Synthesis and Iron-Binding Properties of Protochelin, the Tris(catecholamide) Siderophore of *Azotobacter vinelandii*

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Protochelin, the tris(catecholamide) siderophore recently identified in the culture medium of *A. vinelandii*, can be synthesised by coupling of suitably protected azotochelin and aminochelin derivatives in preparative yields. Based on the ligand protonation constants of $pK_1 = 6.67$, $pK_2 = 8.09$ and $pK_3 = 9.68$, the proton-independent stability constant for ferric protochelin could be estimated to be $10^{44.6}$. The solubilisation of Fe^{III} hydroxide by protochelin has been investigated spectrophotometrically in aqueous solution at pH = 7. Since the production of protochelin by *A. vinelandii* depends on high molybdate levels in the growth medium, the studies have been carried out in the presence as well as in the absence of an equimolar amount of molybdate. Although the protochelin promoted dissolution of Fe^{III} hydroxide is delayed significantly in presence of molybdate, the effect is far less pronounced than the one observed for azotochelin, the bis(catecholamide) siderophore of *A. vinelandii*.

Although iron is abundant in the soil it is relatively inaccessible to microorganisms since it exists predominantly as highly insoluble ferric hydroxides and oxides. In order to satisfy their requirement for iron, many micro-organisms excrete high-affinity iron chelators, so called siderophores, to solubilise Fe^{III} for transport into the cell[1].

The nitrogen-fixing cells of *Azotobacter vinelandii* have a variety of distinct iron uptake mechanisms, the activation of which depends on the concentration of the essential micronutrient in its environment[2]. At relatively high iron concentrations (>7 μM[2,3]), a low affinity uptake system operates, employing 2,3-dihydroxybenzoic acid as chelator. At lower ion concentrations, true siderophores are produced: azotochelin[4] and aminochelin[3] at conentrations below 7 μM, and azotobactin at iron concentrations below 3 μM[5]. Only recently, the siderophore protochelin[6] has been identified in the culture fluid of *A. vinelandii*[2]. This condensation product of azotochelin and aminochelin had already been predicted to be an additional tris(catecholamide) siderophore of *A. vinelandii*[3,6,7] (Figure 1).

Interestingly, protochelin is exclusively secreted by cells grown at high molybdate levels (1 mM), when molybdenum competes for the siderophore ligands, especially the ones of lower denticity[18]: *cis*-dioxomolybdenum(VI) complexes containing two coordinated catecholamide subunits[9,10,11] or 2,3-dihydroxybenzoic acid[12] are well-known. The fact that protochelin is the only siderophore of *A. vinelandii* that incorporates three bidentate catecholamide subunits and therefore matches exactly the sixfold coordination requirements of iron is likely to be related to its specific biofunction. The siderophore enterobactin, one of the most powerful iron chelators known ($K_f = 10^{49}$[13]), is also based on three catecholamide units, and other lysine-containing tris-catecholate derivatives have been exploited as enterobactin analogs[7,14].

In order to investigate the iron binding properties of protochelin, the tris(catecholamide) siderophore was synthesized and the stability constant of its iron complex determined. Furthermore, the protochelin-promoted dissolution of iron(III) hydroxide was followed spectrophotometrically in the presence and in the absence of molybdate. By comparison with the data obtained for the tetradentate siderophore azotochelin[8], it is evident that protochelin not only achieves a higher dissolution rate, but, above all, demonstrates a far superior selectivity for iron in the presence of molybdate.

Results and Discussion

Ligand Synthesis

Our approach to the synthesis of protochelin is outlined in Scheme 1. Benzyl groups were chosen to protect the catecholate oxygens throughout the entire synthetic procedure. The intermediate benzyl protected azotochelin derivative **1**, was obtained in a 4 step procedure as described by Chimiak and Neilands[7]. The stochiometric reaction of one equivalent *O,O*-dibenzyl-2,3-dihydroxybenzoic acid[15] with one equivalent of 1,4-diaminobutane by use of *N,N'*-carbonyldiimidazole (CDI[16]) provided a convenient approach to the key intermediate, the aminochelin derivative **2**, in satisfac-

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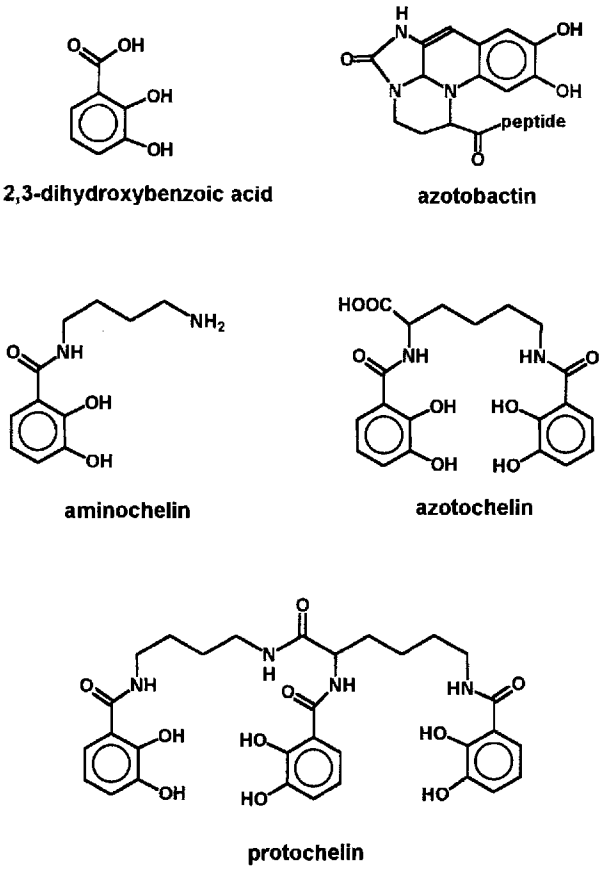
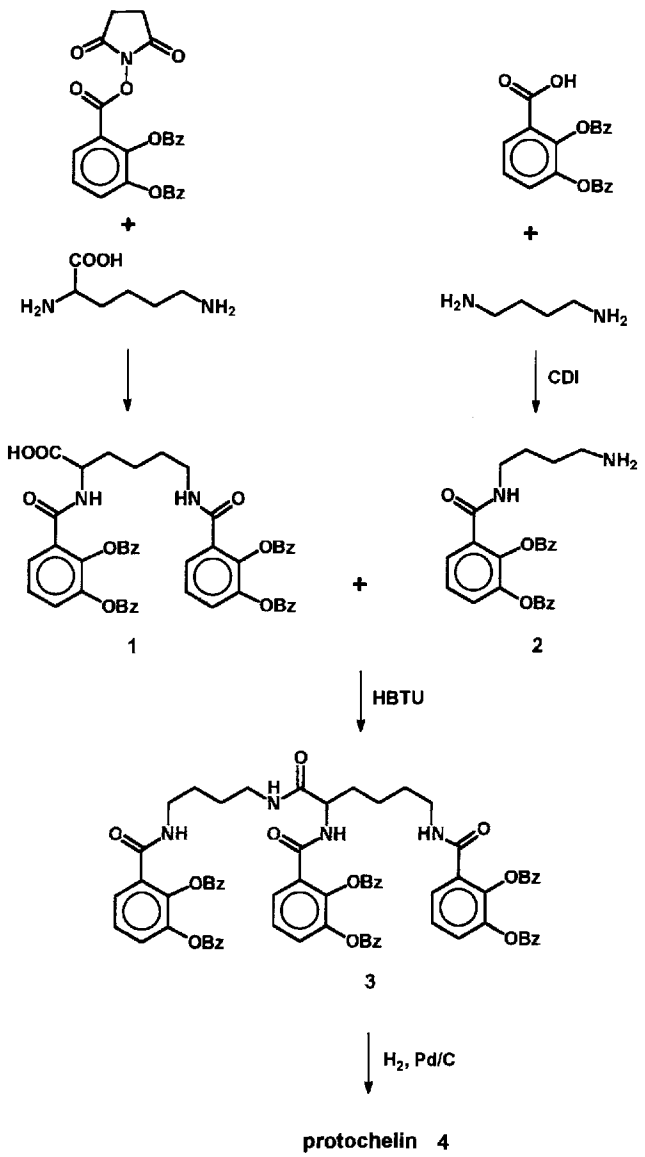
Figure 1. Siderophores produced by *Azotobacter vinelandii*



Scheme 1



tory yields (72%). Due to the insolubility of the protected aminochelin derivative in ethylacetate, the desired mono-(catecholamide) could easily be separated from the corresponding bis(catecholamide) by-product by precipitation from a concentrated chloroform solution by addition of ethylacetate.

The subsequent condensation of **1** and **2** was achieved by HBTU-mediated coupling (HBTU = *O*-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate[17]). The protected protochelin derivative **3** was isolated in 84% yield after column chromatography of the crude product. Removal of the protecting groups by hydrogenation afforded protochelin (**4**) in almost quantitative yield in the form of an amorphous solid. The ^{1}H-NMR and UV/Vis spectra of the synthetic product were identical to those reported for the natural material[6].

Stability of the Iron Protochelin Complex

The three lower protonation constants of protochelin (PROT) were determined by a simultaneous spectrophotometric and potentiometric titration and found to be pK_4 = 9.68, pK_5 = 8.09, and pK_6 = 6.67 respectively. The proton dependent stability constant K^*_{FePROT} = $[Fe(PROT)^{3-}] \cdot [H^+]^3/[Fe^{3+}] \cdot [H_3\text{-}PROT^{3-}]$ for the iron protochelin complex was determined spectrophotometrically by means of competitive complex formation with EDTA[18] in aqueous solution at pH = 6.9 as $10^{8.3}$, using the known stability constant for iron(III)-EDTA of log K_{FeEDTA} = 25.1[19].

$$Fe(PROT)^{3-} + EDTA^{4-} + 3H^+ \rightleftharpoons Fe(EDTA)^- + H_3PROT^{3-}$$

$$K_{eq} = [Fe(EDTA^-] \cdot [H_3PROT^{3-}]/[Fe(PROT)^{3-}] \cdot [EDTA^{4-}] \cdot [H^+]^3 = K_{FeEDTA}/K^*_{FePROT}$$

To obtain the conventional (proton-independent) formation constant $K_{Fe(PROT)} = [Fe(PROT)^{3-}]/[Fe^{3+}] \cdot [PROT^{6-}]$, an average pK_a value of 12.1[18] was assumed for the experimentally inaccessible three higher protonation constants of protochelin. Based on this figure, a log $K_{Fe(PROT)}$ value of 44.6 has been estimated for ferric protochelin. Thus the stability of ferric protochelin is comparable with the stabilities of iron(III) complexes of other tris(catecholamide) ligands, such as linear enterobactin (log K_{FeL} = 43[20]), the enterobactin analogue mecam (log K_{FeL} = 43[21]), and the lysine derivative described by Akiyama and Ikeda (log K_{FeL} = 46[14]).

Solubilisation of Iron Hydroxide by Protochelin

For the solubilisation studies an initial siderophore concentration of 0.4 mM was used, which lies within the geochemically significant range estimated for soil bacteria[22]. Freshly prepared iron hyroxide was selected as the iron-source. Due to its high surface area and structural disorder it dissolves much faster than, for example, hematite or geothite and should therefore compete most effectively with molybdate for the siderophore ligand. This system is not homogeneous and equilibrium has to be established between the water soluble siderophore, the metal complexes, and the suspended iron hydroxide particles. Since the dimensions of these particles and their degree of crystallinity influence their activity, the iron hydroxide was precipitated under defined and reproducible conditions 30 minutes before start of each experiment.

The spectral changes observed during the solubilisation of iron hydroxide by protochelin in the absence of molybdate at pH = 7 are shown in Figure 2a. The spectra show a maximum absorbance at 495 nm for the broad charge transfer band of the iron siderophore complex which lies in the region characteristic for iron tris(catecholate) species[18]. The solubilisation proceeds rapidly, as evident from Figure 3, with 55% of the equilibrium concentration of the iron protochelin complex being reached within 3 minutes. The reaction is complete after 16 hours.

The addition of an equimolar amount of molybdate to the ligand solution of an otherwise identical experiment results in an instantaneous formation of the orange coloured molybdenum complex and a significant delay in the solubilisation of $Fe(OH)_3$: iron complex formation is 55% complete after 14 minutes and equilibrium is reached after 48 hours (Figures 2b and 3). Evidently, the binding of molybdenum to protochelin reduces the iron binding activity of the siderophore. A similar observation has been made using the Chrome Azurol-S (CAS) assay for determination of the protochelin siderophore activity[2]: while the deferration of the chromogenic CAS ligand by protochelin occurred rapidly as expected, no decolorisation was detected if protochelin was treated with an excess of molybdate before addition of the CAS solution.

Figure 3 compares the time course of the solubilisation of $Fe(OH)_3$ by the hexadentate siderophore protochelin in absence and presence of molybdate with the results obtained for the tetradentate siderophore azotochelin[8]. Although the conditions for the experiments were identical, protochelin achieves a much higher initial dissolution rate than azotochelin, in absence as well as in presence of molybdate. Furthermore, the influence of molybdate on the protochelin-promoted dissolution is far less pronounced than the marked effect observed in the case of azotochelin.

A conceivable explanation for these findings can be given in terms of the composition of the complexes. Whereas protochelin is capable of binding Fe^{III} in a 1:1 stochiometic fashion, azotochelin requires the coordination of additional ligands and/or the formation of dinuclear assemblies to satisfy the preferred octahedral coordination geometry of Fe^{III}[23], and thus represents a less effective siderophore. In

Figure 2. UV-Vis spectral changes accompanying the solubilisation of Fe^{III} hydroxide by protochelin at pH = 7 (a) in the absence and (b) in the presence of an equimolar amount of molybdate; for (a): selected spectra recorded after 3 min, 6 min, 20 min, 60 min, 150 min, 7 h, and 16 h; for (b): spectra recorded in periodic intervals of 3.5 min

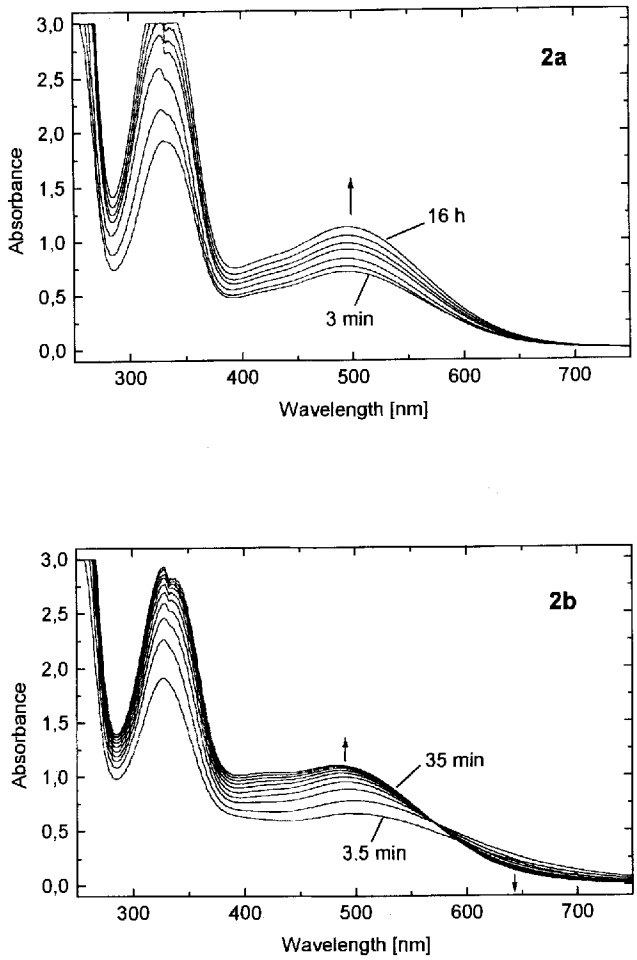
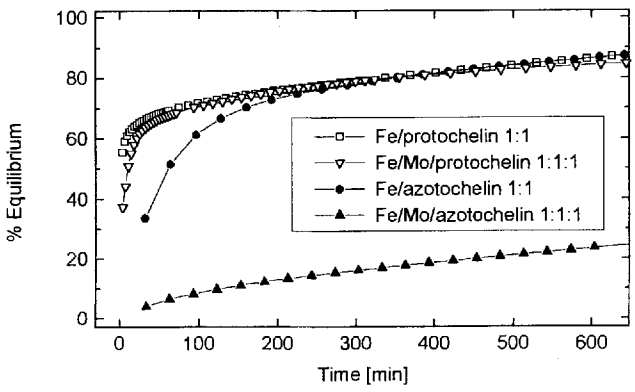
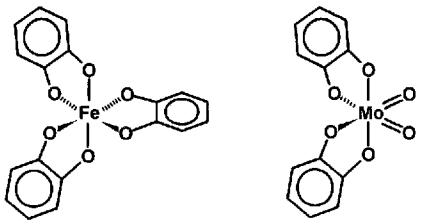


Figure 3. Time course of the siderophore-promoted solubilisation of Fe^{III} hydroxide in absence and presence of an equimolar amount of molybdate in HEPES buffer at pH = 7; the per cent equilibrium is based on the absorbance at 560 nm when equilibrium was reached



contrast, the bis(bidentate) ligand azotochelin is well suited for complexation of the MoO_2^{2+} unit, which has four vacant coordination sites (Figure 4). Since the molybdenum

Figure 4. Schematic representation of Fe^{3+} and *cis*-MoO_2^{2+} catecholate complexes, shown in Λ configuration



complex of azotochelin is formed almost instantaneously in solution, the solubilisation of $Fe(OH)_3$ involves a metal exchange reaction that requires the partial unwrapping of the tetradentate ligand prior to iron binding and thereby complicates $Fe(OH)_3$ dissolution. Metal ion exchange reactions between siderophore complexes in which the denticity of the ligand matches the coordination number of the metal ion are known to be extremely slow[24]. However, in the *cis*-dioxomolybdenum protochelin 1:1 complex only two catecholamide subunits are likely to be coordinated, so that the third subunit remains available for iron binding. Evidence for the formation of monocatecholate iron species (λ_{max} ca. 600 nm[18]) can be derived from the relatively high absorbance observed between 550 and 700 nm in case of the initial UV/Vis spectra shown in Figure 2b. In view of this observation we conclude that protochelin is able to retain its siderophore activity up to the presence of an equimolar quantity of molybdate by means of the third catecholamide subunit which is not involved in molybdenum binding.

Conclusions

Protochelin shows all characteristics required of an excellent iron siderophore. Its high affinity and, in particular, its selectivity for ferric ion in presence of molybdate distinguishes protochelin from azotochelin, the bis(catecholamide) siderophore of *A. vinelandii*.

The distribution of molybdenum in the soil is uneven. Consequently, the production of protochelin will no doubt provide a competitive advantage in molybdate-rich environments. Yet, the question arises why such an efficient siderophore is not generally found in culture media of iron-limited cells of *A. vinelandii*. On the one hand, there may be additional factors operating in the soil which induce protochelin production and which have yet to be identified. On the other hand, it might be advantageous not to have the regulation of siderophore production controlled by iron alone: iron as well as molybdenum are essential for the optimal growth of *A. vinelandii* and it has been suggested that siderophores may also be involved in molybdenum uptake[1b,25,26].

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Experimental Section

Commercially available reagents (Aldrich, Fluka) were used without further purification. Solvents were dried by standard procedures. – NMR data were recorded on a Bruker AM 400 spectrometer. For $CDCl_3$ and [D_4]methanol solvents, TMS was used as an internal standard. – Mass spectra were measured on a Varian MAT 212 mass spectrometer (70 eV), and UV-visible spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer. – Melting points were determined on a Kofler micro-hotplate and are uncorrected. – Elemental analyses were performed by the Microanalytical Service, Organic Chemistry Department, University of Münster.

N^2,N^6-Bis(2,3-dibenzyloxybenzoyl)-L-lysine (**1**): **1** was obtained from lysine hydrochloride and 2,3-dibenzoxybenzoic acid[15] as described by Chimiak and Neilands[7] in the form of a chromatographically pure oil. – ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.23–1.46 (m, 4H, $CH_2CH_2CH_2$), 1.48–1.62 (m, 1H, CH_2CH_2CH), 1.80–1.95 (m, 1H, CH_2CH_2CH), 3.31 (m, 2H, CH_2CH_2NH), 4.66 (m, 1H, CH), 5.20 (s, 2H, benzylic CH_2), 5.31 (s, 6H, benzylic CH_2), 7.25–7.68 (m, 24H, arom. H), 7.85–7.93 (m, 2H, arom. H), 8.06 (t, 1H, NH), 8.69 (d, 1H, NH).

N-(2,3-Dibenzyloxybenzoyl)diaminobutane Hydrochloride (**2**): Solid *N,N'*-carbonyldiimidazole (1.622 g, 10 mmol) was added to a solution of 2,3-dibenzoxybenzoic acid (3.344 g, 10 mmol) in 15 ml of anhydrous THF. After CO_2 evolution had ceased, the resulting clear solution was added dropwise over 2 h to a vigorously stirred solution of 1,4-diaminobutane (1.01 ml, 10 mmol) in 30 ml of anhydrous THF. Stirring was continued overnight at room temperature. After solvent evaporation the residue was dissolved in 150 ml of chloroform and washed successively with saturated $NaHCO_3$ solution (100 ml), brine (100 ml), 2 N HCl (100 ml), and brine (100 ml). The organic layer was collected and dried with $MgSO_4$. The solvent was then partially removed by evaporation and ethyl acetate was added to precipitate the product. The white precipitate was collected, washed with ethyl acetate, and dried under vacuum. Recrystallisation of the crude product from chloroform/ethyl acetate yielded 3.175 g (72%) of pure **1** as a white solid; m.p. 137°C. – ^{1}H NMR (400 MHz, D_2O): δ = 0.98 (m, 2H, $CH_2CH_2CH_2$), 1.21 (m, 2H, $CH_2CH_2CH_2$), 2.55 (t, 2H, $CH_2CH_2NH_3$), 2.77 (t, 2H, CH_2CH_2NH), 4.33 (s, 2H, benzylic CH_2), 4.37 (s, 2H, benzylic CH_2), 6.45–6.98 (m, 13H, arom. H). – MS (70 eV); *m/z* (%): 404 (5) [M$^+$ – HCl]. – $C_{25}H_{29}ClN_2O_3$ (440.97): calcd. C 68.09, H 6.63, N 6.35; found C 68.12, H 6.69, N 6.34.

N^1-[N^2,N^6-Bis(2,3-dibenzyloxybenzoyl)-L-lysyl]-N^4-(2,3-dibenzyloxybenzoyl)-1,4-diaminobutane (**3**): 350 µl (2 mmol) of diisopropylethylamine were added to a solution of 779 mg (1 mmol) of *N,N'*-bis(2,3-dibenzyloxybenzoyl)lysine and 379 mg (1 mmol) of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[16] in 6 ml of anhydrous THF and 3 ml of anhydrous acetonitrile. 441 mg (1 mmol) of **2** were then added and the resulting clear solution was stirred at room temperature for 5 h. After removal of the solvent under reduced pressure saturated sodium chloride solution (30 ml) was added and the product was extracted with ethyl acetate (3 × 25 ml). The organic phase was washed successively with 2 N HCl (5 ml), saturated sodium chloride solution (5 ml), 5% sodium hydrogen carbonate solution (5 ml), and saturated sodium chloride solution (5 ml), followed by drying with $MgSO_4$ and solvent evaporation. Column chromatography on silica gel using ethyl acetate as solvent afforded 979 mg (84%) of pure protochelin derivative **3** as a hard, amorphous solid. – ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.08–1.19 (m, 2H, $CH_2CH_2CH_2$), 1.31–1.40 (m, 6H, $CH_2CH_2CH_2$), 1.64 (m, 2H, CH_2CH_2CH), 3.06–3.28 (m, 6H, CH_2CH_2NH), 4.35 (m, 1H, CH), 5.04–5.15 (m, 12H, benzylic CH_2), 6.46 (t, 1H, NH) 7.10–7.48 (m, 36H, arom. H), 7.64–7.71 (m, 3H, arom. H), 7.85 (t, 1H, NH), 7.89 (t, 1H,

NH), 8.30 (d, 1H, NH). – MS (70 eV); *m/z* (%): 1165 (4) [M^+]. – $C_{73}H_{72}N_4O_{10}$ (1165.39): calcd. C 75.24, H 6.23, N 4.81; found C 75.19, H 6.27, N 4.78.

N^1-[N^2,N^6-Bis(2,3-dihydroxybenzoyl)-L-lysyl]-N^4-(2,3-dihydroxybenzoyl)-1,4-diaminobutane (Protochelin, **4**): All glassware necessary for the deprotection reaction was acid-washed with 2 N HCl prior to use to remove contaminating iron. A solution of **3** (583 mg, 0.5 mmol) in 2.5 ml of benzene was diluted with 70 ml of absolute ethanol and hydrogenated at room temperature over 5% Pd on charcoal catalyst until no *O*-protected material was detectable by TLC (solvent: ethyl acetate). The reaction mixture was filtered through a glass microfibre filter (Whatman). Evaporation of the solvent afforded **4** (306 mg, 98%) as a colourless solid. – ^{1}H NMR (400 MHz, [D$_4$]methanol): δ = 1.44–1.72 (m, 8H, $CH_2CH_2CH_2$), 1.78–1.98 (m, 2H, CH_2CH_2CH), 3.21–3.29 (m, 2H, CH_2CH_2NH), 3.33–3.42 (m, 4H, CH_2CH_2NH), 4.53 (m, 1H, CH), 6.65–6.74 (m, 3H, arom. H), 6.88–6.95 (m, 3H, arom. H), 7.17 (dd, 1H, arom. H), 7.19 (dd, 1H, arom. H), 7.29 (dd, 1H, arom. H). – MS (70 eV); *m/z* (%): 624 (4) [M^+]. – $C_{31}H_{36}N_4O_{10}$ (624.65): C 59.61, H 5.81, N 8.97; found C 59.57, H 5.83, N 9.00.

Determination of the Physical Constants of Protochelin: All reagents were of analytical grade and all solutions were prepared using 18 MΩ water from a Millipore Milli-Q system.

Protonation Constants Determination: The protonation constants of protochelin were obtained by a combined potentiometric and spectrophotometric titration using an automated system[27] equipped with a standard glass electrode and a silver chloride electrode (Metrohm). A blank titration of 25 ml of a 0.1 M aqueous KCl solution was carried out to determine the electrode zero using Gran's method[28]. The solution was kept in a jacketed titration cell at 25 ± 0.5°C and under an atmosphere of argon. After acidification with 150 µl of 0.2 M HCl, the titration was carried out against 0.3 M KOH using 10 µl increments dispensed from a Metrohm 665 dosimat. The titration was repeated in the presence of protochelin (1.19106×10^{-4} M) and 300 µl of 0.2 M HCl. The titration data were analysed using the computer program TITRFIT[29].

Stability Constant Determination: The formation constant of the iron(III)–protochelin complex was determined by a spectrophotometric competition study of the system protochelin–Fe^{III}–EDTA using an automated system[27]. The iron(III) complex of protochelin was prepared using a 2.323×10^{-5} M iron(III) concentration and a 5 molar ligand excess in 0.1 M MOPS buffer at pH = 7. This solution was titrated against 0.40174 M EDTA, pH 7, using 200 µl increments dispensed from a Metrohm 665 dosimat. The resulting data were analysed by the computer program COMPTI[27]. A formation constant for the iron–protochelin complex of log $K_{Fe/Prot}$ = 44.6 was obtained based on the formation constant of log $K_{Fe/EDTA}$ = 25.1.

Ligand-Promoted Solubilisation of Iron Hydroxide: All solutions were prepared using 18 MΩ/cm water from a Millipore Milli-Q system. Stock solutions were prepared by dissolving weighed amounts of the reagents in deionized water. Iron was introduced into the solutions by using an AAS iron standard solution. The electronic absorption spectra were recorded on a Perkin-Elmer Lambda 5 UV-VIS spectrophotometer, equipped with a PC for data collection and evaluation. The temperature for the study was maintained at 25 ± 0.5°C. To precipitate ferric hydroxide the pH value of a solution of 6 µmol Fe^{III} in 10 ml of 0.1 M HEPES buffer was adjusted to pH = 7 with concentrated HCl and carbonate free KOH in a thermostated titration vessel. After 30 min, 60 µl of a 0.1 M K_2MoO_4 solution and 3.75 mg (6 µmol) of protochelin dissolved in 5 ml of HEPES pH = 7 buffer were added, resulting in a mixture 0.4 m M each in molybdate, total iron, and protochelin. The time of mixing was recorded as zero time and the first scan obtained after 3.5 minutes. Further spectra were recorded at periodic intervals. A peristaltic pump was used to circulate the reaction mixture from the vessel through a filter probe to a flow-through cuvette of 1 cm optical pathlength at a flow rate of 1.25 ml/min. The pH value was checked throughout the experiments and it remained at 7.00 ± 0.02. The control experiments were carried out analogously in absence of molybdate. In such cases spectra were taken initially at intervals of 3 min because of the faster dissolution rate. Further spectra were recorded in longer intervals until there was no further change in absorbance. All dissolution experiments were performed twice with the suspensions being stirred throughout the entire experiments.

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