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Synthesis and iron-binding properties of quinolobactin, a siderophore from a pyoverdine-deficient *Pseudomonas fluorescens*

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Abstract

Quinolobactin is a new siderophore produced by a pyoverdine deficient mutant of *Pseudomonas fluorescens*. A simple and efficient synthesis of quinolobactin is described, starting from xanthurenic acid. The protonation constants of quinolobactin were determined by potentiometric titrations as $pK_{a2} = 5.50 \pm 0.07$, $pK_{a1} = 10.30 \pm 0.05$. The equilibria of the metal complexes were studied by means of spectrophotometric and potentiometric titrations. The overall stability constants of the quinolobactin-Fe^{III} complexes was found to be $\log \beta_{111} = 18.60 \pm 0.10$, $\log \beta_{121} = 32.60 \pm 0.20$, $\log \beta_{120} = 28.20 \pm 0.25$ resulting in a pFe^{III} value of 18.2 at pH 7.4. The UV-visible spectral parameters of the $[FeL_2]$ are in agreement with a complex containing two ligands coordinated to one Fe^{3+} cation through the oxygen and nitrogen quinoline atoms.

Introduction

Siderophores are iron-chelating agents that are excreted by micro-organisms to render iron soluble in the environment and favour the uptake of this metal (Neilands 1995). Almost of the natural siderophores are hexadentate ligands and contain a hydroxamic acid, catechol and/or α-hydroxycarboxylic acid metal-binding group (Albrecht & Crumbliss 1998). Synthetic iron chelators based on 8-hydroxyquinoline chelating subunits have been described and have revealed promizing properties in iron chelating therapy as well as in iron nutrition (Pierre *et al.* 2003). Siderophores of the pyoverdin family, produced by fluorescent *Pseudomonas* are characterized by a chromophore derived from 2,3-amino-6, 7-dihydroxyquinoline (Abdallah 1991). These siderophore are not, truely speaking, hydroxyquinoline chelators, the chromophoric chelating unit acting in a catecholate coordination mode (Albrecht & Crumbliss 1998). Recently, a true 8-hydroxyquinoline siderophore has been identified from transposon mutant strain 3G6 of *Pseudomonas fluorescens* ATCC 17400 which was deficient in pyoverdine production (Mossialos *et al.* 2000). This iron-chelating molecule was identified as 8-hydroxy-4-methoxy-quinaldic acid and designated quinolobactin (Figure 1). It has been shawn that quinolobactin-mediated iron uptake system is repressed by the cognate pyoverdine. The structure of the iron complex and the thermodynamics of complexation have not been described so far. In this paper, we describe a synthesis and the iron-binding properties of quinolobactin.

Materials and methods

Synthesis

NMR spectral data (^1H and ^{13}C) were recorded on a Brucker Avance 300 spectrometer at 300 MHz for ^1H and 75 MHz for ^{13}C. 1D (^1H, ^{13}C, DEPT.120, QUATC) and 2D (gHMQC, gHMBC) spectra were acquired and processed using the standard sofware implented in the spectrometer. Chemical shifts δ are given in ppm. Mass spectra were recorded on a Thermo Quest Finnigan Polaris Q mass spectrometer. Melting points are uncorrected. The solvents were purified and dried by standard methods. Xanthurenic acid

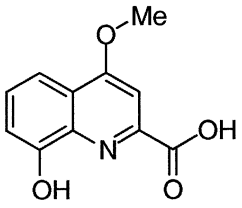


Figure 1. Structure of Quinolobactin **3**

was purchased from Acros Organics. Other reagents were from Lancaster.

8-hydroxy-4-methoxyquinoline-2-carboxylic acid methyl ester **2c** $C_{12}H_{11}NO_4$

A suspension of xanthurenic acid **1** (2.05 g, 10 mmol) in thionyl chloride (10 ml) was refluxed during 14 h. The homogeneous dark green solution was then concentrated under reduced pressure until a dark red solid was obtained. This residue was very cautiously treated with dry methanol (10 ml). The mixture was refluxed for 10 mn, cooled at ambient temperature and then concentrated under *vacuo*. The solid then obtained was suspended in dry merthanol (50 ml) and powdered sodium methylate (2.5 g, 46 mmol) was added with care (exothermic reaction). The resulting orange-red suspension was heated to reflux temperature for 2 h. After cooling to room temperature, the residue was poured in iced water (200 ml) and acidified with concentrated HCl to pH 3. The resulting suspension was repeatedly extracted with ethyl acetate/CH_2Cl_2 (3/1, v/v). The organic layer was washed with brine, dried over Na_2SO_4 and evaporated under *vacuo* to give the quinolobactine methyl ester **2c** (65%) as an orange solid.

^{1}H NMR ($CDCl_3$), δ: 4.05 (s, 3H, O-CH_3); 4.12 (s, 3H, O-CH_3); 7.21 (dd, 1H, aromatic H, $^3j_{HH}$ = 7.5 Hz, $^4j_{HH}$ = 1.3 Hz); 7.49 (*pseudo* t, 1H, aromatic H); 7.64 (s, 1H$_{ar}$); 7.65 (dd, 1H, aromatic H, $^3j_{HH}$ = 8.4 Hz, $^4j_{HH}$ = 1.3 Hz).

MS (DCI, NH_3/*iso*butane, 150 °C): 234 $[M+H]^+$ 100%.

8-hydroxy-4-methoxyquinoline-2-carboxylic acid (quinolobactin ***3****)* $C_{11}H_9NO_4$

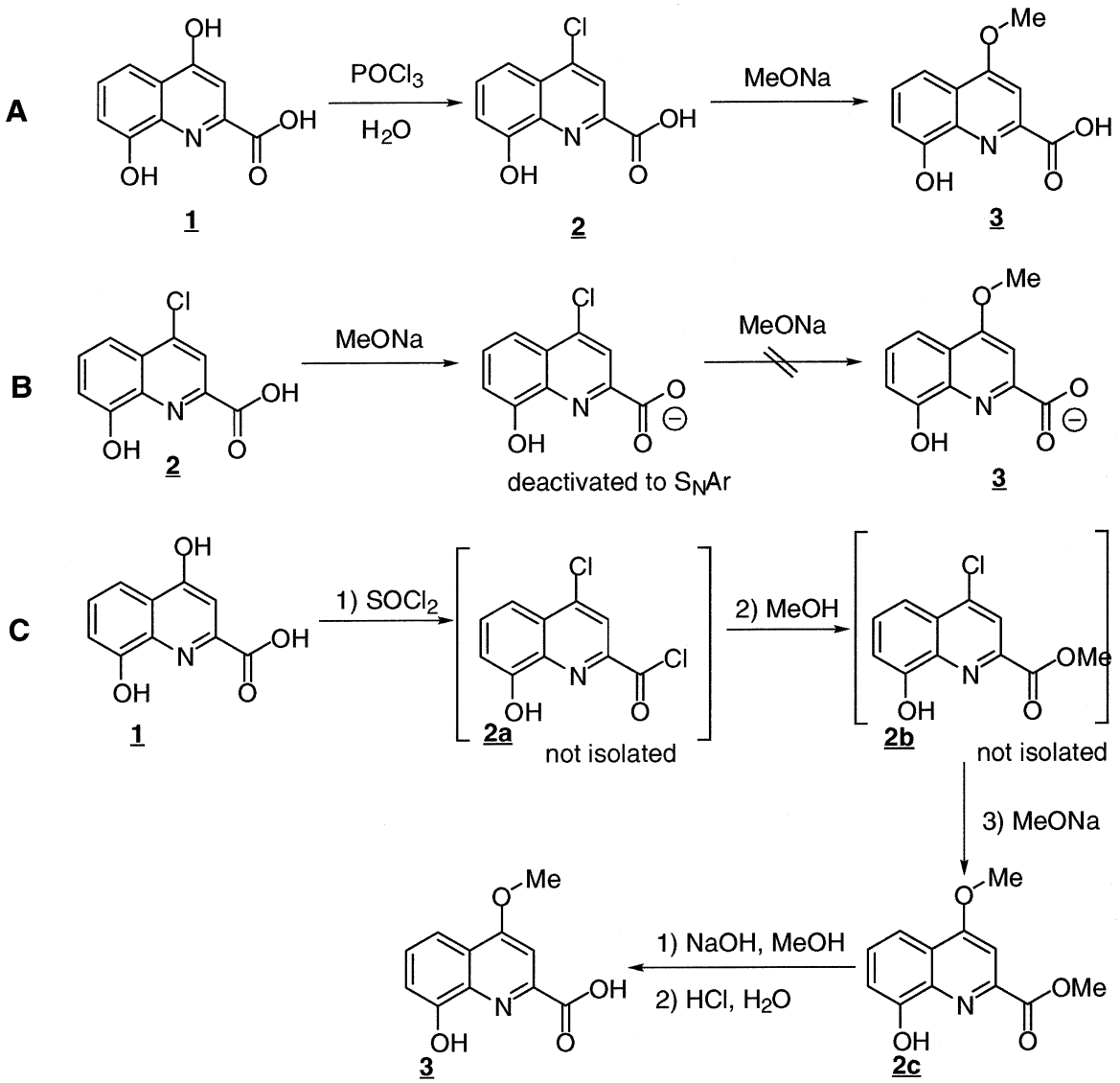
A suspension of 8-hydroxy-4-methoxyquinoline-2-carboxylic acid methyl ester **2c** (0.40 g, 1.71 mmol) in methanol (5 ml) was saponified with a solution of sodium hydroxyde (0.274 g, 6.8 mmol) in water (0.5 mL) at reflux temperature during 30 mn. The solution was poured in water and the pH was lowered to 2 with hydrochloric acid. The mixture was carefully extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 and the solvent was evaporated *in vacuo* to give the quinolobactin as a yellow solid (0.35 g, 93%).

M.p. = 252 °C (248 °C, Neuenhaus *et al.* 1980); ^{1}H NMR (D_2O, NaOD, pD $\approx$ 13), δ: 4,1 (s, 3H, O-CH_3); 6,85 (dd, 1H, aromatic H, $^3j_{HH}$ = 11.43 Hz and $^4j_{HH}$ = 1.83 Hz); 7,27 (dd, 1H, aromatic H, $^3j_{HH}$ = 12.81 Hz and $^4j_{HH}$ = 1.83 Hz); 7.42 (*pseudo* t, 1H, aromatic H); 7.45 (s, 1H, aromatic H). ^{13}C NMR (D_2O, NaOD), δ: 56.1 (O-$\underline{C}H_3$), 99.9, 104.8, 115.2 (aromatic $\underline{C}$-H), 123.6 (quaternary aromatic $\underline{C}$), 129.8 (aromatic $\underline{C}$-H), 143.9, 151.2, 163.4 and 165.4 (quaternary aromatic $\underline{C}$), 174.4 (-$\underline{C}O_2^-$).

MS (DCI, NH_3/*iso*butane, 150°C): 220 $[M+H]^+$ 100%.

Physicochemical measurements

Iron (III) stock solutions were prepared by dissolving appropriate amounts of ferric perchlorate hydrate (Aldrich) in standardized $HClO_4$, $NaClO_4$ solutions. The solutions were standardized for ferric ion spectrophotometrically by using a molar extinction coefficient of 4160 M^{-1} cm^{-1} at 240 nm (Bastian *et al.* 1956). Spectrophotometric titrations were carried out on a Varian Cary 50 UV-visible spectrophotometer equipped with a Peltier thermostatting accessory, and using an optic fiber of 1.000 cm path length plunged in the titrated solution and connected to a microcomputer for data acquisition. Potentiometric experiments were carried at 25 °C out in water-DMSO 95/5 v/v solutions. The ionic strength was fixed at 0.1 M with sodium perchlorate (Prolabo puriss). The potentiometric titrations were performed using a DMS 716 Titrino (Metrohm) equipped with a combined glass electrode (Metrohm, filled with NaCl saturated solution) and connected to an IBM Aptiva microcomputer. The electrodes were calibrated to read p[H] according to the classical method (Martell & Motekaitis1992) from titration of 0.01 M $HClO_4$ by 0.02 M NaOH: the data were analyzed using the program GLEE (Gans & O'Sullivan 2000) allowing refinement of E_0 and slope and the determination of the ionic product of water pK_w = 14.01. The ligand and its Fe^{III} complexes of ca 0.001 M were titrated with standardized 0.02 M sodium hydroxide. Argon was bubbled through the solutions to exclude CO_2 and O_2. Sodium hydroxide was prepared from 0.1 M NaOH and was standardized against potassium hydrogen phtalate. Carbonate content was checked by Gran's method. The titra-



Scheme 1. Synthetic pathway proposed by Neuenhaus *et al.* (A), the cause of its failing (B) and the successful synthesis (C).

tion data (140 points collected over the pH range 4.8-10.9 for the ligand solution and 90 points collected over the pH range 3.4-7 for the Fe^{3+}-ligand solution) were refined by the nonlinear least-squares refinement program HYPERQUAD (Gans *et al.* 1996) to determine the equilibrium constants (ligand protonation and complexation). The pK values have been calculated from the cumulative constants determined with the program. The incertainties in the pK values correspond to the added standard deviations in the cumulative constants.

The ferric complexes were investigated by spectrophotometry: the UV-visible spectra of a water-dmso 95/5 v/v solution containing ligand (0.00048 M) and Fe^{III} (0.00016 M) in the molar ratio 3 : 1 were recorded as a function of pH over the range 1-9 (adjusted with NaOH) using an optic fiber probe plunged in the solution and using an automatic titrator system (Metrohm, DMS Titrino 716) for the addition of NaOH and the measure of pH. The ionic strength was fixed at 0.1 M with $NaClO_4/HClO_4$. The spectrophotometric data were processed with the LETAGROP-SPEFO program (Silen & Warnqvist 1969). The program uses a non linear least-squares method and calculates the thermodynamic constants of the absorbing species and their corresponding electronic spectra. The

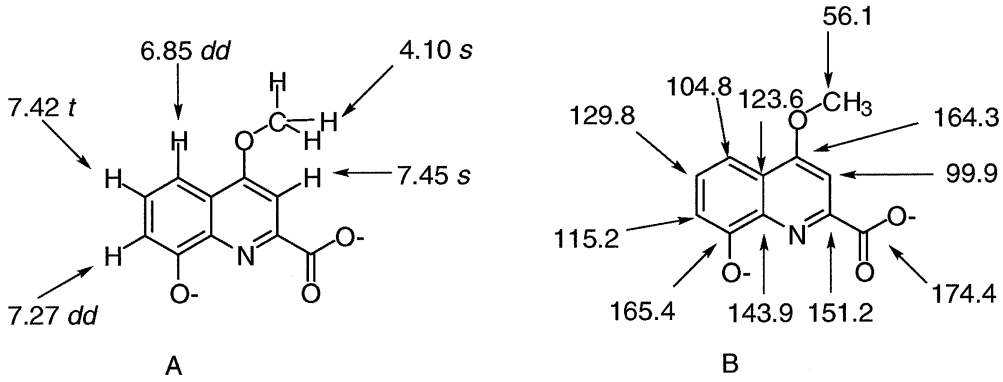


Figure 2. Spectroscopic assignement for ^{1}H NMR (A) and for ^{13}C NMR (B), in D_2O/NaOD

calculations were done from absorbance data of 5 wavelengths (between 500 and 700 nm) for solutions in each pH range. The range of values for the residual-squares sum ($\Sigma(A_{exp}-A_{calc})^2$) of the fits was 10^{-2}–10^{-3}.

Results and discussion

Synthesis of quinolobactin

Since Quinolobactin **3** was first obtained in minute amount from a strain of *Pseudomonas fluorescens*, such a low quantity precludes the determination of complexing abilities. To circumvent this availability problem, a straightforward synthetic pathway (Scheme 1A) has been proposed (Neuenhaus *et al.* 1980), Starting from commercialy xanthurenic acid **1**, but no experimental detail was provided. We have firstly attempted to reproduce this synthesis. If the chloro-compound **2** was easily obtained and characterized, we were unable to displace the chlorine atom from **2**. The direct conversion of the chloro acid **2** into quinolobactin **3** proved to be impossible. Changing the solvent from dry methanol to dry DMF or DMSO at ambient or at reflux temperature, increasing heating time from 12 h to 5 days, or/and using a great excess of fresh MeONa (2 to 40 equivalents), were inefficient to afford the desired compound **3**. Furthermore, in all experiments the chloro compound **2** was recovered unchanged and in high yield (98 to 99%)! This lack of reactivity can be easily explained: when the chloro compound **2** is treated with sodium methylate, the first reaction occurred at the carboxylic acid developing a delocalised negative charge which imped a second approach of a negatively charged species i.e. the methylate ion in the key aromatic nucleophilic substitution step (Scheme 1B).

We have successfully prepared quinolobactin according to the scheme depicted in Scheme 1C. In this pathway, the quinolobactin methyl ester **2c** could be obtained in one-pot three steps starting from xanthurenic acid **1**. Thionyl chloride could be used as an effective chlorating agent producing the chloro acid chloride **2a** (not isolated). When reacting with dry methanol and then with sodium methylate, esterification (producing **2b**, not isolated) and subsequent chloro displacement occurred readily, leading to the methyl ester of quinolobactin **2c** in a satisfactory yield of 64% (3 steps). Quinolobactin **3** was finally obtained in excellent yield by saponification of the ester moiety and then acidification. The quinolobactin **3** was studied by NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra of quinolobactin (^{1}H-^{1}H-COSY, direct ^{1}H-^{13}C-correlation gHMQC and long-range ^{1}H-^{13}C correlation gHMBC) allowed the full atribution of the signals, especially in the case of the so called difficult to characterised quaternary carbons (the proposed attribution is depicted Figure 2, A for ^{1}H and B for ^{13}C).

All the spectroscopic datas are consistent with the quinolobactin structure and was confirmed by SM.

Stability constants

The potentiometric titration of the protonated ligand with NaOH (Figure 3, curve a) allowed to determine the two deprotonation constants of the quinoline moiety. The pK_a of the carboxylic group is below 2 and could not be determined from this method. Analysis of the titration curve by the HYPERQUAD program (σ_{fit} = 3.5) yielded the pK_a values defined by Equation 1

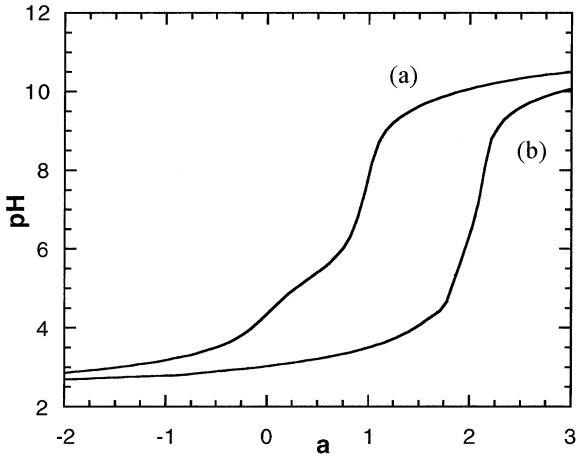


Figure 3. Potentiometric titration curves for (a) 1 mM ligand quinolobactine; (b) 1 mM quinolobactine + Fe^{3+} 2:1; a = mole of base added per mole of ligand. Solvent: water:dmso 95:5 v:v; $I = 0.10$ M ($NaClO_4$), $T = (25.0 \pm 0.2)$ °C. The data were refined by the Hyperquad program.

and 2

$$LH_n \rightleftharpoons LH_{n-1} + H^+ \quad (1)$$

$$K_{an} = [LH_{n-1}][H^+]/[LH_n] \quad (2)$$

to be of: $pK_{a2} = 5.50 \pm 0.07$, $pK_{a1} = 10.30 \pm 0.05$.

The value of 5.50 and 10.30 are attributed to the pyridine nitrogen and to the hydroxy group respectively. These pKa's are slightly higher than those, 5.08 and 9.67, of the 8-hydroxyquinoline (Boukhalfa *et al.* 2002). They reflect the electron-donor effect of the methoxy group.

The equilibria of the metal complexes were studied by means of spectrophotometric and potentiometric titrations allowing the determination of their stability constants. It was not possible to calculate the constant for the complexes formation directly from the potentiometric titration since complexation occurs at low pH (Figure 3, curve b). The spectrophotometric titration of a solution containing Fe^{III} and quinolobactin in a 1:3 metal-to-ligand molar ratio was carried out from pH 1 to 9 in order to determine the formation constants of the different complex formed upon increasing the pH. The spectra are shown in Figure 4. Increasing the pH from 1 to 6.1 resulted in the appearance of charge-transfer bands at $\lambda_{max} = 460$ and 615 nm. No spectral change was observed over the pH range 6.1-8.4. The absorbance data were processed with the LETAGROP-SPEFO program (Sillen & Warnqvist 1969). The best fit ($\Sigma(A_{exp}-A_{calc})^2 = 2\ 10^{-3}$) was obtained by considering the formation of the $[FeLH]^-$, $[FeL_2H]^-$ and $[FeL_2]^-$ species, providing the values $\log \beta_{111} = 18.60 \pm 0.10$, $\log \beta_{121} = 32.60 \pm 0.20$, $\log \beta_{120} = 28.20 \pm 0.25$ and the molar absorptivities at 615 nm $\varepsilon = 1250$, 1750 and 2980 M^{-1} cm^{-1} for the $[FeLH]^-$, $[FeL_2H]^-$ and $[FeL_2]^-$ complexes respectively.

β_{mlh} is defined by the Equations 3 and 4:

$$mFe^{3+} + nL + H^+ \rightleftharpoons Fe_mL_lH_h \quad (3)$$

$$\beta_{mlh} = [Fe_mL_lH_h]/[Fe^{3+}][L][H^+] \quad (4)$$

Titration of the ligand with Fe^{III} in a buffer solution at pH 7.4 was carried out. Plot of the absorbance at $\lambda = 615$ nm versus $[Fe^{3+}]/[L]$ ratio shown in Figure 5 exhibits a plateau for $[Fe^{3+}]/[L] > 0.5$. This clearly indicates the formation of a complex containing two ligands coordinated to one Fe^{3+} cation in agreement with fit of the absorbance data. The spectral parameters of the $[FeLH]^-$ and $[FeL_2]^-$ complexes are similar to those of the mono- and bis(8-hydroxyquinoline-5-sulfonate) Fe^{III} complexes ($\lambda_{max} = 617$ nm, $\varepsilon = 1280$ and $\lambda_{max} = 600$ nm, $\varepsilon = 3180$ M^{-1} cm^{-1} respectively) (Boukhalfa *et al.* 2002). This reflects that the cation Fe^{3+} is coordinated through the nitrogen and the oxygen quinoline atoms and confirms that quinolobactin forms Fe^{III} complexes in the 1:2 Fe:ligand stoichiometry. There is no evidence of coordination by the carboxylate group. Presumably, the carboxylate coordination could be unfavored to achieve the octahedral environment around Fe^{III} but remains close to the coordination sphere.

Since the ligand is a weak acid, proton competition occurs depending on its protonation constants and the p[H]. The $p[Fe^{III}] = -\log [Fe^{III}]$ is thus a better measure of the relative efficiency of the ligand under given conditions of p[H], $[Fe^{III}]_{tot}$ and $[L]_{tot}$. The $p[Fe^{III}]$ value at physiological p[H] = 7.4 and for $[Fe^{III}]_{tot} = 0.001$ mM and $[L]_{tot} = 0.01$ mM has been calculated to be 18.2. This value is higher that those calculated from literature equilibrium constants for bidentate ligands such as acetohydroxamic acid (pFe = 13) (Kotrly S, Sucha L, 1985) or 2,3-dihydroxy-*N*,*N*-dimethylbenzamide (pFe = 15) (Garett *et al.* 1989) and slightly lower than those for 8-hydroxyquinoline-5-sulfonic acid (pFe = 19.5) (Boukhafla *et al.* 2002) or for Tiron (19.7) (Kotrly S, Sucha L, 1985). This value is notably lower than the pFe^{III} values of almost bacterial siderophores (Albrecht-Gary & Crumbliss 1998) which are tetra- or hexadentate ligands.

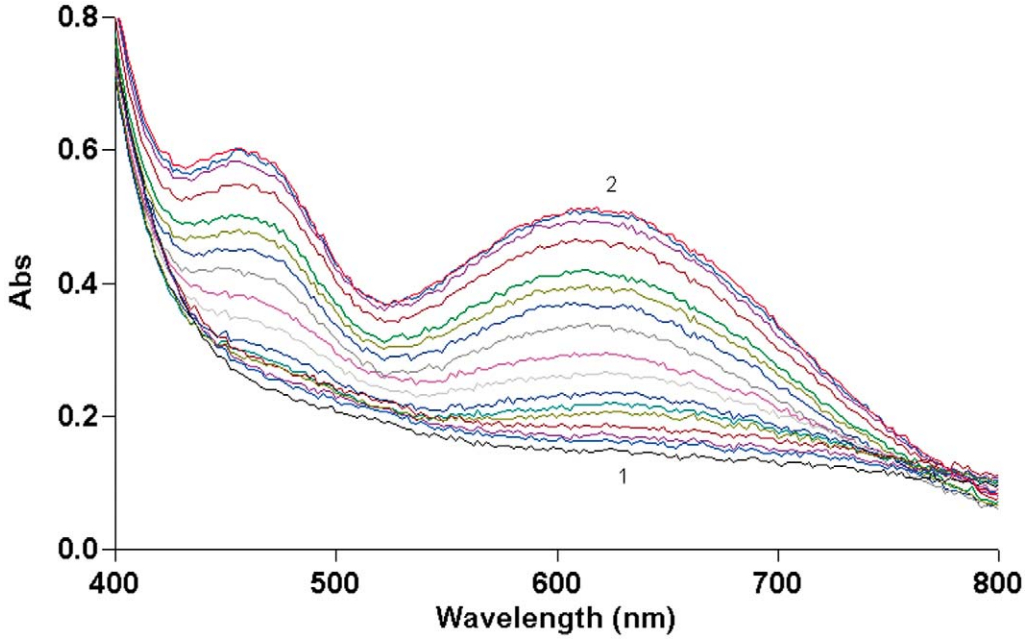


Figure 4. UV-vis absorption spectra of Fe^{III}-quinolobactin as a function of pH. $[quinolobactin]_{tot} = 0.48$ mM ; $[Fe^{III}]_{tot} = 0.16$ mM; Solvent: water:dmso 95:5 v:v, $I = 0.10$ M ($NaClO_4$), $T = (25.0 \pm 0.2)\,°C$; from 1 to 2 : p[H] = 1.08 (1), 1.35, 1.45, 1.67, 2.38, 2.52, 2.68, 2.95, 3.16, 3.59, 3.80, 4.15, 4.45, 4.70, 5.20, 5.52, 6.06, 7.42 (2).

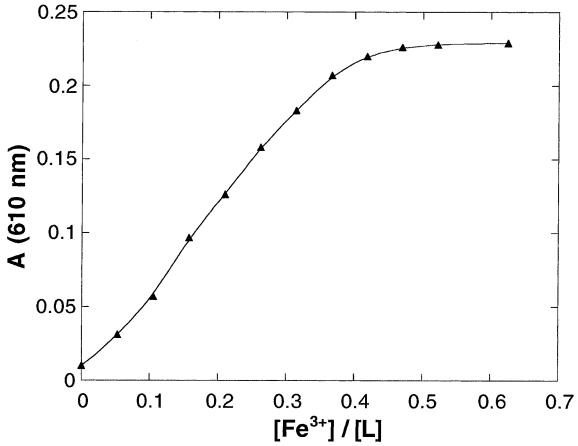


Figure 5. Absorbance at ? = 610 nm as a function of the molar ratio Fe^{3+}: quinolobactin. $[quinolobactin]_{tot} = 0.16$ mM Solvent: water:dmso 95:5 v:v, pH = 7.4 (MOPS buffer 0.05 M), $I = 0.10$ M ($NaClO_4$), $T = (25.0 \pm 0.2)\,°C$.

Conclusions

The relatively low pFe value determined for quinolobactin confirms the conclusion of the biological studies (Mossialos *et al.* 2000): 'quinolobactin can be considered a low-affinity siderophore since the pyoverdine-negative mutant which produces it is not able to grow in the presence of the strong iron chelator EDDHA unless the homologous pyoverdine or a heterologous pyoverdine is added'. Thermodynamic data are in accordance with this assertion. Quinolobactin forms complex of the 2:1 stoichiometry (two ligands for one iron) at physiological pH, having a 8-hydroxyquinolinate coordination and a pFe value lower than that of the complex with 8-hydroxyquinoline-5-sulfonate salt. This indicate that the carboxyl group do not enhance the efficiency of the ligand to chelate Fe^{III}. We believe that its presence favor aqueous solubilisation of the ligand and its ferric complex.

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