Kinetics and Thermodynamics of Complex Formation between Fe^{III} and Two Synthetic Chelators of the Dicatecholspermidine Family

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This report deals with a kinetic and thermodynamic study of complex formation between Fe^{III} and a new series of synthetic chelators, 2,3- (FR401) and 3,4-dicatecholspermidine (FR295). For the following data, the first value concerns FR295 and the second FR401. Each ligand undergoes five acid-base dissociations, four of which involve the hydroxy groups of the catechol moieties and the final one the central ammonium group. They occur with average at pK_{2a} = 8.40 and 7.95 for the first two hydroxy groups, pK_{3a} = 12.6 and 12.20 for the two remaining hydroxy groups and pK_{1a} = 5.50 and 4.90 for the ammonium group. Complex formation with both chelators occurs rapidly in acidic media (pH < 2), resulting in the second-order complex formation rate constants k_1 = 450 M^{-1} s^{-1} and 500 M^{-1} s^{-1} and first-order complex dissociation rate constants k_{-1} = 0.82 s^{-1} and 0.28 s^{-1}. From these two elementary rates, the complex dissociation constant is determined in acidic media: $K_1 = 1.82 \cdot 10^{-3}$ M and $5.6 \cdot 10^{-4}$ M. The Fe^{III} complexes also undergo five proton dissociations with marked decreases in the catechol pK_a value: ΔpK_{1a} = 1.25 and 1.25, ΔpK_{2a} = 6.60 and 6.50, ΔpK_{3a} = 6.55 and 6.95. From ΔpK_a and K_1, the affinity of both FR295 and FR401 for Fe^{III} is determined: log β = 29 and 31 with pFe = 22 and 25. This new series of chelators is aimed for antibiotic targeting. It proved efficient in inhibiting *Plasmodium falciparum* growth and delivered iron ions to Gram-negative bacteria.

Introduction

Iron is one of the most essential elements for practically all life forms. In its most common state of oxidation, Fe^{III} is insoluble in neutral media. It is, therefore, directly unavailable for the living species.[1] Consequently, this element is solubilised and regulated in physiological media by several iron transport and acquisition systems. In microorganisms, one of the most important is constituted by siderophores.[2] Siderophores are very efficient low molecular mass iron chelators used and or synthesized when iron is required. Once in the chelate form these iron transport systems are recognized by the cell, where they are admitted across the membranes by specific paths, and iron is delivered.[3]

Synthetic chelators can be siderophore-like and, therefore, used by some cells for the acquisition of iron.[3] Considerable effort to synthesize siderophore-like and other high-efficiency iron chelators has been made in the last twenty years. Most of these molecules were synthesised for the therapy of iron-overload which can be partially eliminated by chelation.[4,5] Other approaches were investigated to use the siderophore for drug delivery inside pathogenic microorganisms.[6] Siderophores can also be effective against malaria.[7]

Most of the iron ligands in siderophores are catechols and/or hydroxamates.[1,2] A series of synthetic chelators based on a spermidine skeleton bearing catechols behaved as siderophores and as iron scavengers. They inhibited *Plasmodium falciparum* proliferation in vitro and delivered iron to several Gram-negative bacteria, thereby, promoting their growth.[8,10]

This inhibition of *Plasmodium falciparum* is of interest as malaria is still the major cause of human mortality in the tropics where an increasing prevalence of strains of *Plasmodium falciparum* resistant to chloroquine and other antimalarial drugs.[7,11] As for the siderophore activity, it opens up ideas of using these molecules for drug delivery. Indeed, with the emergence of AIDS, and the consequent increase in the number of immunodepressive patients, opportunistic diseases such as tuberculosis with new forms of resistance to antibiotics have appeared.[12] Some of these forms of resistance are mostly related to impermeability of the microorganism's membrane to the drug than to a the classical deactivating enzymes.[12] This impermeability can be overcome by delivering the drug as an antibiotic−siderophore adduct using the microorganism's iron acquisition pathway.[3,8,12,13] Macrolide antibiotics linked to catecholspermidines proved to be effective against antibiotic-resistant mycobacteria.[8,13] These promising results may lead to a new series of antibiotic carriers and antimalarial drugs based on the catecholspermidine series.

Fe^{III} is insoluble in neutral media. It can be complexed to other chelators, in physiological media in the so-called low molecular mass iron pool.[2] However, in vertebrates and some invertebrates, it is regulated by the transferrins, which possess very high affinities for the metal (> 10^{20}

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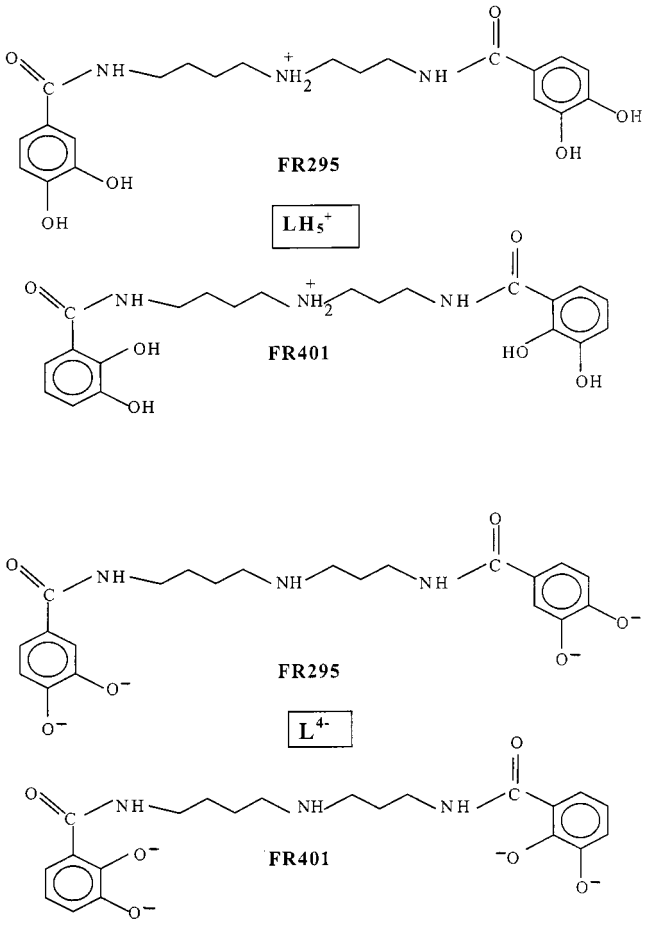
M^{-1}).[14] Therefore, in order to be competitive and to keep their iron charge, synthetic siderophores must possess extremely high affinities for Fe^{III}.[15−18] The affinity constants of natural or synthetic siderophores for iron range from 10^{20} M^{-1} to $> 10^{40}$ M^{-1}.[15−18] These extremely high affinities render any thermodynamic or kinetic analysis of complex formation with Fe^{III} difficult.[18] In this article we analyse, by the chemical relaxation approach and analytical chemistry,[14,19−21] the kinetic and thermodynamic behaviour of the siderophore-like 2,3 (FR401) and 3,4-dicatecholspermidines (FR295, Scheme 1) during complex formation with Fe^{III}.



Scheme 1

Results and Discussion

All our experiments were performed at an ionic strength of μ = 0.2 in the presence of Cl^-. At pH = 2−4, the absorption spectra of both FR295 and FR401 are independent of acidity, which indicates the presence of a single prototropic species for each of them. From pH = 4 to 7, the absorption spectra vary to attain a first plateau at pH = 7−8. They vary again to attain a second plateau at pH = 9−10. From pH = 10−13, the spectra vary again to reach a third plateau in very basic media (Figures 1, 2, and 3).

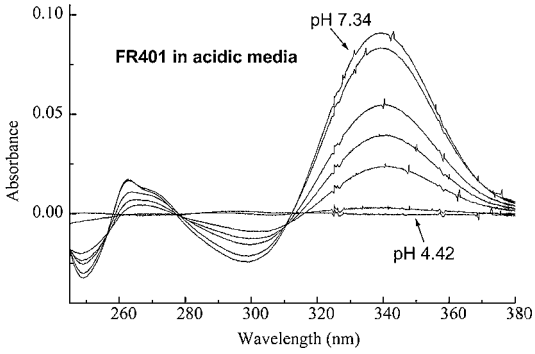


Figure 1. Differential absorption spectra of FR401 between pH = 4.42 and 7.34 with $c_0 = 4.5 \cdot 10^{-5}$ M and μ = 0.2 at 25±0.5 °C

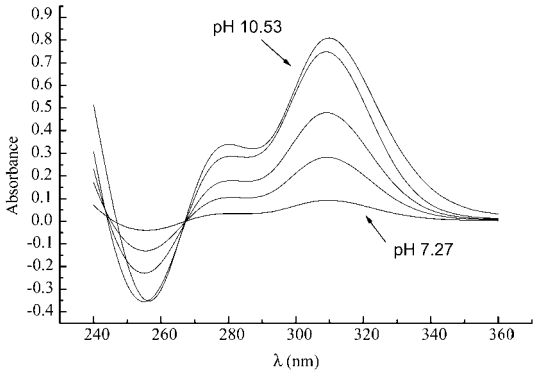


Figure 2. Differential absorption spectra of FR295 between pH = 7.27 and pH 10.53 with $c_0 = 4.5 \cdot 10^{-5}$ M and μ = 0.2 at 25±0.5 °C

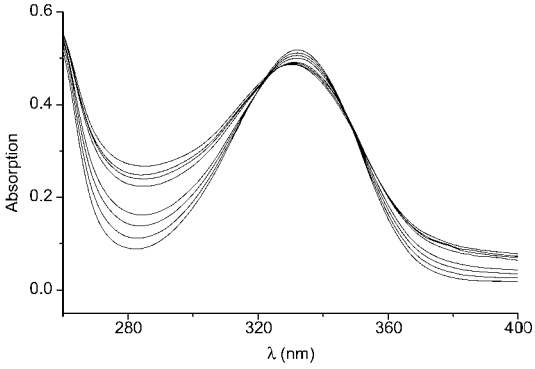


Figure 3. Absorption spectra of FR401 between pH = 10.56 and 13.15 with $c_0 = 5.0 \cdot 10^{-5}$ M and μ = 0.2 at 25 °C

When $FeCl_3$ is added to an acidic solution (pH ≈ 2) of FR295 or FR401, marked modifications in the absorption spectra of both ligands occur (Figure 4). This implies the formation of an iron(III) complex with FR295 and FR401.

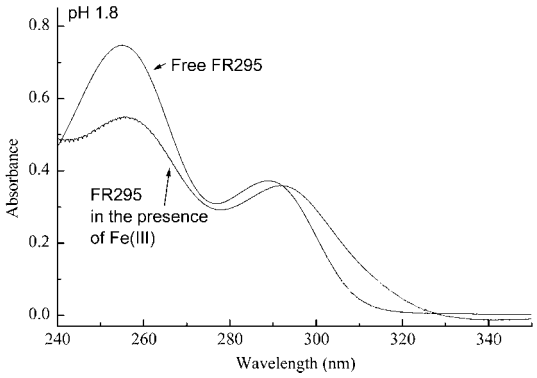


Figure 4. Absorption spectra of FR295 at pH = 1.80 in the absence and presence of 1 equiv. of $FeCl_3$ with $c_0 = c_1 = 4.5 \cdot 10^{-5}$ M and $\mu = 0.2$ at 25±0.5 °C

When the pH of solutions of FR295 and FR401 containing 1 equiv. of $FeCl_3$ are varied from acidic to basic ($2 < pH < 10$), modifications in the absorption spectra occur (Figure 5). Adding another equivalent of Fe^{III} does not have any effect on these absorption spectra and leads to the precipitation of iron hydroxide at $pH > 2.5$. All these experiments were performed under argon in order to avoid catechol oxidation and all the processes observed were reversible.

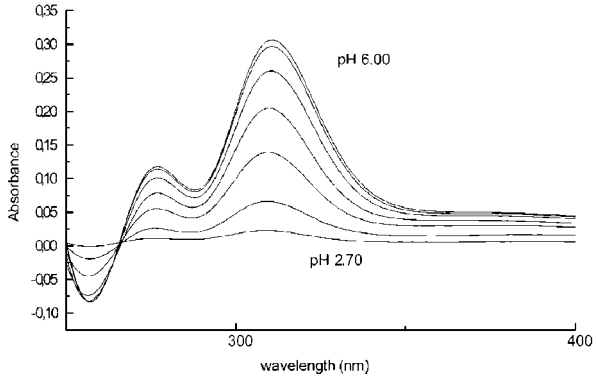


Figure 5. Differential absorption spectra of FR295 in the presence of 1 equiv. of $FeCl_3$ between pH = 2.70 and 6.00 with $c_0 = c_1$ and $\mu = 0.2$ at 25±0.5 °C

Free Ligand Proton Dissociations

The two dicatecholspermidine ligands FR401 and FR295 can exist as several prototropic species (Scheme 1).[22] When the pH of FR401 and FR295 solutions is increased, the variations in the absorption spectra of the ligands indicate the presence of one or more prototropic species in each of the 3−7, 7−9.5 and 9.5−13 pH ranges (Figures 1, 2, 3). To a first approximation, we assume that in each pH range, a ligand species can deprotonate according to Equation (1) where ion charges are not written.

$$LH_{(m-n)} + n\,H^+ \rightleftarrows LH_m \quad (1)$$

The Beer−Lambert law can be expressed in each of the pH ranges as in Equation (2) where A is the absorbance at a given wavelength, with $l = 1$ cm for all of the experiments, m is 5 in acidic media, n is the number of the protons involved in the acid-base reactions, $\varepsilon_{(m-n)}$ is the molecular extinction coefficient of the $LH_{(m-n)}$ species and ε_m is the molecular extinction coefficient of the of the LH_m species.

$$A = \{\varepsilon_{(m-n)}[LH_{(m-n)}] + \varepsilon_m[LH_m]\}l \quad (2)$$

Equation (2) can also be expressed as Equation (3)[23,24] where $\Delta A = A - A_0$, $\Delta\varepsilon = \varepsilon_{(m-n)} - \varepsilon_m$ and c_0 is the ligand concentration, with A_0 the absorbance due to the LH_m species and $\boldsymbol{K}_a = [LH_m][H^+]^n/[LH_{(m-n)}]$.

$$\Delta\varepsilon/\Delta A = 1/c_0 + [H^+]^n/(K_a c_0) \quad (3)$$

The best linear regression of the experimental $\Delta\varepsilon/\Delta A$ against $[H^+]^n$ is obtained for $n = 1$ in all of the pH ranges (Figures 6, 7, and 8 and Table 1).

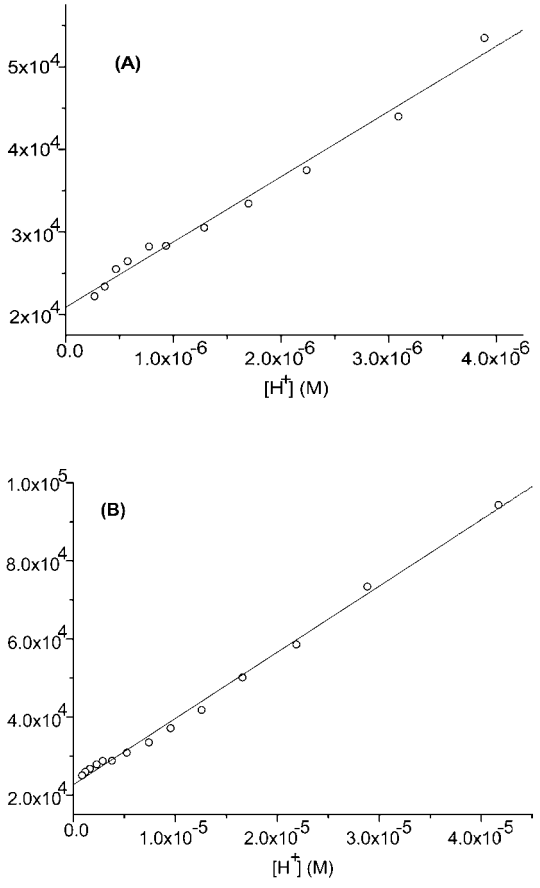


Figure 6. Plot of $\Delta\varepsilon/\Delta A$ against $[H^+]$ at pH ≤ 7: A) FR295, intercept, $(2\pm0.05)\cdot10^4$ M^{-1}; slope $(7.90\pm0.50)\cdot10^9$ M^{-2}; $r = 0.99357$; B) FR401, intercept, $(2.25\pm0.05)\cdot10^4$ M^{-1}; slope, $(1.70\pm0.05)\cdot10^9$ M^{-1}

In both ligands, there are five deprotonation sites; four proton losses can occur from the two dicatechol moieties of FR295 and FR401 and another proton loss from the central quaternary ammonium group. However, we only detected three proton dissociations, each of which seems to

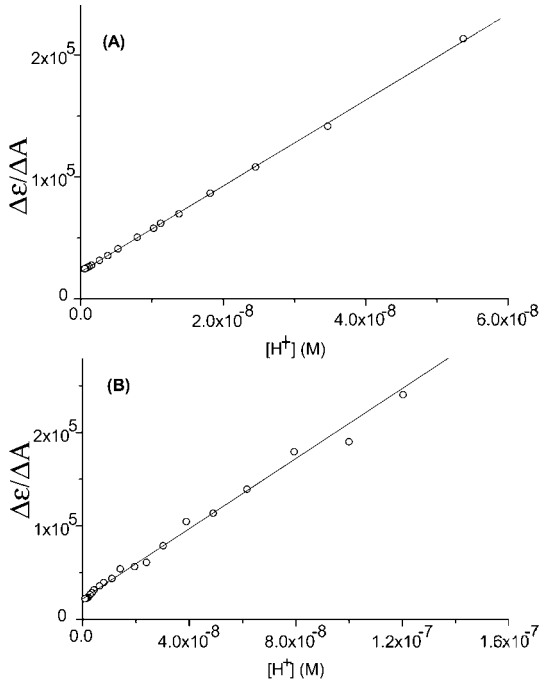


Figure 7. Plot of $\Delta\varepsilon/\Delta A$ against $[H^+]$ at 10. 50 ≥ pH ≥ 7: A) FR295, intercept, $(2.20\pm0.5)\cdot10^4$ M^{-1}; slope, $(3.50\pm0.05)\cdot10^{12}$ M^{-1}; r = 0.99983; B) FR401, intercept, $(2.20\pm0.05)\cdot10^4$ M^{-1}; slope, $(1.90\pm0.05)\cdot10^{12}$ M^{-1}

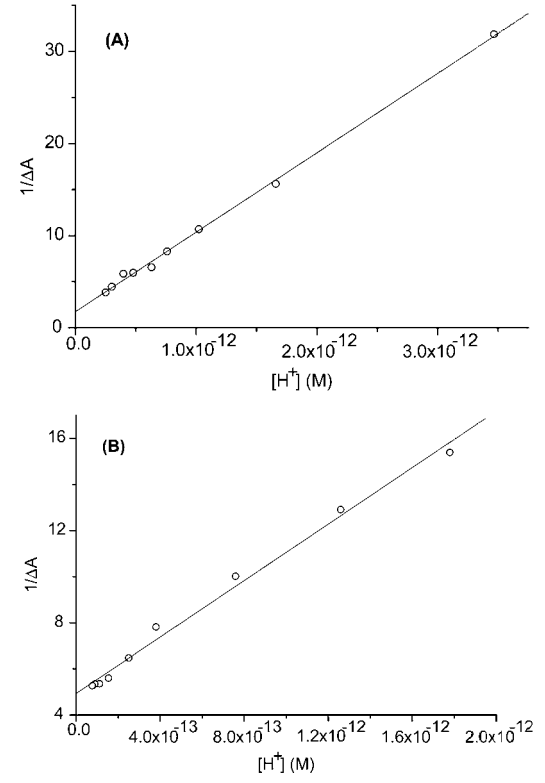


Figure 8. Plot of $1/\Delta A$ against $[H^+]$ at pH ≥ 10.50: A) FR295, intercept, 1.80±0.2; slope, $(8.60\pm0.15)\cdot10^{12}$ M^{-1}; r = 0.99912; B) FR401, intercept, 4.90±0.30; slope, $(6.20\pm0.30)\cdot10^{12}$ M^{-1}; r = 0.99569

Table 1. An approximation of the overall proton dissociation constants involved in the acid-base equilibria of FR295 and FR401

Ligand	3 < pH < 6 pK_{1a}	6 < pH <9 pK_{2a}	9 < pH < 13 pK_{3a}
FR295	5.50 ± 0.02	8.40 ± 0.02	12.60 ± 0.02
FR401	4.90 ± 0.02	7.95 ± 0.02	12.10 ± 0.02

involve a single proton transfer. Although the two dicatecholspermidine ligands are not symmetrical, in each of the ligands, the catechol moieties have the same environment and are practically identical. Therefore, these dicatechols should behave in the same way towards proton dissociation that can explain the presence of what seems to be isosbestic points in Figures 1 to 3 and 5. An isosbestic point may imply the presence of two species in the medium. Nonetheless, it cannot constitute as evidence for this presence.[25] Subsequently, we can assume that the three measured pK_a values of Table 1 can describe five proton dissociations. We can, therefore, write Equations (4) to (8) with L^{4-}, the completely deprotonated species, $K_{1a} = [LH_4][H^+]/[(LH_5)^+]$, $K_{2a} = [(LH_3)^-][H^+]/[LH_4]$, $K_{3a} = [(LH_2)^{2-}][H^+]/[(LH_3)^-]$, $K_{4a} = [(LH)^{3-}][H^+]/[(LH_2)^{2-}]$ and $K_{5a} = [L^{4-}][H^+]/[(LH)^{3-}]$.

$$LH_4 + H^+ \rightleftarrows (LH_5)^+ \quad (4)$$

$$(LH_3)^- + H^+ \rightleftarrows LH_4 \quad (5)$$

$$(LH_2)^{2-} + H^+ \rightleftarrows (LH_3)^- \quad (6)$$

$$(LH)^{3-} + H^+ \rightleftarrows (LH_2)^{2-} \quad (7)$$

$$L^{4-} + H^+ \rightleftarrows (LH)^{3-} \quad (8)$$

At pH < 9.5, three protons are involved in acid-base reactions with both ligands (Table 1) In this case the Beer−Lambert law can be expressed as in Equation (9).

$$A = \varepsilon_1[LH_5] + \varepsilon_2[LH_4] + \varepsilon_3[LH_3] + \varepsilon_4[LH_2] + \varepsilon_5[LH^{3-}] + \varepsilon_6[L^{4-}] \quad (9)$$

At 6 < pH < 9.5 $[LH_5^+]$, $[LH_4]$, $[LH_3^-]$ >> $[LH_2^{2-}]$, $[LH^{3-}]$, $[L^{4-}]$ and at pH > 9.5 $[LH_5^+]$, $[LH_4]$, $[LH_3^-]$ << $[LH_2^{2-}]$, $[LH^{3-}]$, $[L^{4-}]$. In this case, with K_{1a}, K_{2a}, K_{3a}, K_{4a}, and K_{5a} and the conservation of mass, we can write Equations (10) and (11) at 6 < pH <9.5 and pH > 9.5, respectively.

$$A = \frac{\{\varepsilon_1[H]^3 + \varepsilon_2[H]^2K_{1a} + \varepsilon_3[H]K_{1a}K_{2a} + \varepsilon_4K_{1a}K_{2a}K_{3a}\}c_0}{([H]^3 + [H]^2K_{1a} + [H]K_{1a}K_{2a} + K_{1a}K_{2a}K_{3a})} \quad (10)$$

$$A = \frac{c_0\{\varepsilon_4[H^+]^2 + \varepsilon_5[H^+]K_{4a} + \varepsilon_6 K_{4a}K_{5a}\}}{[H^+]^2 + [H^+]K_{4a} + K_{4a}K_{5a}} \quad (11)$$

Two non-linear least-squares regressions were obtained for each of the two ligands. The first two were for the data collected at pH < 9.5 [Equation (10)] and the other two for the data collected at pH > 9.5 [Equation (11)]. They gave very close $K_{2a} \approx K_{3a}$ and $K_{4a} \approx K_{5a}$ values. This implies that the values reported in Table 1 can be expressed as $pK_{2a} \approx (pK_{2a} + pK_{3a})/2$ and $pK_{3a} \approx (pK_{4a} + pK_{5a})/2$. All these pK_a values are in agreement with those reported for catechol proton dissociations. These occur in the vicinity of neutrality for one hydroxy group and at pH > 10 for the second.[22] As for the ammonium group of the spermidine, the values of the proton dissociation constant are highly dependent on the nature of the associated moieties and can vary from about 2 to more than 10.[26]

Complex Proton Dissociation

If a monocomplex is formed between Fe^{III} and FR295 or FR401, as Figure 4 seems to imply, it can also deprotonate at the catechol moieties and the central ammonium group. The variation of the absorption spectra of the two complexes seems to indicate the presence of at least three prototropic species in the pH = 3−6 area (Figure 5).

As in the case of the free ligands, we assume to a first approximation that in each pH range when at least one species deprotonates to yield the other, we can write Equation (12).

$$FeLH_{(m-n)} + nH^+ \leftrightarrows FeLH_m \quad (12)$$

As m is unknown, ion charges are not written. The Beer−Lambert law can be expressed in each of the pH ranges as Equation (13) [where A is absorbance at a given wavelength, with l = 1 cm for all our experiments; $\varepsilon'_{(m-n)}$ is the molecular extinction coefficient of the $FeLH_{(m-n)}$ species and ε''_m is the molecular extinction coefficient of the of $FeLH_m$] and Equation (14) {where $\Delta A = A - A_0$ and $\Delta\varepsilon' = \varepsilon'_{(m-n)} - \varepsilon''_m$, with A_0 the absorbance due to the LH_m species and $K_n = [FeLH_{(m-n)}][H^+]^n/[FeLH_m]$}.

$$A = \{\varepsilon'_{(m-n)}[LH_{(m-n)}] + \varepsilon''_m[LH_m]\}l \quad (13)$$

$$\Delta\varepsilon'/\Delta A = 1/c_0 + [H^+]^n/(K_n c_0) \quad (14)$$

Equation (14) is never obeyed by our experimental data. However, the plot of $1/\Delta A$ against $[H^+]$ can be roughly simulated by three lines, each of which obeys Equation (15) with $K'_{na} = [FeLH_{(m-1)}][H^+]/[FeLH_m]$, where ΔA_{n0} is the experimental differential absorption determined from the intercept of each of the three regression lines.

$$1/\Delta A = 1/\Delta A n_0 + [H^+]/\Delta A_0 K'_{na} \quad (15)$$

We, therefore, assumed that the Fe^{III} complex undergoes at least three proton losses when the pH range is ca. 2 to ca. 8 [Equations (16) to (18)].

$$FeLH_{(m-3)} + H^+ \leftrightarrows FeLH_{(m-2)} \quad (16)$$

$$FeLH_{(m-2)} + H^+ \leftrightarrows FeLH_{(m-1)} \quad (17)$$

$$FeLH_{(m-1)} + H^+ \leftrightarrows FeLH_m \quad (18)$$

In this case the Beer−Lambert relation can be expressed as Equation (19) in which, ε'_3, ε'_2, ε'_1, and ε' are the molecular extinction coefficients of species $FeLH_{(m-3)}$, $FeLH_{(m-2)}$, $FeLH_{(m-1)}$, and $FeLH_m$, respectively.

$$A = \varepsilon'_3[FeLH_{(m-3)}] + \varepsilon'_2[FeLH_{(m-2)}] + \varepsilon'_1[FeLH_{(m-1)}] + \varepsilon'[FeLH_m] \quad (19)$$

Equation (19) can be expressed as a function of c_0, $K'_{1a} = [FeLH_{(m-1)}][H^+]/[FeLH_m]$, $K'_{2a} = [FeLH_{(m-2)}][H^+]/[FeLH_{(m-1)}]$ and $K'_{3a} = [FeLH_{(m-3)}][H^+]/[FeLH_{(m-2)}]$ as in Equation (20).

$$\Delta A = \frac{(\varepsilon'_3 - \varepsilon')K_{1a}K'_{2a}K'_{3a} + (\varepsilon'_2 - \varepsilon')K'_{1a}K'_{2a}[H^+] + (\varepsilon'_1 - \varepsilon')K'_{1a}[H^+]^2}{K'_{1a}K'_{2a}K'_{3a} + K'_{1a}K'_{2a}[H^+] + K'_{1a}[H^+]^2} \times c_0 \quad (20)$$

A non-linear least-squares regression of the data against Equation (20), where $(\varepsilon'_3 - \varepsilon')$ is determined experimentally and the equilibrium constants are roughly estimated from the three lines related to Equation (15) in the three different pH ranges, gave the K'_{1a}, K'_{2a}, and K'_{3a} values with about 10 to 15% of uncertainty (Figure 9, Table 2).

Therefore, the proton dissociation constants of the FR295 and FR401 ligands are shifted from basic to acidic when the ligands are complexed by Fe^{III}. As for the free ligand, we will assume that the apparent pK_a values reported in Table 2 concern the deprotonation of the ligands engaged in the complex. Complex formation can be accompanied by the deprotonation of the ligands involved which stabilises the complex.[27−29] Catechols are very good chelators for Fe^{III} whereas amines are not.[27,29] Since the affinity of a ligand for a metal increases upon proton loss,[28] we assume that pK'_{1a} and pK'_{3a} are related to catechol deprotonation. Consequently, each of pK'_{1a} or pK'_{3a} involves two proton dissociations, whereas pK'_{2a} involves the amine single proton dissociation.

Kinetics of Complex Formation

When acidic solutions of FR295 or FR401 are mixed rapidly with a solution of $FeCl_3$, a single kinetic process is observed. It appears as an exponential increase of absorption at λ = 290 nm for each of the ligands (Figures 10 and 11). The rate of this process depends on the $FeCl_3$ concentration and the pH value for 1.5 ≤ pH ≤ 2.2. At pH > 2.2 iron hydroxides start appearing.[30]

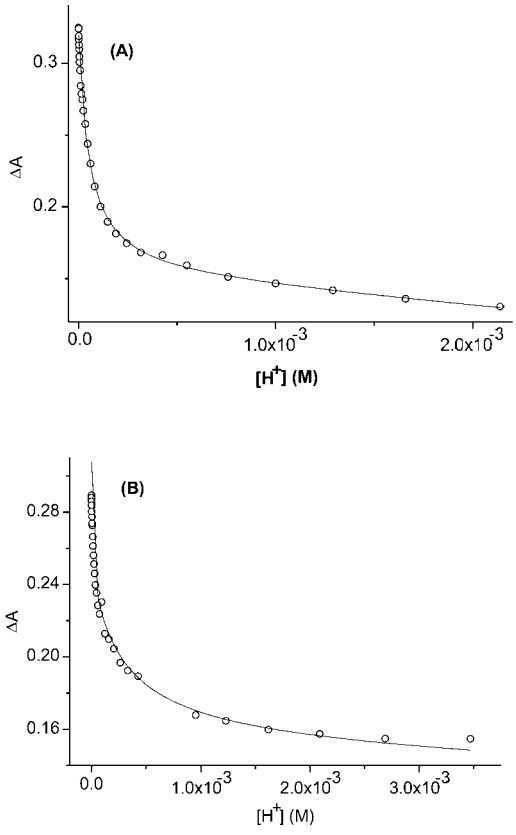


Figure 9. Plot of ΔA against $[H^+]$ at 25±0.5 °C for $c_1 = 4.5\cdot10^{-5}$ M and $\mu = 0.2$, and the non-linear least-squares regression fit according to Equation (20): A) FR295 with $K'_{1a} = (9.0\pm1.0)\cdot10^{-7}$ M, $K'_{2a} = (5.0\pm0.3)\cdot10^{-5}$ M and $K'_{3a} = (1.60\pm0.15)\cdot10^{-2}$ M; B) FR401 with $K'_{1a} = (7\pm1)\cdot10^{-6}$ M, $K'_{2a} = (2.30\pm0.30)\cdot10^{-4}$ M and $K'_{3a} = (3.5\pm0.3)\cdot10^{-2}$ M

Table 2. An approximation of the overall proton dissociation constants involved in the acid-base equilibria of the Fe^{III} monocomplexes of FR295 and FR401

Ligands	pK'_{2a} ammonium	pK'_{1a} catechol	pK'_{3a} catechol
FR295	4.25 ± 0.05	1.80 ± 0.05	6.05 ± 0.05
FR401	3.65 ± 0.05	1.50 ± 0.05	5.15 ± 0.05

In FR295 and FR401, the pK_a values of the catechol and central ammonium groups are both higher than 3 (Table 1). Subsequently, under our experimental conditions (pH ≤ 2), both chelators are in the protonated form $(LH_5)^+$ (Scheme 1). In this case, the chemical relaxation approximation will only be relevant for $c_1 \geq 2c_0$,[21] where c_1 is the analytical $FeCl_3$ concentration. The kinetic processes of Figures 10 and 11 always appear as a single exponential phenomenon that may imply the formation of a single complex. All this suggests that, because the two kinetic phenomena are pH-dependent, and because there is an excess of iron along with the detection of a single relaxation mode,[19–21,23,24,27] $(LH_5)^+$ may react with one Fe^{III} with

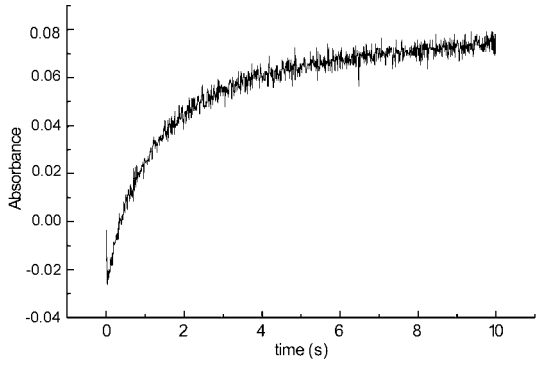


Figure 10. Exponential increase of the absorption with time at λ = 290 nm when an FR295 solution is rapidly mixed with a $FeCl_3$ solution with $c_0 = 4\cdot10^{-4}$ M, $c_1 = 5\cdot10^{-3}$ M and $\mu = 0.2$ at pH = 1.90 and 25±0.5 °C

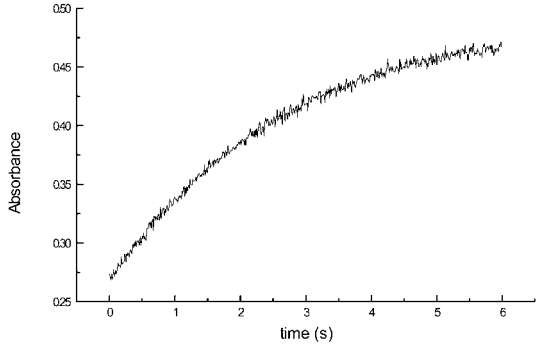


Figure 11. Exponential increase of the absorption with time at λ = 290 nm when a FR401 solution is rapidly mixed with a $FeCl_3$ solution with $c_0 = 4\cdot10^{-4}$ M, $c_1 = 3.6\cdot10^{-3}$ M and $\mu = 0.2$ at pH = 1.47 and 25±0.5 °C

the involvement of a proton transfer process. This can occur according to three possible mechanisms in which $FeLH_3^{2+}$ is the iron complex with each of the catechol moieties deprotonated on one hydroxy group and LH_3^- is the ligand species, also deprotonated on one hydroxy group.[27]

Mechanism I

$$Fe^{3+} + LH_5^+ \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} FeLH_5^{4+} \text{ (rate-limiting)} \quad (21)$$

$$Fe\,LH_{(5-n)}^{(n+2)+} + nH^+ \leftrightarrows FeLH_5^{4+} \quad (22)$$

Mechanism II

$$LH_{(5-n)}^{(n-1)-} + nH^+ \leftrightarrows LH_5^+ \quad (23)$$

$$Fe^{3+} + LH_{(5-n)}^{(n-1)-} \underset{k_{-1}}{\overset{k_2}{\rightleftarrows}} FeLH_{(5-n)}^{(n+2)+} \text{ (rate-limiting)} \quad (24)$$

Mechanism III

$$Fe^{3+} + LH_5^+ \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} FeLH_{(5-n)}^{(n+2)+} + nH^+ \quad (25)$$

A proton dissociation is usually diffusion-controlled and occurs in the μs range.[19,20,23,24] Therefore the phenomena of Figures 10 and 11 must involve rate-limiting complex formation. The reciprocal relaxation time equations associated with rate-limiting Equation (21) and Equation (24) and with Equation (25) are expressed as Equations (26) to (28) where $\boldsymbol{K}^2_{2a} = [LH_{(5-n)}{}^{(n-1)-}][H^+]^n/[LH_5{}^+]$ and $\boldsymbol{K}'^2_{1a} = [Fe LH_{(5-n)}{}^{(n+2)+}][H^+]^n/[FeLH_5{}^{4+}]$.[19,20,27]

$$\tau_1^{-1} = k_{-1}[H^+]^n/([H^+]^n + \boldsymbol{K}'_{1a}) + k_1([LH_5{}^+] + [Fe^{3+}]) \quad (26)$$

$$\tau_2^{-1} = k_{-2} + k_2([LH_5{}^+] + [Fe^{3+}])\boldsymbol{K}^2_{2a}/[H^+]^2 \quad (27)$$

$$\tau_3^{-1} = k_3([Fe^{3+}] + [LH_5{}^+]) + k_{-3}[H^+]^n \quad (28)$$

Under our experimental conditions where $c_1 \geq 2c_0$, and where $\boldsymbol{K}'_{1a}$, $[H^+] >> c_1$, c_0 and $\boldsymbol{K}_{2a}$, Equations (26), (27), and (28) can be simplified into Equations (29), (30), and (31), respectively.

$$\tau_1^{-1}(1 + \boldsymbol{K}'^2_{1a}/[H^+]^n) \approx k_{-1} + k_1(c_1 - c_0)(1 + \boldsymbol{K}'^2_{1a}/[H^+]^n) \quad (29)$$

$$\tau_2^{-1} \approx k_{-2} + k_2(c_1 - c_0)\boldsymbol{K}_{2a}{}^n/[H^+]^n \quad (30)$$

$$\tau_3^{-1} \approx k_3(c_1 - c_0) + k_{-3}[H^+]^n \quad (31)$$

Equations (30) and (31) are not obeyed by the experimental data. This eliminates Mechanisms II and III. Two good linear least-squares regressions of the experimental relaxation times against Equation (29) are obtained for FR295 and FR401 when $n = 2$ (Figures 12 and 13). From the slope and intercept of these two regression lines k_1, k_{-1} and $\boldsymbol{K}_1 = k_{-1}/k_1 = [Fe^{3+}][LH_5{}^+]/[FeLH_5{}^{4+}]$ are determined (Table 3).These rate constants are of the same order of magnitude as those reported for complex formation with Fe^{3+}.[18,27,32,33]

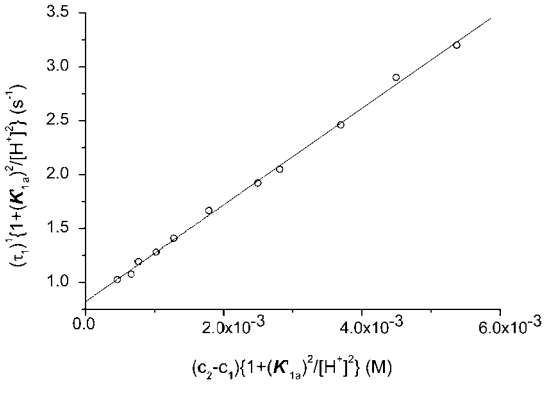


Figure 12. Plot of $\tau^{-1}(1 + \boldsymbol{K}'^2_{1a}/[H^+]^2)$ against $(c_1 - c_0)(1 + \boldsymbol{K}'^2_{1a}/[H^+]^2)$ in acidic media ($1.47 \leq pH \leq 2.20$): intercept, 0.82 ± 0.04 s^{-1}; slope, 450 ± 20 M^{-1} s^{-1}; $r = 0.99905$

If the fast formation of another complex following that of $FeLH_3{}^{2+}$ is involved in Mechanism I, to a first approximation, we can write at a fixed pH value reactions in Equations (32) and (33).

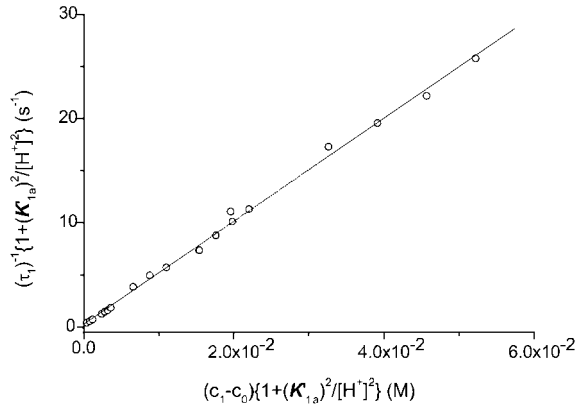


Figure 13. Plot of $\tau^{-1}(1 + \boldsymbol{K}'^2_{1a}/[H^+]^2)$ against $(c_1 - c_0)(1 + \boldsymbol{K}'^2_{1a}/[H^+]^2)$ in acidic media ($1.50 \leq pH \leq 2.20$) for FR401: intercept, 0.28 ± 0.03 s^{-1}; slope, 500 ± 20 $M^{-1} \cdot s^{-1}$; $r = 0.99849$

Table 3. The rate and equilibrium constants involved in complex formation between FR295 and FR401 with Fe ^{III} in acidic media

Ligand	k_1 [$M^{-1}s^{-1}$]	k_{-1} [s^{-1}]	$K_1 \times 10^4$ [M]	$K_1^{-1} \times 10^{-3}$ [M^{-1}]
FR295	450 ± 20	0.82 ± 0.04	18.2	0.55
FR401	500 ± 20	0.28 ± 0.03	5.6	1.80

$$Fe^{3+} + \mathcal{L} \underset{k_{-(obs)}}{\overset{k_{(obs)}}{\rightleftharpoons}} Fe\mathcal{L} \text{ (rate-limiting)} \quad (32)$$

$$Fe\mathcal{L} + L \leftrightarrows Fe\mathcal{L}_2 \quad (33)$$

In these reactions, the state of protonation of the ligand L and the complexes $Fe\mathcal{L}$ and $Fe\mathcal{L}_2$ are not known. Under our experimental conditions ($c_1 >> c_0$) with a fixed pH, the expression of the reciprocal relaxation time associated with rate limiting Equation (32) can be estimated by Equation (34) where the apparent equilibrium constant $K = [L][Fe\mathcal{L}]/[Fe\mathcal{L}_2]$.[20]

$$\tau_1^{-1} = k_{-obs}(K/[L]) + k_{obs}(c_1 - c_0) \quad (34)$$

This last equation is not obeyed by our experimental data. Furthermore, in acidic media, at $pH \approx 2$, Fe^{III} starts being present in the $FeOH^{2+}$ form [Equation (35)][31] with an equilibrium constant $K_{(FeOH)} = [H^+][FeOH^{2+}]/[Fe^{3+}] \approx 2 \cdot 10^{-3}$ M.[31]

$$FeOH^{2+} + H^+ \leftrightarrows Fe^{3+} + H_2O \quad (35)$$

If complex formation occurs between $FeOH^{2+}$, and FR401or FR295 the reciprocal relaxation time equation associated with Equation (36) will be expressed as Equation (37).[24]

$$LH_5^+ + FeOH^{2+} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} FeOHLH_5^{3+} \quad (36)$$

$$\tau_3^{-1} = k_{-4} + k_4\{c_1 K_{FeOH}/(K_{FeOH} + [H^+]) - c_0\} \quad (37)$$

Equation (37) is not obeyed by our experimental data. In contrast, if we take into account the presence of $FeOH^{2+}$ in the experimental medium, the reciprocal relaxation equation associated with the reaction in Equation (21) of Mechanism I will be expressed as Equation (38).

$$\tau^{-1} = k_1\{c_1[H^+]/([H^+] + K_{FeOH}) - c_0\} + k_{-1}[H^+]^n/([H^+]^n + K'_{1a}) \quad (38)$$

Under our experimental conditions ($1.4 < pH < 2$, $[FeOH^{2+}]$) Equation (38) simplifies into Equation (29). In this case, the presence of $FeOH^{2+}$ will not affect our kinetic analysis.

With dicatechols in non-hexadentate chelators such as amonabactins, more than one chelator can be engaged in complex formation with Fe^{III}.[22] Under our experimental conditions, we only detected a single 1:1 complex. This, however, does not exclude the formation of complexes with different stoichiometries (Fe_2L_3) under other experimental conditions where for instance $c_0 \leq c_1$. Moreover, as in the case of enterobactin, in FR401, the protonation of the oxygen ligand at position 2 may lead to a monosalicylate-type coordination with Fe^{III}.[34] Nevertheless, this coordination cannot occur with FR295 because it does not have a hydroxy group at position 2. Furthermore, the fact that the behaviours of both the FR401 and FR295 ligands towards complex formation are identical tends to exclude the salicylate-type coordination with FR401. In acidic media, for both FR295 and FR401, a complex is formed between Fe^{3+} and $L(H_5)^+$. This complex loses five protons from pH = 2 to 9. If we assume that, as in the case of the free ligands, the proton dissociation constants reported in Table 3 refer to two proton losses from the equivalent hydroxy groups in each of the two catechol moieties and from the central ammonium group, as shown for the free ligands, then $p\boldsymbol{K}'_{1a} = (pK_{2a} + pK'_{3a})/2$, $p\boldsymbol{K}'_{3a} = (pK'_{4a} + pK'_{5a})/2$ and $p\boldsymbol{K}'_{2a} = pK'_{1a}$ with $K'_{1a} = [(FeLH_4)^{3+}][H^+]/[(FeLH_5)^{4+}]$, $K'_{2a} = [(FeLH_3)^{2+}][H^+]/[(FeLH_4)^{3+}]$, $K'_{3a} = [(FeLH_2)^+][H^+]/[(FeLH_3)^{2+}]$, $K'_{4a} = [FeLH][H^+][(FeLH_2)^+]$ and $K'_{5a} = [(FeL)^-][H^+][FeLH]$.

$$(FeLH_4)^{3+} + H^+ \leftrightarrows (FeLH_5)^{4+} \quad (39)$$

$$(FeLH_3)^{2+} + H^+ \leftrightarrows (FeLH_4)^{3+} \quad (40)$$

$$(FeLH_2)^+ + H^+ \leftrightarrows (FeLH_3)^{2+} \quad (41)$$

$$FeLH + H^+ \leftrightarrows (FeLH_2)^+ \quad (42)$$

$$(FeL)^- + H^+ \leftrightarrows FeLH \quad (43)$$

The affinities of catechols for iron are extremely high.[16,18,22,28,29,31] We may, therefore, assume that with the analytical Fe^{III} concentration $c_1 \approx c_0$, the complexes of FR295 and FR401 are the predominant species from mildly acidic to basic media. This is always required with Fe^{III} complexes in order to avoid ferric hydroxide precipitation.

The Affinities of FR295 and FR401 for Fe^{III}

The affinity of a ligand for a metal increases upon proton loss.[28] This is manifested by the so-called chelation effect in which the apparent pK_a of the ligand engaged in the coordination with the metal decreases upon complex formation.[29]. The greater the pK_a difference (ΔpK_a) is between the free ligand proton dissociation and that of the same ligand engaged in the complex media, the higher is the affinity for the metal.[14,23,27] In Mechanism I, upon complex formation [Equation (20)], $FeLH_5^{4+}$ readily deprotonates by a diffusion-controlled acid-base dissociation to produce $FeLH_3^{2+}$ [Equation (21)]. The proton loss increases the affinities of both FR401 and FR295 for the metal by more than 12 orders of magnitude ($[Fe^{3+}][LH_3^-]/[FeLH_3^{2+}] = K_1K'_{1a}K'_{2a}/K_{1a}K_{2a}$). $FeLH_5^{4+}$ is, therefore, practically inexistent in the experimental medium. $FeLH_3^{2+}$ in turn deprotonates to finally yield FeL^- with another increase in the affinity of several orders of magnitude (Table 4). This is deduced from Equation (1) and the proton dissociation constants of the free ligand and of the complex [Equations (44) and (45)]. This yields $\log\beta$ ($-\log K$) values of Equations (29) and (31) for FR295 and FR401, respectively.

$$Fe^{3+} + L^{4-} \leftrightarrows FeL^- \quad (44)$$

$$1/\beta = K = [L^{4-}][Fe^{3+}]/[FeL^-] = K_1(K'_{1a}K'_{2a}K'_{3a}K'_{4a}K'_{5a})/(K_{1a}K_{2a}K_{3a}K_{4a}K_{5a}) \quad (45)$$

Table 4. The affinity constants and the pFe involved in complex formation between Fe^{III} and FR295 and FR401

Ligand	log β	pFe
FR295	29	22
FR401	31	25

These values are high and place both ligands in the classical range of affinities expected for dicatechols. Indeed, with monocatechols, the affinities for iron are about 37 ($\log\beta_3$) and for a tricatechol they are about 40.[16,18,22] The pH increase stabilises the complex by the consequent deprotonation of the hydroxy groups and allows it to achieve the $\log\beta$ value of 30 with the completely deprotonated ligands. An amine is not a particularly good ligand for Fe^{III}.[30] However, the pK_a we ascribed to the deprotonation of the ammonium group in the free ligand is shifted by more than 1 pK_a unit in the complex. This implies that the central

amine is also engaged in complex formation. This engagement is not, however, as strong as that observed for the catechol moieties where the ΔpK_a value exceeds 6 pK_a units implying, as expected, a very strong coordination to the metal centre.[28,29] Fe^{III} can then be considered to be coordinated to two catechol moieties and one amine group with the sixth coordination site probably occupied by water. Although we succeeded in synthesising the Fe^{III} and Ga^{III} complexes of FR401 and FR295 (Exp. Sect.), we do not yet have direct evidence to confirm such a structure. All that we can say is, that under our experimental conditions, with $c_1 \geq c_0$ a monoferric complex is formed between Fe^{III} and one FR295 or FR401 ligand and that in both cases five donor atoms seem to be engaged in the formation of this complex.

The affinities of FR295 and FR401 for iron are reported for complex formation with the fully ionised ligand L^{4-} [Equation (46)]. The FeL^- form is not the only one present in the vicinity of neutrality. Therefore, in order to have a scale of efficiency for iron chelators, Raymond et al. defined the pFe ($-\log[Fe^{3+}]$) scale. The pFe is determined for an analytical ligand concentration of 10^{-4} M and an analytical Fe^{III} concentration of 10^{-6} M at pH = 7.4, which is equivalent to that of physiological media.[15] From the different proton dissociation constants of Tables 2 and 3 and from mass conservation [Equations (46) and (47)] we can determine the analytical expression of $[Fe^{3+}]$ [Equation (48)] with $c_0 << [L] \approx c_1$.

$$c_1 = [FeL^-] + [FeLH_2^+] + [FeLH_4^+] + [Fe] \quad (46)$$

$$c_0 = [L] + [FeL^-] + [FeLH_2^+] + [FeLH_4^+] \quad (47)$$

$$[Fe^{3+}] = \frac{K\{[H^+]^5 + (K_{2a})^2[H^+]^3 + (K_{2a}K_{3a})^2[H^+] + (K_{2a}K_{3a})^2K_{1a}\}c_1}{\{[H^+]^5 + (K'_{1a})^2[H^+]^3 + (K'_{1a}K'_{3a})^2[H^+] + (K'_{1a}K'_{3a})^2K_{2a}\}c_0} \quad (48)$$

Table 4 represents the affinities of both ligands for iron and the relevant pFe. These values indicate that FR295 and FR401 can be considered as very good ligands for Fe^{III}.[15–18,22,34] This can explain their siderophore-like role with mycobacteria and their capacity to inhibit the growth of *Plasmodium falciparum* in vitro, where they probably act as iron sequestering agents depriving the medium of this element essential for the development of the microorganism.

Experimental Section

KCl (Merck Suprapur), NaOH, and HCl (Merck Titrisol), EDTA (Merck Titriplex), $FeCl_3$, sodium hydrogen carbonate (Normapur), EtOH, acetic acid (100%), DMSO, sodium acetate, sodium formate and sodium citrate (Merck), gallium nitrate (ACROS), and Hepes (Aldrich) were used without further purification. Water and glassware were prepared as described previously.[14] FR295 and FR401 were synthesized and purified according to published procedures and were kept under vacuum at −20 °C.[8,10,13]

Stock Solutions: All stock solutions were used fresh. Because of the chelator's slight solubility in aqueous media ($< 10^{-3}$ M), FR295 and FR401 were first dissolved in ethanol at 10^{-2} M. These solutions were later diluted to the 10^{-4} to 10^{-5} M range in the final buffers. The solutions at pH < 2.5 were not buffered; those at 2.5 < pH < 3.5 were buffered with formate, those at 3.5 < pH < 6 with acetate, those at 6 < pH < 8.5 with Hepes and those at pH > 8.5 with carbonate. Buffer salt concentration was $5 \cdot 10^{-2}$ M and the final pH was attained by microinjections of concentrated HCl, NaOH, or AcOH. $FeCl_3$ solutions were prepared in acidic media at pH < 2. The Fe^{III} complexes of FR401 and FR295 were prepared in acidic media by adding the required amount of $FeCl_3$ to the ligands. The pH was then gently raised to final values by microinjections of concentrated NaOH. The final ionic strength of all solutions was adjusted to 0.2 with KCl. For pH > 5, all solutions were prepared under argon in a glove bag.

pH Measurements: The pH values were measured at 25±0.5 °C with a Jenco pH-meter equipped with an "Ingold" combined microelectrode calomel/glass. Buffers used for pH standardization were of pH = 4.00, 7.00, and 10.01 (Sigma).

Spectrophotometric Measurements: Spectrophotometric measurements were performed in anaerobic conditions under argon at 25±0.5 °C with a Cary C210 or a Perkin–Elmer Lambda 2 spectrophotometer equipped with a magnetic stirring device and thermostatted cell carrier. For each set of experiments, the spectra were recorded under different pH values. The reversibility of the observed processes with $[H^+]$ was checked at the end of each series of measurements by lowering the final pH to the initial values and by comparing the final to the initial absorption spectra. Values were discarded throughout the series where there was a difference of > 3% between the spectra of identical pH values. This was done in order to avoid the risk of catechol oxidation mainly in basic media.

Stopped-Flow Measurements: Kinetic measurements were performed by mixing acidic solutions of FR401 or FR295 with solutions of $FeCl_3$ under argon in an SF 3 L Hi-Tech stopped-flow spectrophotometer equipped with a thermostatted bath (25±0.5) °C.

Mathematical Formalism and Signal Analysis: The experimental conditions were set so as to permit the use of chemical relaxation formalism.[21] All experimental signals were analysed as described elsewhere.[14] They were all pure exponentials and were dealt with as relaxation modes.[14,19–21]

Solubility of the Ligands and Synthesis of the Fe^{III} and Ga^{III} Complexes with the FR295 and FR401 Ligands: Saturated ethanol solutions of FR401 and FR295 were added to solutions of $FeCl_3$ in 50% water and ethanol. The colour of the mixture immediately turned dark and a precipitate appeared. The pH was then gently raised to neutrality, and the precipitate filtered, washed, and dried under vacuum. It was slightly soluble in DMSO, ethanol, and methanol. Electrospray mass spectrometry experiments performed on the methanolic solutions of the precipitate showed only the presence of FR401 and FR295 ligands and iron. The same experiments were repeated by mixing the same alcoholic solutions of FR295 and FR401 with solutions of gallium nitrate. The solubility of the dark precipitate was too low to allow any NMR spectroscopic measurements. Both FR295 and FR401 are scarcely soluble in purely aqueous media. Under our experimental conditions the highest concentration attained was $< 10^{-3}$ M. This concentration did not allow the use of other measurement techniques such as potentiometric p***K*** determination.

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