the siderophores

Further evidence in support of multiple equilibria, namely, the formation of both 1:1 and 2:1 complexes with Fe(III), was provided by reverse titration. When constant ligand at a sufficiently high siderophore concentration was titrated with increasing Fe(III) concentrations, the species with the higher ligand-to-metal stoichiometry should have been formed first; and this species would gradually give way to the species with the lower ligand-to-metal stoichiometry as the Fe(III) concentration is increased. In Fig. 2a, the spectral changes obtained for a 0.23 mM pyochelin solution in methanol upon the addition of varying aliquots of FeCl<sub>3</sub> in methanol are compared. From the  $\lambda_{\max}$  observed initially at approximately 500 nm, it is evident that the observed absorbance changes are dominated by the formation of the limiting 2:1 complex when the pyochelin-to-Fe(III) concentration ratio is much greater than 1. The addition of additional increments of Fe(III) was attended by an increase in the absorbance at approximately 525 nm owing to formation of the complex with the lower ligand-to-metal stoichiometry.

### 3''-nor-NH-Pyochelin

We repeated the same series of experiments with 3''-nor-NH-pyochelin. The UV-vis data obtained for a series of solutions containing FeCl<sub>3</sub> at a fixed concentration of 0.25 mM in buffered (pH 2.7) 50:50 (v/v) methanol-water mixtures and 3''-nor-NH-pyochelin at varying ligand-to-metal concentration ratios are summarized in Fig. 1b. Unlike the case of pyochelin, there was no apparent shift in  $\lambda_{\max}$  (approximately 510 nm) with increasing increments of 3''-nor-NH-pyochelin.

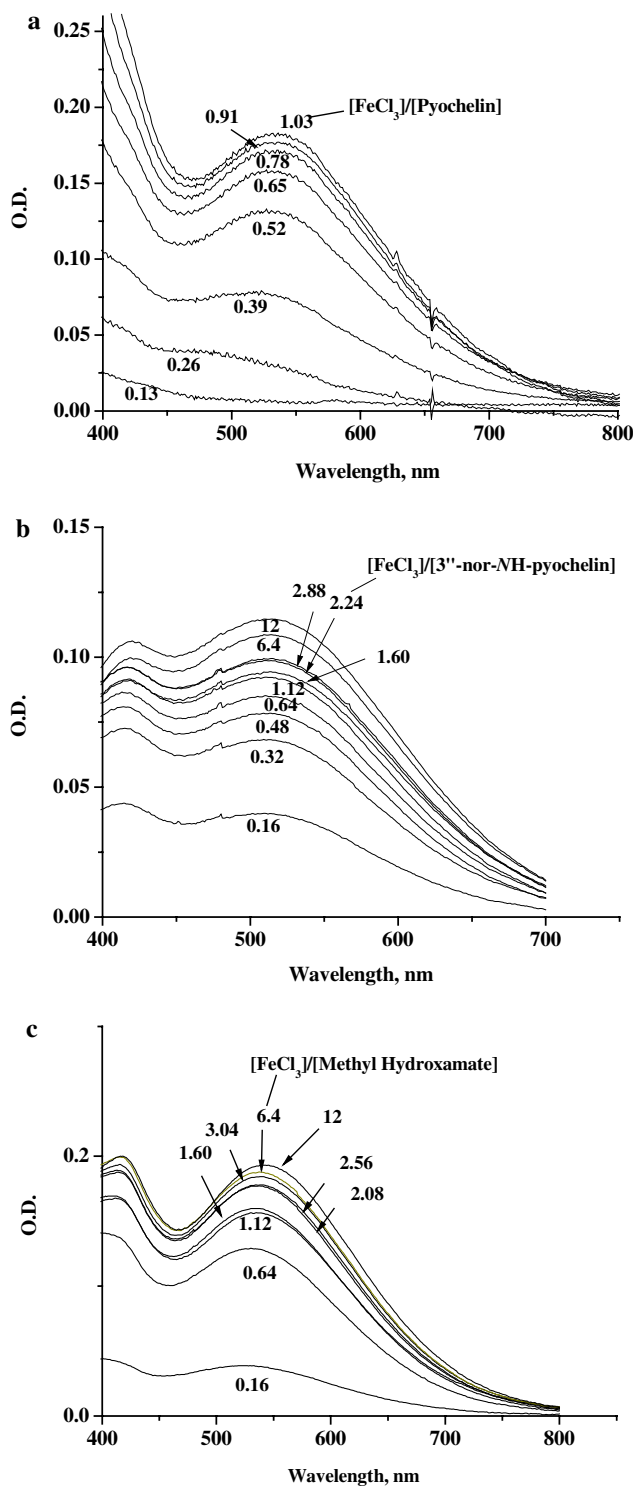
Figure 2b compares the spectral changes obtained in the reverse titration. Here, the results were obtained for buffered (pH 2.7) 50:50 (v/v) methanol-water solutions containing 0.107 mM 3''-nor-NH-pyochelin and varying levels of FeCl<sub>3</sub>. As in the forward titration, no apparent shift in  $\lambda_{\max}$  (approximately 510 nm) was noted as the level of FeCl<sub>3</sub> in the solution was increased.

From the absorbance changes in the forward and reverse experiments, we found that the limiting complex formation was thermodynamically complete at a ligand-to-metal ratio of approximately 5 when the 3''-nor-NH-pyochelin concentration was varied at constant Fe(III) concentration (Fig. 4a), or a metal-to-ligand ratio of approximately 1 when the Fe(III) concentration was varied at fixed 3''-nor-NH-pyochelin concentration (Fig. 4c). These results are consistent with a limiting ligand-to-metal stoichiometry of 1:1 in the reverse titration, and a stoichiometry higher than 1:1 for the limiting complex in the forward titration.

### Methyl hydroxamate

Experiments with methyl hydroxamate as the ligand yielded similar spectral changes in the UV-vis region.





**Fig. 2** a UV-vis spectra obtained for a series of solutions containing pyochelin at a fixed concentration of 0.23 mM and increasing concentrations of  $\text{FeCl}_3$ . b UV-vis spectra obtained for a series of solutions containing 3''-nor-NH-pyochelin at a fixed concentration of 0.107 mM and increasing concentrations of  $\text{FeCl}_3$ . c UV-vis spectra obtained for a series of solutions containing methyl hydroxamate at a fixed concentration of 0.125 mM and  $\text{FeCl}_3$  at varying concentration ratios. As in Fig. 1, all the spectra were corrected for the baseline. The contribution of the free ligand to the absorbance in the vicinity of 500 nm is negligible for all three siderophores

When solutions were prepared with 0.25 mM  $\text{FeCl}_3$  and varying concentrations of the methyl hydroxamate in buffered (pH 2.7) 50:50 (v/v) methanol-water mixtures, the  $\lambda_{\text{max}}$  of the complexes formed shifted from approximately 540 nm to approximately 510 nm with increasing levels of the ligand (Fig. 1c). When the solutions contained 0.125 mM methyl hydroxamate with varying  $\text{Fe(III)}$  concentrations, the  $\lambda_{\text{max}}$  of the complexes formed moved from approximately 510 to 540 nm with increasing  $\text{Fe(III)}$ -to-methyl hydroxamate concentration ratios (Fig. 2c).

From the absorbance changes in the forward and reverse experiments, we found that the limiting complex formation was thermodynamically complete at a ligand-to-metal ratio of approximately 4 when the methyl hydroxamate concentration was varied at constant  $\text{Fe(III)}$  concentration (Fig. 5a), or at a metal-to-ligand ratio of approximately 1 when the  $\text{Fe(III)}$  concentration was varied at fixed methyl hydroxamate concentration (Fig. 5c). These results are consistent with a limiting ligand-to-metal stoichiometry of 1:1 in the reverse titration, and a stoichiometry higher than 1:1 for the limiting complex in the forward titration.

#### Analysis of the UV-vis titration data

A quantitative analysis of the UV-vis titration data was carried out to obtain the conditional formation constants (pH~2) for the complexes formed between  $\text{Fe(III)}$  and the three ligand systems. In each case, the titration data were fitted within the framework of the formation of 1:1 and 2:1 ligand- $\text{Fe(III)}$  complexes. At each wavelength, we could then write, following the Beer-Lambert law,

$$\text{Absorbance}(\lambda) = \text{OD} = \varepsilon_1(\lambda) \times C_1 + \varepsilon_2(\lambda) \times C_2, \quad (1)$$

where OD is the optical density, subscripts 1 and 2 refer to the 1:1 and 2:1 complexes, respectively, and where the optical cell path length was taken to be 1 cm. The extinction coefficients for the two species could be obtained from the limiting OD in the two titration experiments, where presumably the complex of the limiting stoichiometry would be formed under the conditions of the experiment in each case. Thus, when the titration was carried out by varying the ligand concentration at constant  $\text{Fe(III)}$ , the limiting complex at high ligand-to-metal concentration ratios should have been the 2:1 complex, and the OD would be given by  $\varepsilon_2(\lambda)C_{2\text{max}} = \varepsilon_2(\lambda)M_0$ , where  $M_0$  is the stoichiometric concentration of the  $\text{Fe(III)}$  in the experiment. Similarly, in the reverse titration, the limiting complex at high metal-to-ligand ratios should be the 1:1 complex, and the limiting OD would be given by  $\varepsilon_1(\lambda)C_{1\text{max}} = \varepsilon_1(\lambda)L_0$ , where  $L_0$  is the stoichiometric concentration of the ligand in this experiment. Table 1 lists the extinction coefficients at two wavelengths in the vicinity of  $\lambda_{\text{max}}$  of the 1:1 and the 2:1 ligand- $\text{Fe(III)}$  complexes of pyoch-

**Table 1** Extinction coefficients ( $M^{-1} \text{ cm}^{-1}$ ) for the ligand-to-metal charge transfer bands observed at wavelengths near  $\lambda_{\text{max}}$  for the 1:1 and 2:1 Fe(III) complexes of pyochelin, 3''-nor-NH-pyochelin, and methyl hydroxamate

Ligand	FeL	FeL <sub>2</sub>
Pyochelin	$\epsilon_{488} = 1,297,$ $\epsilon_{526} = 1,417$	$\epsilon_{488} = 1,448,$ $\epsilon_{526} = 922$
3''-nor-NH-Pyochelin	$\epsilon_{510} = 1,187$	$\epsilon_{510} = 2,428$
Methyl hydroxamate	$\epsilon_{510} = 3,552,$ $\epsilon_{540} = 3,657$	$\epsilon_{510} = 3,652,$ $\epsilon_{540} = 3,362$

**Table 2** Conditional equilibrium constants (pH~2) determined for the formation of the Fe(III) complexes of pyochelin and its analogs

Ligand	$K_1 (M^{-1})^a$	$K_2 (M^{-1})^b$	$K_{\text{comp}}^c$
Pyochelin	$6,740 \pm 4,640$	$274 \pm 178$	$29.4 \pm 16.7$
3''-nor-NH-Pyochelin	$33,300 \pm 4,940$	$2,630 \pm 1,070$	$10.3 \pm 7.0$
Methyl hydroxamate	$3,010 \pm 520$	$2,790 \pm 779$	$1.16 \pm 0.95$

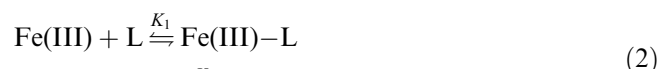
$$^a K_1 = [\text{FeL}]/([\text{Fe}][\text{L}])$$

$$^b K_2 = [\text{FeL}_2]/([\text{FeL}][\text{L}])$$

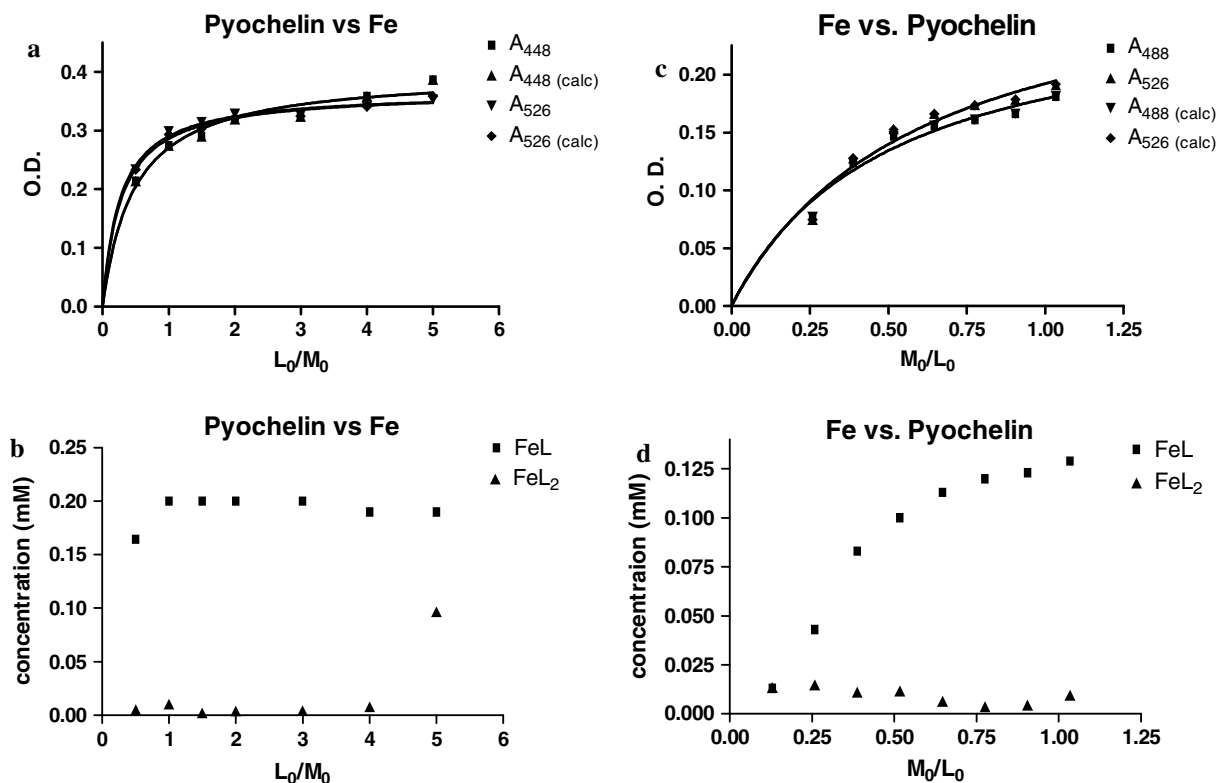
$$^c K_{\text{comp}} = [\text{FeL}]^2/([\text{FeL}_2][\text{Fe}])$$

elin, 3''-nor-NH-pyochelin, and methyl hydroxamate determined in this manner.

Once the extinction coefficients had been determined for the two species at a particular wavelength, the absorbance measured in a given experiment could be used to deduce the concentration of the two species under the conditions of the experiment by invoking the relationship  $C_1 + C_2 = M_0$  in the forward titration, and  $C_1 + 2C_2 = L_0$  in the reverse titration. Finally, after the concentrations of the 1:1 and 2:1 Fe(III) complexes had been determined as a function of  $L_0/M_0$ , the results could be used to obtain the conditional equilibrium constants  $K_1$  and  $K_2$  associated with the successive formation of the two complexes in the following chemical equations for the forward titration:

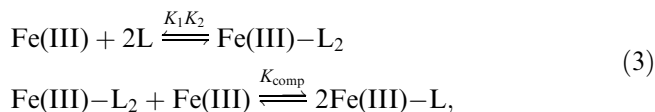


Similarly, in the reverse titration, once the concentrations of the 1:1 and 2:1 Fe(III) complexes had been determined as a function of  $M_0/L_0$ , the results could be used to obtain the product  $K_1K_2$  and  $K_{\text{comp}}$  in the following multiple equilibria:



**Fig. 3 a** Absorbance vs pyochelin-to-Fe(III) concentration ratio ( $L_0/M_0$ ) observed at 448 and 526 nm in a forward titration where a solution of 0.33 mM  $\text{FeCl}_3$  ( $M_0$ ) in methanol was titrated with increasing concentrations of pyochelin ( $L_0$ ). **b** Concentrations of the 1:1 and 2:1 species formed as a function of  $L_0/M_0$ .

**c** Absorbance versus Fe(III)-to-pyochelin concentration ratio ( $M_0/L_0$ ) observed at 448 and 526 nm in a reverse titration where a solution of 0.23 mM pyochelin ( $L_0$ ) in methanol was titrated with increasing concentrations of Fe(III) ( $M_0$ ). **d** Concentrations of the 1:1 and 2:1 species formed as a function of  $M_0/L_0$

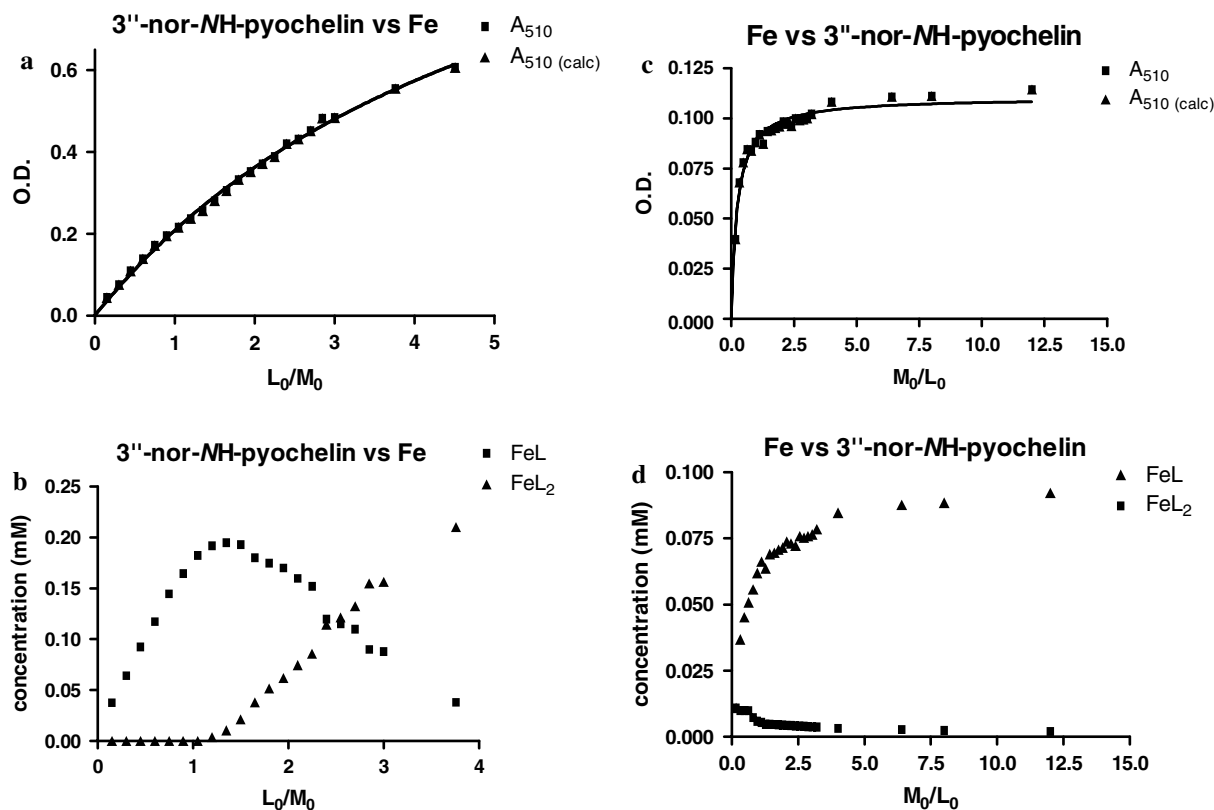


where  $K_{\text{comp}}$  is the conditional equilibrium constant for the comproportionation of the 2:1 complex into two 1:1 species in the presence of Fe(III) at the pH of the reverse titration.

The equilibrium constants  $K_1$ ,  $K_2$ , and  $K_{\text{comp}}$  determined by the aforementioned procedure for the three pyochelin analogs examined in this study are summarized in Table 2. In Figs. 3, 4, and 5, we have also summarized the outcome of our analysis of the absorbance data within the framework of our model. Plots of the absorbance versus pyochelin-to-Fe(III) concentration ratio are compared between the results of fitting the data to the model and the OD data observed at two wavelengths,  $\lambda=448$  and 526 nm in Fig. 3a. Similar comparison between the model and experiment for the absorbance versus Fe(III)-to-pyochelin concentration ratio in the reverse titration is shown in Fig. 3c. The agreement can be seen to be quite satisfactory in both cases. The same comparison is made at  $\lambda=510$  nm for

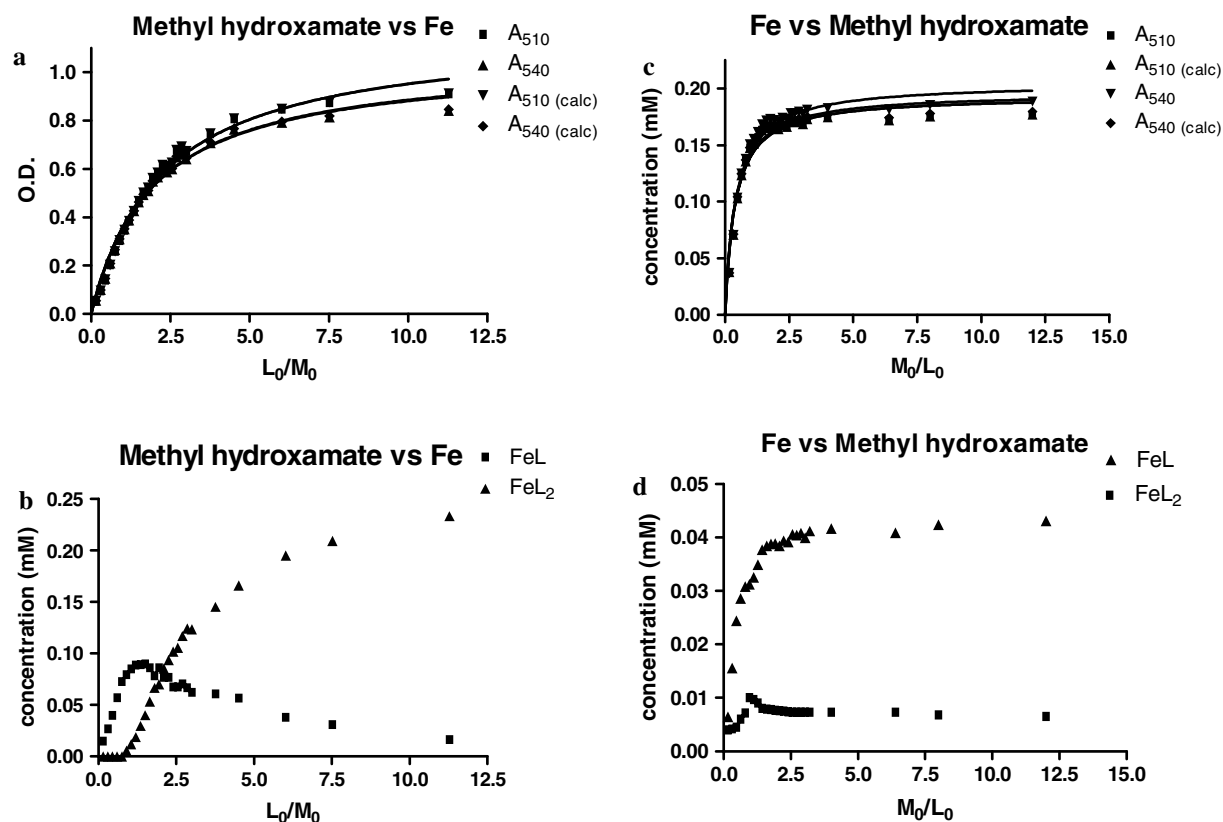
the forward titration in the case of 3''-nor-NH-pyochelin in Fig. 4a, and for the reverse titration in Fig. 4c. Here, the agreement between the model and the observed absorbance is almost perfect. Finally, in Fig. 5, we do the same comparison for methyl hydroxamate at two wavelengths, 510 and 540 nm. Again, the model fits the absorbance data well for both the forward (Fig. 5a) and the reverse (Fig. 5c) titrations.

The concentrations of the 1:1 and 2:1 complexes are summarized for each of the three ligand systems in the various titration experiments in Fig. 3b and d (pyochelin), Fig. 4b and d (3''-nor-NH-pyochelin), and Fig. 5b and d (methyl hydroxamate). In the case of pyochelin, it is clear that the 1:1 complex is formed with a much higher formation constant from the free ligand and Fe(III) than the 2:1 complex is from the 1:1 species. In fact, appreciable concentrations of the 2:1 complex are not formed until  $L_0/M_0 \gg 4$ . The same is also true for 3''-nor-NH-pyochelin, except that the individual equilibrium constants for the two steps are 5–10 times higher than the corresponding constants in the case of pyochelin. As a result, the overall formation constant for the 2:1 complex (product of  $K_1$  and  $K_2$ ) is significantly



**Fig. 4** **a** Absorbance versus 3''-nor-NH-pyochelin-to-/Fe(III) concentration ratio ( $L_0/M_0$ ) observed at 510 nm in a forward titration where a solution of 0.25 mM FeCl<sub>3</sub> ( $M_0$ ) in a buffered (pH 2.7) 50:50 (v/v) methanol–water mixture was titrated with increasing concentration of 3''-nor-NH-pyochelin ( $L_0$ ). **b** Concentrations of the 1:1 and 2:1 species formed as a function of  $L_0/M_0$  in the forward titration. **c** Absorbance versus Fe(III)-to-3''-nor-NH-

pyochelin concentration ratio ( $M_0/L_0$ ) observed at 510 nm in a reverse titration where a solution of 0.107 mM 3''-nor-NH-pyochelin ( $L_0$ ) in a buffered (pH 2.7) 50:50 (v/v) methanol–water mixture was titrated with increasing concentration of FeCl<sub>3</sub> ( $M_0$ ). **d** Concentrations of the 1:1 and 2:1 species formed as a function of  $M_0/L_0$  in the reverse titration



**Fig. 5** **a** Absorbance versus methyl hydroxamate-to-Fe(III) concentration ratio ( $L_0/M_0$ ) observed at 510 and 540 nm in a forward titration where a solution of 0.25 mM  $\text{FeCl}_3$  ( $M_0$ ) in a buffered (pH 2.7) 50:50 (v/v) methanol-water mixture was titrated with increasing concentration of methyl hydroxamate ( $L_0$ ). **b** Concentrations of the 1:1 and 2:1 species formed as a function of  $L_0/M_0$  in the forward titration. **c** Absorbance versus Fe(III)-to-methyl

hydroxamate concentration ratio ( $M_0/L_0$ ) observed at 510 and 540 nm in a reverse titration where a solution of 0.125 mM methyl hydroxamate ( $L_0$ ) in a buffered (pH 2.7) 50:50 (v/v) methanol-water mixture was titrated with increasing concentration of  $\text{FeCl}_3$  ( $M_0$ ). **d** Concentrations of the 1:1 and 2:1 species formed as a function of  $M_0/L_0$  in the reverse titration

higher, but even then the 1:1 complex remains the dominant species until  $L_0/M_0 > 2$ . In other words, the limiting 1:1 complex is readily formed, as we have shown in the reverse titration. In contrast,  $K_1$  and  $K_2$  are essentially equal in the case of methyl hydroxamate.

#### Electron paramagnetic resonance

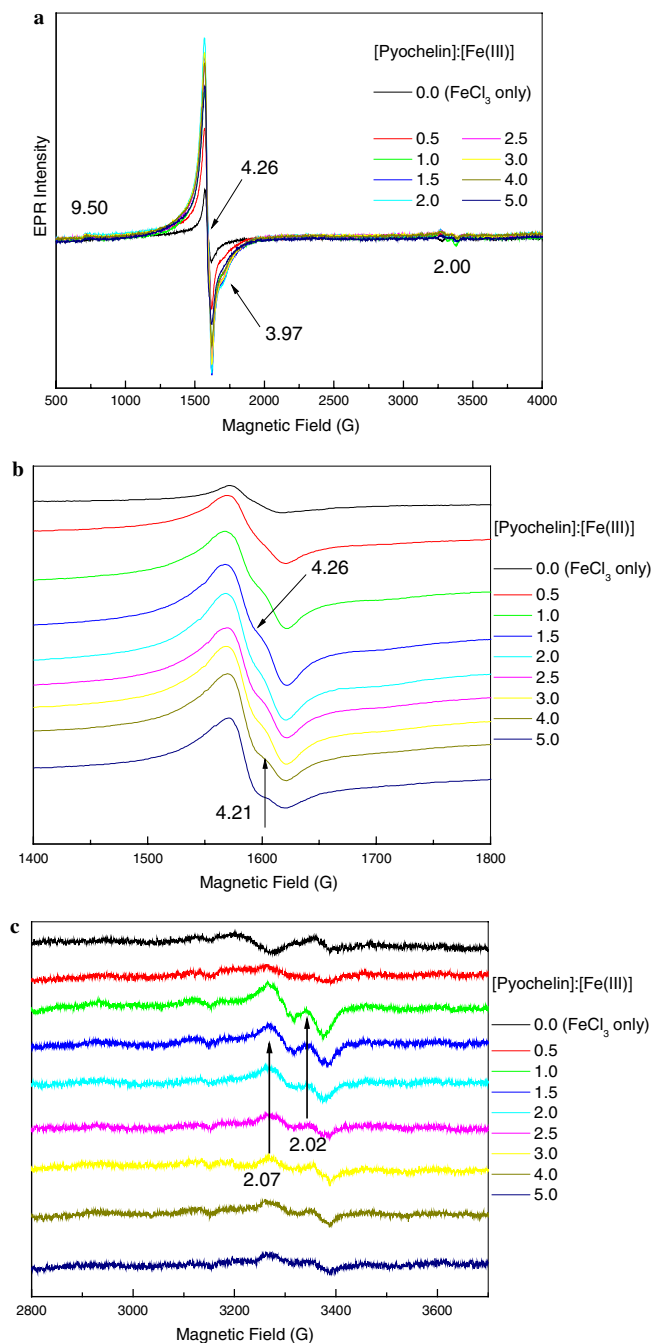
The liquid-helium-temperature EPR spectra observed for the pyochelin-Fe(III), 3''-nor-NH-pyochelin-Fe(III), and methyl hydroxamate-Fe(III) complexes are typical of high-spin ( $S = 5/2$ ) Fe(III) with rhombic ligand fields. The spectra recorded in the case of pyochelin for different pyochelin-to-Fe(III) concentration ratios are summarized in Fig. 6a. The turning points observed in the derivative spectrum at  $g = 9.50$ , 4.26, and 2.00 are consistent with a six-coordinate Fe(III), a coordination environment similar to that previously reported for the *Escherichia coli* siderophore ferrichrome and most other iron transporters [15]. The subtle variations observed in the EPR spectrum with the ligand-to-metal ratio (Fig. 6b, c) merely reflect changes in the

symmetry of the ligand field around the Fe(III) ion, subtle changes in the zero-field parameters (Fig. 6d, e) between the Fe(III)-methanol complex, and the heterogeneity of the ligand-Fe(III) complexes reflecting the different proportions of the 1:1 and 2:1 pyochelin-Fe(III) species formed under different conditions.

#### X-ray absorption experiments

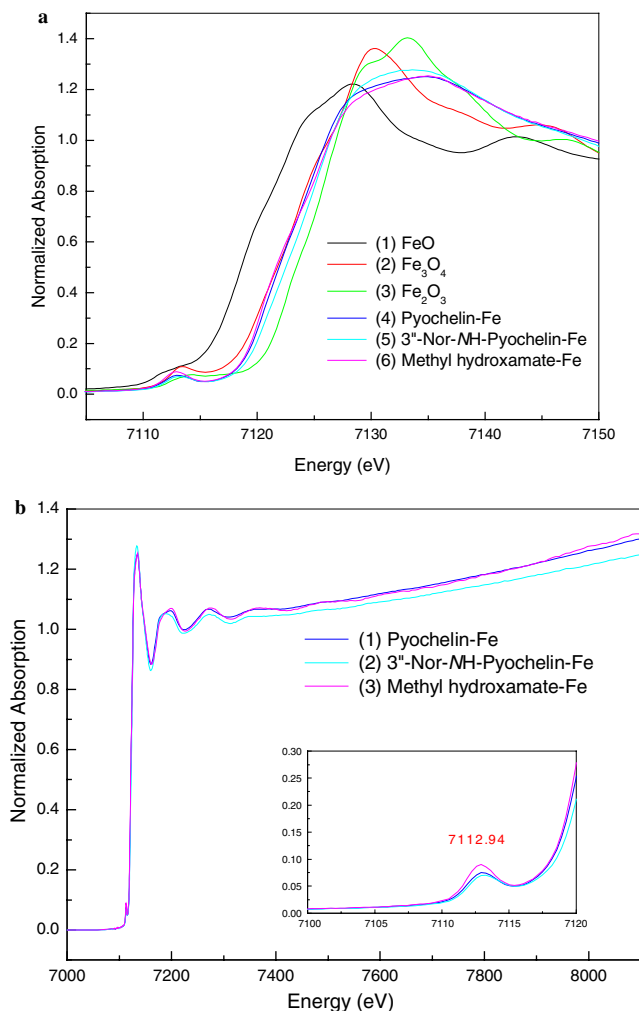
The limiting 2:1 Fe(III) complexes formed with pyochelin, 3''-nor-NH-pyochelin, and the methyl hydroxamate were also subjected to  $K_\alpha$ -edge XAS as well as EXAFS studies. The Fe K-edges observed at 10 K for the three complexes are similar to the corresponding K-edges of  $\text{Fe}_2\text{O}_3$  (7,125 eV), consistent with a +3 oxidation state for the iron complexes (Fig. 7a). The corresponding K-edge of Fe(II) in FeO occurs at approximately 7,120 eV. The normalized pre-edges ( $1s \rightarrow 3d$ ) of the iron complexes at 7,113 eV are less than 0.1 of the absorption intensity (Fig. 7b, inset), indicating that the coordination geometry around the Fe(III) is distorted from octahedral, possibly tetragonal, in each case.





**Fig. 6** **a** X-band electron paramagnetic resonance (EPR) spectra of the pyochelin-Fe(III) complex were obtained for a 0.33 mM  $\text{FeCl}_3$  solution in methanol containing increasing increments of pyochelin. The spectra were recorded at 4 K with a microwave power of 1.263 mW, a modulation amplitude of 10 G, a modulation frequency of 100 kHz, and a microwave frequency of 9.477 GHz. **b** EPR spectra of the pyochelin-Fe(III) complex in the  $g=4.26$  region. **c** EPR spectra of the pyochelin-Fe(III) complex observed in the  $g=2.00$  region

We analyzed the fine structure in the postedge region of the XAS to deduce the first and second coordination shell ligand environment around the Fe(III) in the three 2:1 complexes (Fig. 8a). The locations of the first-shell

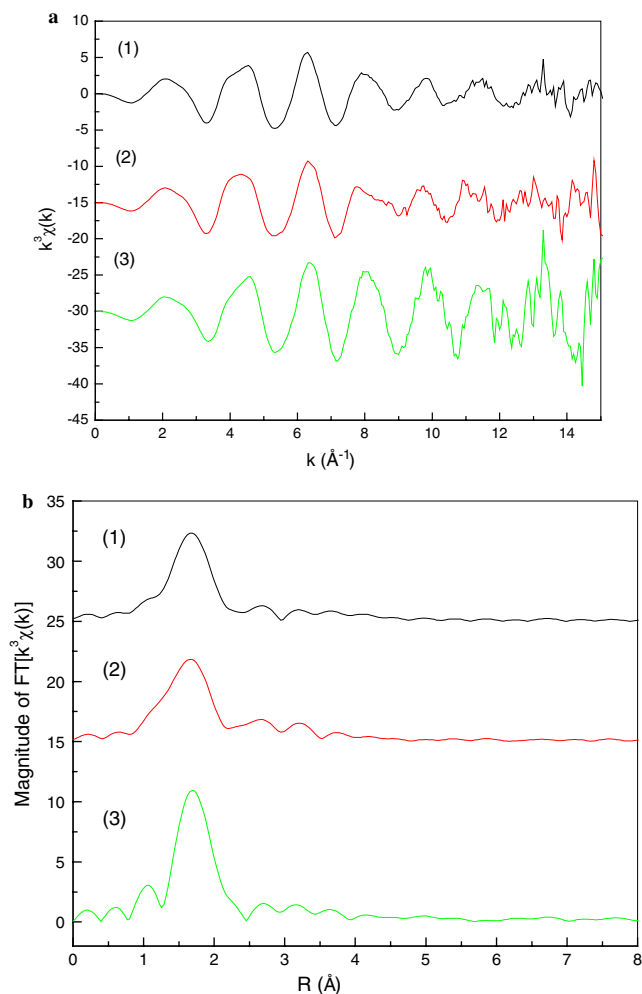


**Fig. 7** **a** X-ray absorption near-edge spectroscopy spectra of iron complexes at 10 K. **b** Fe K-edge X-ray absorption spectroscopy (XAS) spectra of iron complexes at 10 K. The inset displays the XAS spectra in the pre-edge region

ligand atoms are typically in the range 1–2 Å (Fig. 8b). These results are consistent with only oxygen and nitrogen scatterers in the first coordination shells of these complexes, in agreement with the similar  $\lambda_{\text{max}}$  observed for the ligand-to-metal charge transfer bands in the UV-vis spectra of the three 2:1 complexes. No attempt was made to fit the EXAFS data further to extract coordination numbers, as the coordination geometry is already known to be octahedral, and it is not possible to distinguish between N and O ligands in the first coordination shell of the ligand atoms.

#### Theoretical calculations

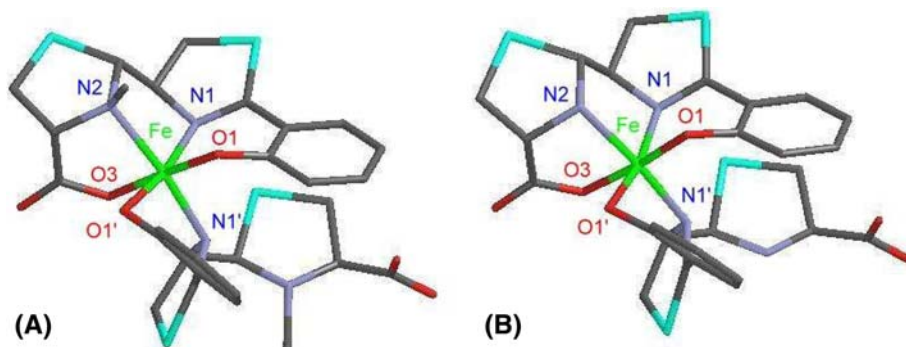
We undertook semiempirical calculations on the 2:1 pyochelin-Fe(III) and 3''-nor-NH-pyochelin-Fe(III) complexes in an attempt to place the aforementioned chemical conclusions on a firmer structural basis, and to



**Fig. 8** **a** Extended X-ray absorption fine structure (EXAFS) of iron complexes at 10 K. All spectra are plotted on the same scale with the middle and upper spectra offset vertically by 15 and 30 units, respectively, for clarity. **b** Fourier transforms of  $k^3\chi(k)$  EXAFS. All spectra are plotted on the same scale with the middle and upper spectra offset vertically by 15 and 25 units, respectively, for clarity. Pyochelin–Fe (1), 3''-nor-NH-pyochelin–Fe (2), and methyl hydroxamate–Fe (3)

distinguish between various modes of coordination. These computations were done using the program Spartan. The results of these computations are summa-

**Scheme 2** Stick presentation views of the stablest pyochelin–Fe (a) and 3''-nor-NH-pyochelin–Fe (b) models

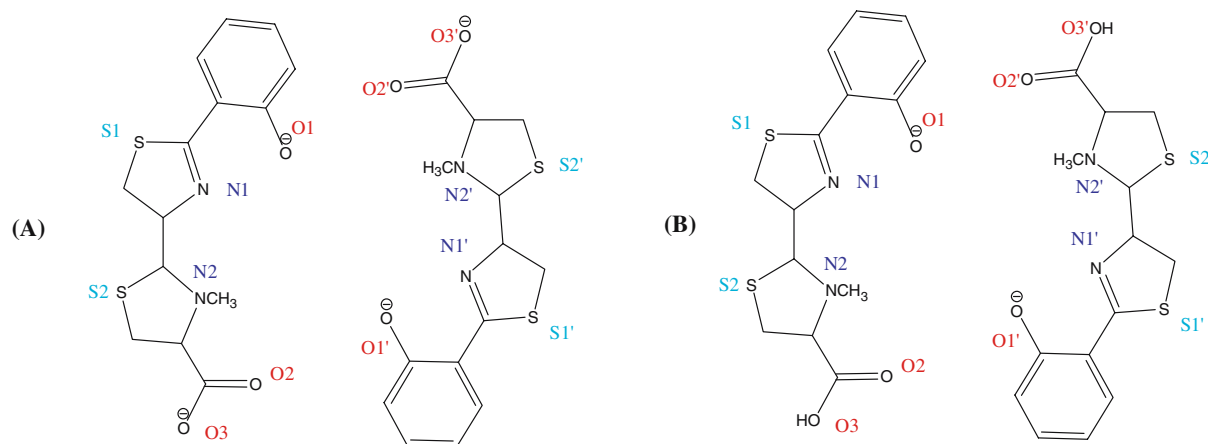


ized in Tables 3 and 4. Only the six stablest conformers are included in each case. It turns out that these conformers involve asymmetric tetradentate and bidentate coordination only; all conformers with symmetric tridentate coordination are much higher in energy. In the case of pyochelin, we found that the stablest geometry of the Fe(III)–pyochelin complexes is that with tetradentate coordination of the iron to the phenol oxygen (O1),  $sp^2$  nitrogen of the thiazole (N1), the  $sp^3$  methyl secondary nitrogen of the thiazoline (N2), and the sidechain carboxylate oxygen (O3) of one pyochelin, and bidentate coordination to the phenol oxygen (O1') and the  $sp^2$  imino nitrogen of the thiazole ring (N1') of the second ligand molecule. In these calculations, we assumed that the phenol oxygen is deprotonated, and although the carboxylate oxygen is protonated at pH~2 in methanol solution, this proton was released in the formation of the complexes. A parallel calculation was also done with the carboxylate oxygen protonated in the Fe(III) complex, but the conformation energy of the complex was significantly less stable. Finally, although other coordination schemes were possible, these conformers were all higher in energy by more than 15 kcal mol<sup>-1</sup>. The same structural conclusions were obtained in the case of 3''-nor-NH-pyochelin, not surprisingly, as the only difference between 3''-nor-NH-pyochelin and pyochelin is the methyl substitution at the nitrogen of the thiazoline ring (N2) in the case of pyochelin. Stick presentation views of the stablest conformer predicted for pyochelin–Fe(III) and the 3''-nor-NH-pyochelin–Fe(III) are shown in Scheme 2.

In the case of methyl hydroxamate, only the phenoxyl and imino groups are retained to provide the original set of oxygen and nitrogen ligands, and two other ligand atoms must be sought to complete the octahedron. The remaining ligand atoms could come from the  $sp^2$  oxygen of the methoxylamide group (O2 or O2') and/or the etheral oxygen of the methoxyl group (O3 or O3'). The coordination to the latter two ligands could either be symmetrical, with both ligands contributing the  $sp^2$  oxygen of the methoxylamide group (i.e., O2 and O2') or the etheral oxygen of the methoxyl group (i.e., O3 or O3'), or asymmetrical, with one ligand contributing the methoxylamide carbonyl oxygen (O2 or O2') and the other ligand the etheral oxygen (O3 or O3'). In any

**Table 3** Semiempirical bond distances and coordination–conformational energies of various pyochelin–Fe(III) models

Model	Bond distances (Å)						Energy (kcal mol <sup>-1</sup> )	
	Fe–O1	Fe–N1	Fe–N2	Fe–O3	Fe–O1'	Fe–N1'		
1	A	1.8559	1.8910	2.1046	1.9029	1.8856	2.0815	–500.33
	B	2.0334	1.8813	2.1126	2.7467	1.8758	2.0500	–348.59
		Fe–O1	Fe–N1	Fe–N2	Fe–O3	Fe–O3'	Fe–N2'	Energy (kcal mol <sup>-1</sup> )
2	A	1.8863	1.8853	2.3762	1.9177	2.0002	1.9515	–485.17
	B	2.5426	2.0706	1.9261	2.1791	4.0978	1.9322	–157.06
		Fe–O1	Fe–N1	Fe–N2	Fe–O2	Fe–O3'	Fe–N2'	Energy (kcal mol <sup>-1</sup> )
3	A	1.9075	2.0090	1.9649	2.2829	1.8970	1.9208	–371.37
	B	2.0669	1.8913	2.2289	2.2714	4.2235	2.2279	–170.31
		Fe–O1	Fe–N1	Fe–N2	Fe–O2	Fe–O1'	Fe–N1'	Energy (kcal mol <sup>-1</sup> )
4	A	1.8837	1.8860	2.1246	2.0414	1.8826	2.0680	–355.26
	B	1.8837	1.8860	2.1246	2.0414	1.8826	2.0680	–355.26
		Fe–O1	Fe–N1	Fe–N2	Fe–O3	Fe–O2'	Fe–N2'	Energy (kcal mol <sup>-1</sup> )
5	A	1.8829	1.9013	2.1781	1.9154	2.2375	1.9536	–353.34
	B	2.0643	1.8890	2.2226	2.5896	2.2258	2.2687	–163.24
		Fe–O1	Fe–N1	Fe–N2	Fe–O2	Fe–O2'	Fe–N2'	Energy (kcal mol <sup>-1</sup> )
6	A	2.1533	2.0595	1.9302	2.2766	2.2267	1.9322	–172.52
	B	2.5426	2.0706	1.9261	2.1791	4.0978	1.9322	–172.52

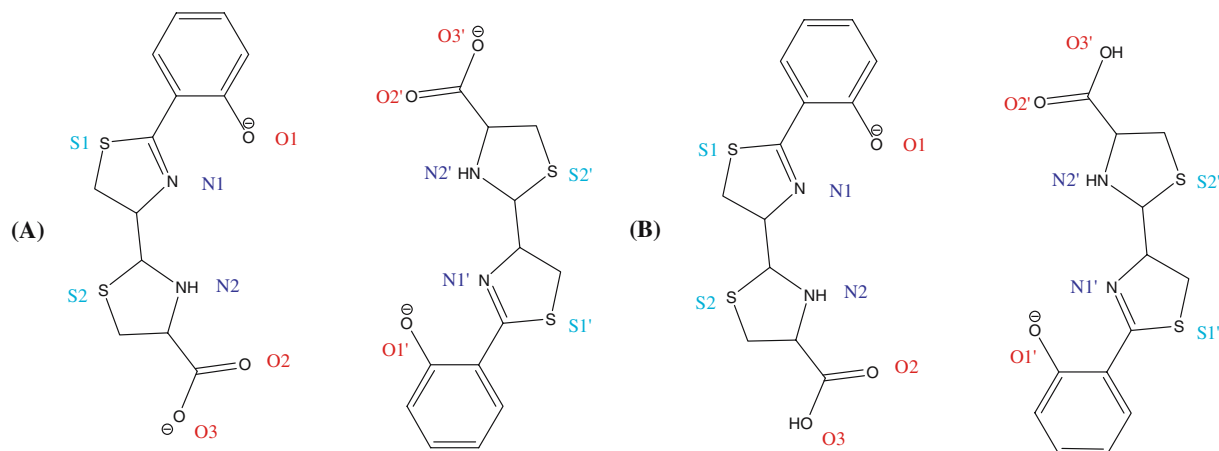


case, each ligand would contribute three ligand atoms for tridentate coordination to form the 2:1 methyl hydroxamate–Fe(III) complex. The energetics predicted by the theoretical calculations favored invoking the phenoxyl oxygen (O1 and O1'), the imino nitrogen (N1 and N1'), and either of the etheral oxygens (O3 and O3'). One of latter etheral oxygens could be replaced by the methoxylamide carbonyl oxygen (O2 or O2'), but this conformer is some 5 kcal mol<sup>-1</sup> higher in energy (Table 5). Stick presentation views of the two most stable conformers predicted for the 2:1 methyl hydroxamate–Fe(III) complex are shown in Scheme 3.

Finally, the results of the coordination–conformational energy calculations for the Fe–pyochelin and Fe–3''-nor-NH-pyochelin complexes, that is, the tetradentate and bidentate coordination predicted for the first and second ligand, respectively, are consistent with the relatively larger formation constant for the 1:1 complex compared with the equilibrium constant for the formation of the 2:1 complex from the 1:1 species. The equal equilibrium constants for the successive formation of the 1:1 and the 2:1 complexes in the case of methyl hydroxamate are also consistent with the tridentate coordination of two ligands to form the 2:1 Fe(III) complex in this system.

**Table 4** Semiempirical bond distances and coordination–conformational energies of various 3''-nor-NH-pyochelein–Fe(III) models

Model		Bond distances (Å)						Energy (kcal mol <sup>-1</sup> )
		Fe–O1	Fe–N1	Fe–N2	Fe–O3	Fe–O1'	Fe–N1'	
1	A	1.8889	1.8911	2.1011	1.9005	1.8821	2.0420	–490.58
	B	1.8875	2.0312	1.9205	2.1691	1.8989	1.8720	–344.44
		Fe–O1	Fe–N1	Fe–N2	Fe–O3	Fe–O3'	Fe–N2'	Energy (kcal mol <sup>-1</sup> )
2	A	1.9036	2.0003	1.9321	1.8866	1.8817	2.2449	–485.11
	B	2.0276	1.8751	2.0401	2.0876	2.7377	1.9482	–127.96
		Fe–O1	Fe–N1	Fe–N2	Fe–O2	Fe–O3'	Fe–N2'	Energy (kcal mol <sup>-1</sup> )
3	A	1.9182	2.0310	1.9383	2.3775	1.8897	1.9225	–377.25
	B	2.1250	2.0217	1.9270	2.1827	2.4596	1.9209	–168.32
		Fe–O1	Fe–N1	Fe–N2	Fe–O2	Fe–O2'	Fe–N1'	Energy (kcal mol <sup>-1</sup> )
4	A	1.8854	1.8839	2.1300	2.0410	1.8800	2.0422	–344.19
	B	1.8854	1.8839	2.1300	2.0410	1.8800	2.0422	–344.19
		Fe–O1	Fe–N1	Fe–N2	Fe–O3	Fe–O2'	Fe–N2'	Energy (kcal mol <sup>-1</sup> )
5	A	1.8957	2.0116	1.9442	1.9115	2.4806	1.9500	–356.17
	B	2.0970	2.0422	1.9151	2.3952	2.2146	1.9264	–163.88
		Fe–O1	Fe–N1	Fe–N2	Fe–O2	Fe–O2'	Fe–N2'	Energy (kcal mol <sup>-1</sup> )
6	A	1.8980	2.0311	1.9351	2.3746	2.0224	1.9329	–169.32
	B	1.8980	2.0311	1.9351	2.3746	2.0224	1.9329	–169.32

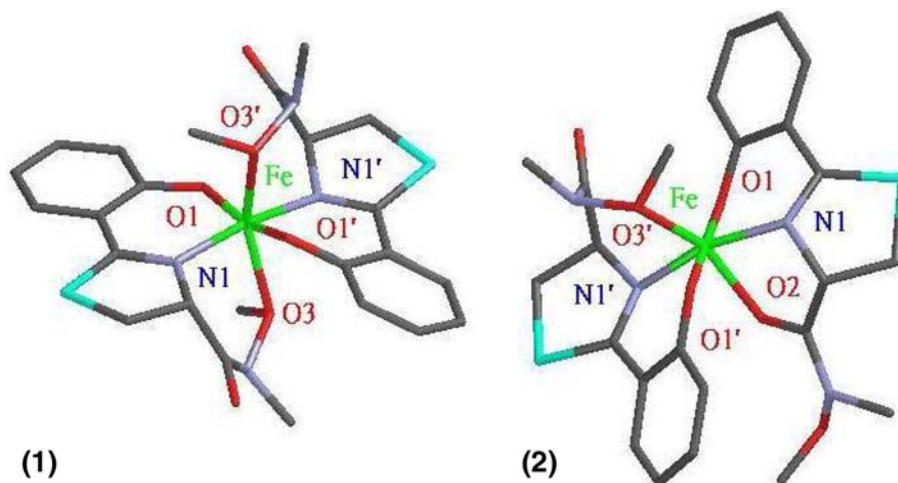


## Discussion

Recently, Klumpp et al. [9] studied the 1:1:1 octahedral complex of Fe(III) with cepabactin and pyochelein in methanol solution by mass spectrometry. On the basis of this study, and the structure of ferric pyochelein co-crystallized with the FptA receptors [10], it was suggested that pyochelein contributed a tetradentate ligand and the cepabactin a bidentate ligand, to form a complex with an overall octahedral coordination. High-spin Fe(III) complexes are typically hexa-coordinated. As in the case of the ternary complex between Fe(III) and

pyochelein and cepabactin, Fe(III) also forms a similar asymmetrical 2:1 complex with pyochelein in methanol solution. In other words, the octahedron is provided by four ligand atoms from one pyochelein and two ligand atoms from a second pyochelein. This tetradentate coordination of one ligand molecule and bidentate coordination of a second to the iron occurs in the case of 3''-nor-NH-pyochelein as well. The recent results for the three-dimensional structure of the specific outer-membrane receptor FptA loaded with ferripyochelein show this same asymmetrical ligand coordination to the Fe(III) in the siderophore–receptor complex, illustrating

**Scheme 3** Stick presentation views of the stablest methyl hydroxamate models (1) and (2)



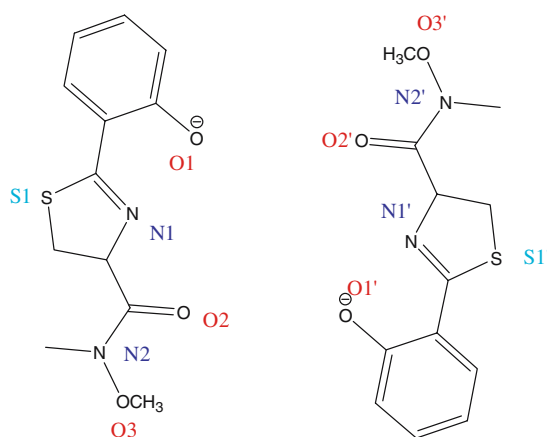
the generality of this type of ligand coordination to Fe(III) with pyochelin and related analogs [10].

The similarity of the EXAFS spectra for the Fe(III) complexes of pyochelin and 3''-nor-NH-pyochelin (Fig. 8a, spectra 1 and 2) indicates that the coordination environments are essentially identical between these two complexes. While the obvious shape differences in spectrum 3 of Fig. 8a observed for the methyl hydroxamate complex and the corresponding sharper

Fourier transform (FT) peak observed in Fig. 8b support the conclusions here that the first coordination sphere of the methyl hydroxamate complex is different from those of pyochelin and 3''-nor-NH-pyochelin, there is no evidence for the direct involvement of sulfur ligand(s) in the methyl hydroxamate. As Fe–S bonds are longer, and sulfur is a much stronger backscatterer than O and N, sulfur coordination would show up as a separate peak in the FT EXAFS. Such a peak was not

**Table 5** Semiempirical bond distances and coordination–conformational energies of various the methyl hydroxamate–Fe(III) models

Model	Bond distances (Å)						Energy (kcal mol <sup>-1</sup> )
	Fe–O1	Fe–N1	Fe–O3	Fe–O1'	Fe–N1'	Fe–O3'	
1	1.8797	1.8769	2.0857	1.8787	1.8708	2.1261	–294.23
2	1.8821	1.8919	2.1207	1.8851	1.8672	2.1030	–289.64
3	1.8596	1.8865	1.9627	1.9779	1.8725	2.1427	–278.21





observed or evident in the FT EXAFS; thus, only N and O ligation is involved in the methyl hydroxamate complex as well. From the structural conclusions derived in this study, it is evident that the phenoxyl group as well as the imino group at the thiazoline ring from pyochelin and its analogs must be critical to the ferric ion coordination. We replaced the phenol substituent by its methoxylated analog on the thiazoline ring; the modified molecule was no longer able to chelate with ferric ion.

In all likelihood, however, the Fe(III) complex exists principally as a 1:1 Fe(III)–pyochelin complex in the extracellular medium with the remaining two coordination sites filled by hydroxides or water molecules. The same scenario occurs for the Fe(III)–pyochelin system in methanol. In fact, the large equilibrium constant (Table 2) measured for the comproportionation of the 2:1 complex of pyochelin (and 3''-nor-NH-pyochelin as well) implies that the 1:1 complex is the predominant species, except under high ligand concentrations. A similar Fe(III)–pyochelin complex is formed in the presence of ethylene glycol, with the diol of the ethylene glycol replacing the two water molecules and serving as the bidentate ligand [10]. In any case, such a 1:1 Fe(III)–pyochelin precursor would then be poised for uptake by a membrane-bound translocation protein such as FptA to deliver the ferric ion into the periplasm of the bacterial cell. This scenario is consistent with the recently reported three-dimensional structure of the ferripyochelin-loaded FptA receptor [10].

## Conclusion

In conclusion, we have studied the coordination properties of pyochelin and two analogs, 3''-nor-NH-pyochelin and methyl hydroxamate, to Fe(III) ion in methanol or buffered methanol–water mixtures. While pyochelin and 3''-nor-NH-pyochelin can serve as tetradentate ligands to coordinate with Fe(III) to form a 1:1 complex, they could also serve as bidentate ligands to complete the octahedral coordination shell by displacing the labile solvent molecules (alkoxide in the case of methanol;

hydroxides in the case of water) to form the corresponding 2:1 ferric complexes. The large value of the comproportionation constant in the case of the pyochelin and 3''-nor-NH-pyochelin complexes supports the predominance of the 1:1 species except when the ligand is in large excess. In contrast, methyl hydroxamate could only serve as tridentate ligands. It is possible here that the two ligands could offer the same set of ligand atoms to coordinate with ferric ions symmetrically. It is hoped that these studies have shed some insight into the nature of the coordination chemistry used by an iron siderophore to facilitate ferric ion transport across the membrane into the cytoplasm of the cell.

**Acknowledgements** We wish to thank Françoise Hoegy for her expert technical assistance. We acknowledge the French Institute of Taipei and the French Minister of Foreign Affairs for a grant and for travel expenses. We also thank Academia Sinica and the National Science Council of Taiwan (Republic of China) for travel expenses.

## References

1. Liu PV, Shokrani F (1978) *Infect Immun* 22:878–890
2. Sokol PA (1986) *J Clin Microbiol* 23:560–562
3. Sokol PA, Woods DE (1983) *Infect Immun* 40:665–669
4. Cox CD, Rinehart KL, Moore ML, Cook JC (1981) *Proc Natl Acad Sci USA* 78: 4256–4260
5. Cox CD, Graham R (1979) *J Bacteriol* 137(1):357–364
6. Cox CD (1980) *J Bacteriol* 142:581–587
7. Beier RC, Stipanovic RD (1989) *Biomed Environ Mass Spectrom* 18:185–191
8. Namiranian S, Richardson DJ, Russell DA, Sodeau JR (1997) *Photochem Photobiol* 65: 777–782
9. Klumpp C, Burger A, Mislin GL, Abdallah MA (2005) *Bioorg Med Chem Lett* 15(6):1721–1724
10. Cobessi D, Celia H, Pattus F (2005) *J Mol Biol* 352: 893–904
11. Zamri A, Abdallah MA (2000) *Tetrahedron* 56:249–256; corrigendum: Zamri A, Abdallah MA (2000) *Tetrahedron* 56:9397
12. Zamri A, Schalk IJ, Pattus F, Abdallah MA (2003) *Bioorg Med Chem Lett* 13:1147–1150
13. Mislin GL, Burger A, Abdallah MA (2004) *Tetrahedron* 60(52):12139–12145
14. Schlegel K, Taraz K, Budzikiewicz H (2004) *BioMetals* 17:409–414
15. Ecker DJ, Lancaster JR Jr, Emery T (1982) *J Biol Chem* 257(15):8623–8626