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Evaluation of 4-hydroxy-6-methyl-3-pyridinecarboxylic acid and 2,6-dimethyl-4-hydroxy-3-pyridinecarboxylic acid as chelating agents for iron and aluminium

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ABSTRACT

4-Hydroxy-6-methyl-3-pyridinecarboxylic acid (DQ6) and the new compound 2,6-dimethyl-4-hydroxy-3-pyridinecarboxylic acid (DQ726) were evaluated for possible application for iron (Fe) and aluminium (Al) chelation therapy. Metal/ligand solution chemistry, cytotoxicity, octanol/water partitioning ($D_{o/w}$), and chelation efficiency were studied. The solution chemistry of the two ligands with Fe(III) and Al(III) was investigated in aqueous 0.6 m (Na)Cl at 25 °C by means of potentiometric titrations, UV–Vis spectrophotometry, and ^{1}H NMR spectroscopy. DQ6 exhibited a high coordination efficiency towards Al(III). Fe(III)/DQ6, Al(III)/DQ726, and Fe(III)/DQ726 complexes were less stable. These results were confirmed by chelation efficiency measurements performed in an octanol/aqueous solution. Accordingly, the effects of the substitution at various ring positions of 4-hydroxy-3-pyridinecarboxylic acid were rationalised. Partitioning experiments at pH 7.4 showed both DQ6 and DQ726, and their Fe(III) and Al(III) complexes, to be hydrophilic. The toxicity of DQ6 and of DQ726 was investigated with human cancer cell lines and normal human primary cells: no cytotoxic effects were observed up to 0.1 mM, following a 3 days exposure. According to our results, DQ6 has the favourable properties to be a chelating agent for Al.

1. Introduction

Hydroxypyridinecarboxylic acids (HPs, Table 1) are new potential chelating agents for iron (Fe) and aluminium (Al) because they display a number of favourable properties. They form strong complexes with both Fe(III) and Al(III) [1–6], and have a very low affinity towards Zn(II) [7,8], which suggests the absence of essential metal decorporation *in vivo* [9]. They have a low molecular weight, which is a prerequisite for oral activity [10]. Toxic side effects induced by redox activity are unlikely for both the free ligands and the Fe(III)/ligand complexes [3,6]. The HPs investigated so far (Table 1) display negligible toxic effects ($IC_{50} > 0.1$ mM) to cancer cell lines and primary human cells, following a 3 days exposure [3,6]. DT0 is non-toxic towards animals, and it was proposed as an aspirin-like drug [11,12]. An analogue, 4-pyridoxic acid (3-hydroxy-5-hydroxymethyl-2-methyl-4-pyridinecarboxylic acid), the main metabolite of vitamin B6, is also non-toxic [13].

The simplest HPs, the unsubstituted DT0 and DQ0, have a distinct disadvantage: although their affinity towards Fe(III) and Al(III) is very high, it is still much lower than that of chelators available presently, such as deferiprone (L1). This affinity was significantly increased by methyl substitutions at the pyridinic ring. DT1 and DT2 showed a higher coordination strength than that of DT0. The complexation strength of DQ1 towards Fe(III) was slightly higher than that of DQ0; that towards Al(III) did not change. The 2-methyl substitution of DQ0 (which gives DQ2) decreased significantly the coordination strength towards Fe(III) and Al(III). The complexes of DQ716 with both Fe(III) and Al(III) are much more stable than those of the other HPs examined so far [6], so that this compound was proposed as a chelating agent for Fe and Al.

More than one HP having the proper chemical requirements would be needed, in order to perform a pharmacological screening (*e.g.* evaluation of chelation efficiency and toxicity *in vivo*) and eventually individuate at least one compound to be used as drug. Moreover, the effects of the methyl substitution at different ring positions on the metal stability of the complexes are still not clear. The metal–ligand solution chemistry of other derivatives should be

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Table 1
Hydroxypyridinecarboxylic acids (HPs) examined so far.

Name (IUPAC)	Acronym	References
3-Hydroxy-4-pyridinecarboxylic acid	DT0 (3H4P)	[1–3]
3-Hydroxy-1-methyl-4-pyridinecarboxylic acid	DT1 (1M3H4P)	[3–5]
3-Hydroxy-2-methyl-4-pyridinecarboxylic acid	DT2	[3,6]
4-Hydroxy-3-pyridinecarboxylic acid	DQ0 (4H3P)	[1,2,6]
4-Hydroxy-1-methyl-3-pyridinecarboxylic acid	DQ1 (1M4H3P)	[4–6]
4-Hydroxy-2-methyl-3-pyridinecarboxylic acid	DQ2	[6]
1,6-Dimethyl-4-hydroxy-3-pyridinecarboxylic acid	DQ716	[6]

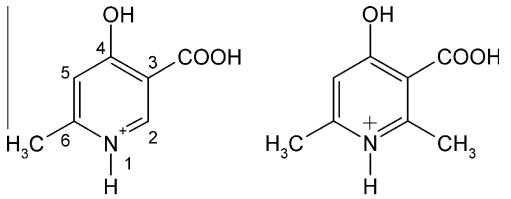


Fig. 1. 4-Hydroxy-6-methyl-3-pyridinecarboxylic acid (DQ6), and 2,6-dimethyl-4-hydroxy-3-pyridinecarboxylic acid (DQ726) shown in their most protonated forms (H_3L^+).

studied, so that the best strategy can be developed for the identification of the strongest Fe(III) and Al(III) chelators.

This paper describes our evaluation of 4-hydroxy-6-methyl-3-pyridinecarboxylic acid (DQ6) and 2,6-dimethyl-4-hydroxy-3-pyridinecarboxylic acid (DQ726) as possible chelating agents for Fe and Al (Fig. 1). DQ6 has been synthesised and characterised previously [14], but its use as chelating agent for iron and aluminium was never explored. According to our knowledge, DQ726 is a new compound and it has never been prepared yet. Both DQ6 and DQ726 were synthesised, and their coordination properties towards Fe(III) and Al(III) were studied by means of potentiometric, UV–Vis, and (in the case of Al(III)) ^{1}H NMR measurements. Their octanol/water partitioning coefficient ($D_{o/w}$), as well as their efficiencies in chelating Fe(III) and Al(III) at physiological pH, were determined *in vitro*. Their cytotoxicity was assessed on human cancer cell lines and primary cultures of human cells.

2. Experimental

2.1. Synthesis

Melting points were determined on a Gallenkamp MFB 595 010M/B capillary melting point apparatus, and are uncorrected. Infrared (IR) spectra were measured on a Perkin–Elmer 1760 FT-IR spectrometer using potassium bromide pressed disks. Values are expressed in cm^{-1}. ^{1}H NMR spectra were recorded on Varian Gemini (200 MHz) and Bruker (300 MHz) spectrometers, using the indicated solvents. NMR data are reported as δ values (ppm) relative to tetramethylsilane as an internal standard. Elemental analyses were performed in the Microanalytical Laboratory, Department of Pharmaceutical Sciences, University of Padova, using a Perkin–Elmer elemental analyser model 240B; results fell in the range of calculated values ±0.4%. Mass spectra were obtained with a Mat 112 Varian Mat Bremen (70Ev) mass spectrometer and Applied Biosystems Mariner System 5220 LC/MS (nozzle potential 250.00). Starting materials as well as solvents were purchased from Sigma (Milan, Italy).

2.1.1. 3-(Dimethylaminomethylene)-4-oxo-6-methyl-2-pyrone (**1**)

About 10 mL (d = 0.89 mg/mL, 74 mmol) *N*,*N*-dimethylformamide dimethyl acetale were slowly added to a stirred suspension of 4-hydroxy-6-methyl-2-pyrone (5 g, 40 mmol) in 10 mL dioxane: the starting material dissolved and the solution became brown. The reaction ran at a temperature of 15 °C for 2 h, when a precipitate formed. The precipitate was collected, washed with cold dioxane and acetone, and dried *in vacuo*. Yield 60% (literature [14] 72%); mp 148–150 °C (literature [14] 152–154 °C); HRMS (ESI) calculated for $[M+H]^+$ *m/z* 179.169, found 180.174. ^{1}H NMR (DMSO-d_2) δ 1.10 (t, 3H, C-CH_3), 2.35 (q, 2H, C-CH_3), 3.22 (s, 3H, N-CH_3), 3.48 (s, 3H, N-CH_3), 5.55 (s, 1H, olefinic proton); 8.22 (s, 1H, olefinic proton).

2.1.2. 4-Hydroxy-6-methylpyridin-3-carboxyl acid (**2**)

About 1 g (5.58 mmol) of pyrone derivative **1** was suspended in 30% aqueous ammonia (20 mL) and 1 mL $NH(CH_3)_2$. After stirring for 30 min at room temperature, the solution was evaporated under reduced pressure to about 1/3 of its volume and the remaining solution cooled (ice-bath) and acidified to pH 3 with HCl 1 M. The formed precipitate was collected and dried yielding a solid product which was re-crystallized from water to give pure product. Yield 64% (literature [14] 49%); mp 264–266 °C (literature [14] 267–268 °C); HRMS (ESI) calculated for $[M+H]^+$ *m/z* 153.125, found 154.258. ^{1}H NMR (D_2O + NaOD) δ 2.15 (s, 3H, C-CH_3), 7.22 (s, 1H), 8.52 (s, 1H). *Anal.* Calc. for $C_8NO_3H_9$: C, 57.48; H, 5.43; N, 8.38. Found: C, 51.30; H, 5.60; N, 7.40%.

2.1.3. Ethyl 4-hydroxy-2,6-dimethylpyridin-3-carboxylate (**3**)

About 5 g (35 mmol) of 2,2,6-trimethyl-4(1*H*)-1,3-dioxin-4-one and 2.5 g (19 mmol) of ethyl-(*Z*)-3-aminobut-2-enoate (ethyl-3-crotonate) were heated in a flask at 120–130 °C for 1 h, until no more water formed (Dean–Stark apparatus). On cooling, diethyl ether (20 mL) was added to the reaction mixture and a precipitate formed, which was collected and washed with a small amount of diethyl ether and dried to give solid product; yield 45% (literature [15] 40%); mp 162 °C (literature [15] 168 °C); HRMS (ESI) calculated for $[M+H]^+$ *m/z* 167.160, found 168.178. ^{1}H NMR (D_2O + NaOH) δ 1.30 (t, 3H, C-CH_3), 2.55 (s, 6H, CH_3), 4.29 (q, 2H, C-CH_2), 6.81 (s, 1H).

2.1.4. 4-Hydroxy-2,6-dimethylpyridin-3-carboxyl acid (**4**)

About 1 g (5.98 mmol) of pyridin-carboxylate derivative **3** was suspended in NaOH 0.5 M (20 mL) and refluxed for 4 h. Then, the resulting solution was cooled, acidified with aqueous HCl 2 M to pH 6 and extracted with chloroform to remove the unreacted ester. Further acidification of aqueous solution to pH 3 and cooling (ice-bath) gave a white solid which was re-crystallized from water to yield pure crystalline product. Yield 90%; mp = 320–325 °C (decomp.); HRMS (ESI) calculated for $[M+H]^+$ *m/z* 167.160, found 168.178; ^{1}H NMR (DMSO-d_6) δ 2.33 (s, 3H, CH_3), 2.71 (s, 3H, CH_3), 6.66 (s, 1H, H-5), 12.82 (s, 1H, N*H*), 16.15 (s, 1H, COO*H*). *Anal.* Calc. for $C_8NO_3H_9$: C, 57.33; H, 5.42; N, 8.36. Found: C, 54.40; H, 5.05; N, 7.80%.

2.2. Thermodynamic study

All potentiometric measurements were performed using a Radiometer ABU93 tri-burette apparatus. UV–Vis and ^{1}H NMR spectra were recorded using a Perkin–Elmer Lambda 20 spectrophotometer and a Bruker DRX-400 spectrometer operating at 400.13 MHz, respectively. All analyte concentrations were expressed in the molality scale (mol/kg of water). For potentiometric and UV–Vis

measurements, working solutions of HCl (0.13 m), NaOH (0.13 m), $FeCl_3$ (0.045 m, containing HCl 0.33 m), and $AlCl_3$ (0.11 m, containing HCl 0.40 m), were prepared and standardised as described previously [16,17]. Both ligands were used as synthesised to prepare 0.0051 m (DQ6) and 0.0041 m (DQ726) working solutions. The ionic strength of all solutions was adjusted to 0.6 m (0.594 M) (Na)Cl [16]. Solutions for ^{1}H NMR measurements were prepared by dissolving weighed amounts of ligand and $AlCl_3$ (Carlo Erba, 98% min) in D_2O (Aldrich, 99.9% atom D). The internal reference was $Me_3SiCH_2CH_2COOH$ (TSP, Aldrich 99%+).

Potentiometric measurements were carried out at 25.0 ± 0.1 °C; duplicate potentiometric measurements were performed using two glass electrodes (VWR 662-1792) and an Ag/AgCl/0.6 m NaCl reference electrode [18,19] with a J-shaped junction. In addition to glass electrode calibration, base standardisation, and ligand standardisation experiments [16], titrations of metal/ligand mixtures were performed. Metal ion to ligand ratios were from 1:1 to 1:9, Fe(III) concentrations ranged from 1.8×10^{-4} to 1.1×10^{-3} m, Al(III) concentrations ranged from 2.7×10^{-4} to 7.6×10^{-4} m. Experimental points were regarded as non-equilibrium points and rejected in subsequent data analysis if the potential did not stabilize within 6 min after each addition of titrant (maximum allowed rate of e.m.f. change: 0.05 mV/min). In the acidic pH range of Al(III)/DQ6 titrations, the measured e.m.f. drifted and reached a constant value only after *ca.* 2 h, suggesting a low complex formation rate. This finding is in agreement with previous results with other HPs [1,3,4,6,20]. Experimental details regarding the handling of the slow kinetics during the titrations are reported [20].

All stability constants were calculated using the computer program PITMAP [21] by simultaneous fitting of all data sets. The program minimises the sum of the squares of the differences between experimental and calculated e.m.f. values. Optimisation is performed using pitmapping [22] or simplex [23] as nonlinear least squares algorithms. Mass balance equations were solved, *i.e.* species concentrations at equilibrium were obtained, by means of the Newton–Raphson method [23]. The stability constants for metal/hydroxo complexes have been taken from the literature: log $\beta_{FeOH} = -2.87$, log $\beta_{Fe(OH)2} = -6.16$ [17], log $\beta_{Fe(OH)3} = -12.16$, log $\beta_{Fe(OH)4} = -22.16$, log $\beta_{Fe2(OH)2} = -2.9$, log $\beta_{Fe3(OH)4} = -6.3$, log $\beta_{Fe12(OH)34} = -48.9$ [24], log $\beta_{AlOH} = -5.52$, log $\beta_{Al(OH)2} = -11.3$, log $\beta_{Al(OH)3} = -17.3$, log $\beta_{Al(OH)4} = -23.46$, log $\beta_{Al3(OH)4} = -13.57$, log $\beta_{Al13(OH)32} = -109.2$ [25].

The UV–Vis measurements are summarised in Table 2. The pH was measured with the same electrodes and procedures as for potentiometric titrations. pH values below 2 were computed from the stoichiometric concentration of HCl, because the [H$^+$] modifications produced by the other species were negligible under these conditions. In other cases the pK_{a1} for the ligand (Table 3), the log β values for some metal/ligand complexes (Table 4) and the values of ε (molal absorbivity coefficient, Table 2) at the given wavelengths were computed by the program PITMAP.

^{1}H NMR spectra were obtained at 25 °C. Chemical shift values are given in δ units with reference to internal TSP. Suitable integral values for the proton signals were obtained by a pre-scan delay of 10 s. The assignment of the proton resonances was performed by standard chemical shift correlations and NOESY measurements when necessary. Spectra were recorded in D_2O solutions containing free ligand, and in solutions containing Al(III) and the ligand. The pH was measured with a Crison 5014 combined glass electrode previously calibrated in buffered aqueous solutions at pH = 4 and 7. The values of pD were computed by adding 0.41 pH units to the pH meter readings [26] in order to correct for isotopic and solvent effects due to the use of D_2O instead of H_2O.

Table 3
Acidic properties of DQ6 and DQ726. For the definition of L see caption of Fig. 1.

Species	DQ6		DQ726	
	pK_a	n	pK_a	n
H_3L^+	0.40 ± 0.01[a]	1	0.59 ± 0.02[a]	1
H_2L	6.32 ± 0.01	10	5.32 ± 0.02	12
HL^-	11.20 ± 0.04	10	11.83 ± 0.01	12

n = Number of UV–Vis wavelengths or number of potentiometric titrations used for data elaboration. Uncertainty is given by the fitting algorithm and represents an estimate of the reproducibility of the given value. In the case of UV–Vis data, it is a "within sample" and "within wavelength" reproducibility.
[a] Values obtained by UV–Vis.

Table 4
Stability constants for metal/ligand complexes, at 25 °C in aqueous (Na)Cl 0.6 m (reactions: $m\,M^{3+} + l\,L^{2-} + h\,H^+ \rightleftarrows M_mL_lH_h^{3m-2l+h}$). For the definition of n see footnote of Table 3.

Species	Fe(III)/DQ6 Log β	n	Al(III)/DQ6 Log β	n
MLH^{2+}	20.51 ± 0.03[a]	1	18.65 ± 0.01	10
			18.76 ± 0.09[a]	2
ML^+	17.00 ± 0.03	4	–	
$ML_2H_2^+$	38.93 ± 0.04	8	36.24 ± 0.06	10
			36.9 ± 0.1[a]	2
ML_2H	34.31 ± 0.03	8	30.1 ± 0.1	4
ML_2^-	–		21.75 ± 0.05	4
ML_3H_3	55.80 ± 0.04	8	52.8 ± 0.1	6
$ML_3H_2^-$	47.99 ± 0.07	8	–	
	Fe(III)/DQ726 Log β	n	Al(III)/DQ726 Log β	n
MLH^{2+}	19.19 ± 0.05[a]	1	17.70 ± 0.01	6
			17.49 ± 0.05[a]	1
ML^+	16.51 ± 0.08	2	–	
$ML_2H_2^+$	36.84 ± 0.06	4	34.37 ± 0.03	6
ML_2H	32.9 ± 0.1	4	27.8 ± 0.1	4
ML_2^-	26.98 ± 0.05	4	–	
ML_3H_3	53.12 ± 0.04	4	49.47 ± 0.06	4
$ML_3H_2^-$	47.6 ± 0.1	3	–	

[a] Values obtained by UV–Vis.

2.3. n-Octanol/water distribution and chelation efficacy

The methods were described in Ref. [27] for Al(III) and in Ref. [2] for Fe(III). DQ6 and DQ726 efficiencies were determined at pH = 7.4

Table 2
Experimental details for UV–Vis measurements. In the last column, the optimised ε values for some species are reported.

Solutions	$C_M \times 10^3$ (m)	$C_L \times 10^3$ (m)	pH	λ (nm)	Path cell (cm)	$\varepsilon \times 10^{-3}$ (mol^{-1} × kg cm^{-1})
DQ6	–	0.118	0.26–1.17	227, 250, 280	1.0	2.48 ± 0.02 (H_2L^+)5.51 ± 0.01 (HL) (at 250 nm)
Fe^{3+} + DQ6	0.537	0.682	0.30–1.27	400	1.0	1.60 ± 0.07 ($FeLH^{2+}$)
Al^{3+} + DQ6	1.172	0.861	2.29–2.94	244, 249	0.1	6.47 ± 0.07 ($AlLH^{2+}$) (at 249 nm)
Al^{3+} + DQ6	0.595	0.876	2.74–3.94	244, 249	0.1	14.9 ± 0.1 ($AlL_2H_2^+$) (at 249 nm)
DQ726	–	0.303	0.25–1.25	280	1.0	0.19 ± 0.02 (H_3L^+)1.13 ± 0.02 (H_2L)
Fe^{3+} + DQ726	0.650	0.646	0.28–1.43	400	1.0	0.80 ± 0.04 ($FeLH^{2+}$)
Al^{3+} + DQ726	0.518	0.508	2.58–4.02	244	0.2	4.43 ± 0.04 ($AlLH^{2+}$)

in a system containing 2 mL *n*-octanol, 2 mL of an aqueous solution containing 1×10^{-3} mol/L ligand, and a form of Al or Fe that has very limited solubility (aluminium oxide or ferric acetate basic) added in excess of the ability of the ligand to bind the metal if all ligand associated with metal. Approximately 5 mg of aluminium oxide or ferric acetate basic was added, introducing ~50 or 25×10^{-6} moles of Al or Fe; ~75 and 40-fold more Al or Fe, respectively, than the 2 mL of 1×10^{-3} mol/L ligand (2×10^{-6} moles) could complex, considering a 1:3 metal:ligand stoichiometry.

Efficiency was calculated as the sum of the increased molar concentration of metal in the presence minus the absence of the ligand, divided by the molar concentration of the ligand × 100%, assuming a 1:3 metal–ligand complex for these bidentate ligands. When the stoichiometry was different from 1:3, the efficiency was corrected. Four replicate trials, each condition with duplicate observations, were conducted.

2.4. Cytotoxicity assays

Tests were carried out with DQ6 and DQ726, following the procedure previously described [3]. Briefly, the cytotoxic activity was determined using a standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazodium bromide (MTT)-based colorimetric assay (Sigma). Quadruplicate cultures were employed for each treatment using human cancer cell lines (OVCAR, OE33, A549, and HeLa; obtained from American Type Culture Collection, ATCC) and normal human cells (keratinocytes, fibroblasts). The cytotoxic effect of each tested compound was evaluated by the ratio between the number of living cells present in the sample and in a blank treated with the solvent only, following a 3 days exposure.

3. Results and discussion

3.1. Synthesis of DQ6 and DQ726

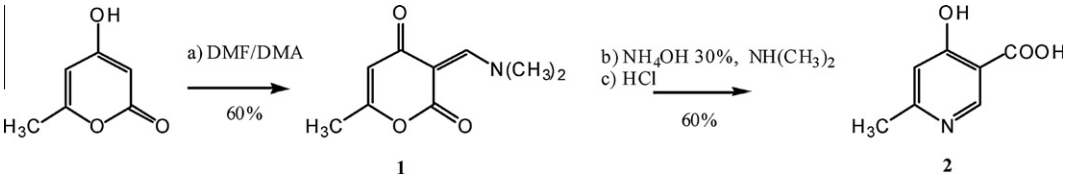
Following a previously described route depicted in Scheme 1 and 4-hydroxy-6-methylnicotinic acid DQ6 (**2**) was synthesised starting from commercial 4-hydroxy-6-methyl-2-pyrone [14], which was reacted with *N*,*N*-dimethylformamide dimethyl acetale to give the 3-dimethylaminomethylene derivative **1** (60% yield). The latter, by treatment with aqueous ammonia (NH_4OH 30%), dimethylamine as catalyst and then with HCl 1 M, provided the desired acid **2** in a 60% yield. ^1H NMR, melting point and elemental analysis data were in agreement with reported values.

For the synthesis of the new compound DQ726 (**4**), a two steps procedure was adopted similarly as previously reported [15] (Scheme 2). The reaction between commercial 2,2,6-trimethyl-4(1*H*)-1,3-dioxin-4-one and ethyl-(*Z*)-3-aminobut-2-enoate (ethyl 3-aminocrotonate) in a 1:1 ratio yielded the known ethyl ester **3** in a 40% yield. The latter was then transformed into the new acid **4** by treatment with NaOH 0.5 M followed by acidification with HCl 2 M at pH 3 (90% yield).

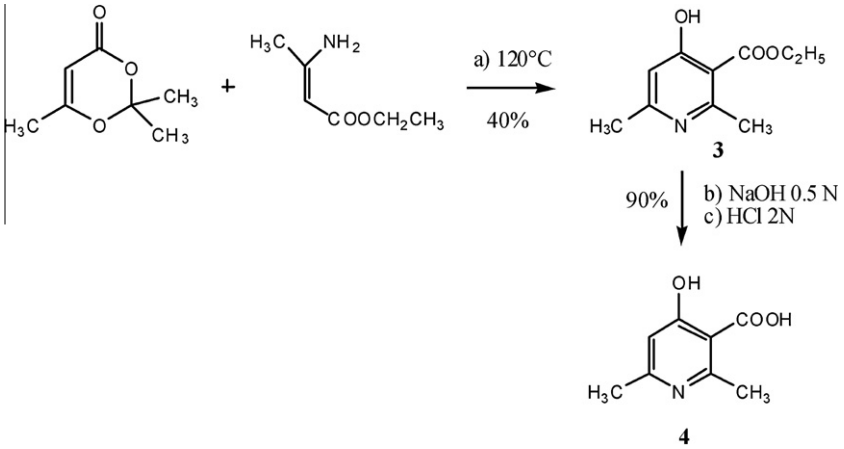
3.2. Acidity constants of DQ6 and DQ726

Potentiometric titrations of each ligand allowed the determination of some pK_a values, which are reported in Table 3. Accurate acidity constants at pH values lower than *ca.* 1.5–2 could not be obtained from potentiometric measurements, because in these conditions the pH modification due to the acid–base equilibria was negligible. For each ligand, the pK_a value for the most protonated form (H_3L^+) was determined by means of UV–Vis measurements. All pK_a values are in agreement with those previously observed for other HPs [1–6].

An unequivocal assignment of the pK_a values of DQ6 and DQ726 is not possible, because 4-hydroxypyridine derivatives can adopt a chinoid electronic configuration in tautomeric equilibrium with the corresponding aromatic form. However, the formation of a strong intramolecular bond between the deprotonated COO^- and the protonated phenolic OH is expected to shift the tautomeric equilibrium towards the aromatic form. Therefore, the pK_{a1} ($H_3L^+ \rightarrow H_2L$), pK_{a2} ($H_2L \rightarrow HL^-$), and pK_{a3} ($HL^- \rightarrow L^{2-}$) of both ligands can be confidently assigned to the carboxylic COOH, to the pyridinic NH, and to the phenolic OH, respectively.



Scheme 1. Synthesis of DQ6 (**2**) [14].



Scheme 2. Synthesis of DQ726 (**4**).

3.3. Metal/ligand complexes: potentiometric results

Potentiometric data were elaborated in a sequential manner, as described previously [1], to avoid the difficulties produced by the strong correlation between the parameters to be optimised in the presence of a large number of metal/ligand species. The stoichiometry and the values of the stability constants of the metal/ligand complexes identified in solution are shown in Table 4. The distribution diagrams for metal/ligand solutions, computed for solutions containing 5×10^{-4} m metal ion and 2×10^{-3} m ligand, are reported in Fig. 2 (for DQ6) and in Fig. S1 of Supplementary material (for DQ726).

In the case of DQ6, the predominant species for both metal ions are MLH, ML_2H_2 and ML_3H_3 (for simplicity, charges will be generally omitted in the formulae, except in Figures and Tables). The deprotonation products of the main metal/DQ6 complexes can be also detected. For DQ726, the species ML_3H_3 is less important for both metal ions, and a number of deprotonation products become significant if not predominant (*e.g.* FeL_2H). Some of them (*e.g.* FeL_2) were observed only at very large ligand-to-metal ratios, when the pH of $Fe(OH)_3$ precipitation was shifted towards more basic values. In all cases, apart for Al(III)/DQ6, the precipitation of the metal hydroxide begins below physiological pH.

According to the hard-soft rule and due to the chelation principle, the metal coordination occurs most likely via the carboxylate and the deprotonated hydroxylic groups, whereas the nitrogen atom remains protonated in the MLH-type species. Deprotonation products of the Fe(III) complexes are probably hydroxo species, *i.e.* the deprotonation occurs at the Fe(III)-coordinated water molecules. The pK_a values of the complexes FeLH and FeL_2H_2, which can be easily computed from data in Table 4, are similar to the pK_a of free Fe(III) (2.87) (*e.g.* for Fe(III)/DQ6 $pK_a(FeLH) = 3.51$, $pK_a(FeL_2H_2) = 4.62$), whereas for the species FeL_3H_3, which likely has no coordinated water, $pK_a = 7.81$. Thus, for example, the complex FeL_2H should be better referred to as $FeL_2H_2(OH)$. In the case of Al(III) complexes, the intrinsic acidity of the coordinated water molecules (pK_a for $Al(H_2O)_6 = 5.52$) is comparable to that of the pyridinic nitrogen of DQ6 and DQ726. Therefore, the deprotonated Al(III) complexes are likely mixtures of non-hydroxo and hydroxo species, *e.g.* $AlL_2H + AlL_2H_2(OH)$.

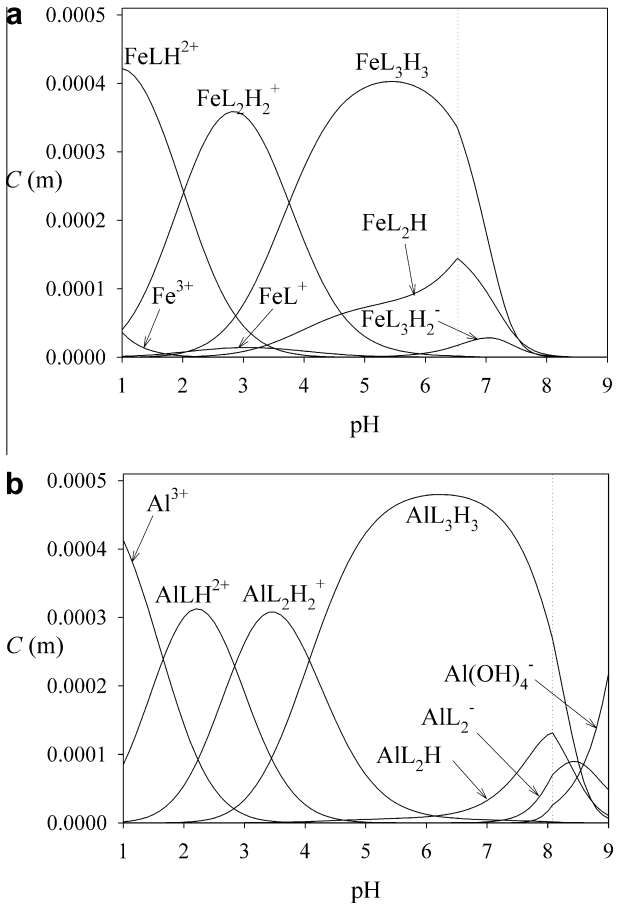


Fig. 2. Distribution diagrams of the most important Fe(III) (a) and Al(III) (b) species in the presence of DQ6 in aqueous (Na)Cl 0.6 m, $T = 25$ °C; $C_M = 5 \times 10^{-4}$ m, $C_L = 2 \times 10^{-3}$ m. Dashed lines show the theoretical starting pH for hydroxide precipitation.

3.4. Metal/ligand complexes: UV–Vis results

UV–Vis spectra for Fe(III)/DQ6 and Al(III)/DQ6 solutions at several pH values are shown in Fig. S2 in the Supplementary material. All spectra show the typical absorption of substituted pyridinic rings. A weak charge-transfer peak appears at around 400 nm when Fe(III) coordinates to each ligand. Al(III) complexation produces an increase of the absorption coefficients at almost all UV wavelengths.

UV–Vis data allowed the determination of the stability constants of some complexes (Table 4). The log β values of the MLH complexes formed in Fe(III)/DQ6 and Fe(III)/DQ726 solutions cannot be obtained by potentiometric titrations because these species are already formed at very acidic pH values (Figs. 2a and S1a). In these cases, the UV–Vis technique was necessary to compute this value, which was kept constant during the elaboration of the potentiometric data. In the case of Al(III), UV–Vis log β values were obtained to confirm the potentiometric results. The agreement of the stability constant values determined by potentiometry and UV–Vis for $AlLH^{2+}$ (Table 4) is very good. For $AlL_2H_2^+$ the difference is larger and should be attributed to the UV–Vis uncertainty in measuring Al(III)/ligand absorption spectra. Different Al(III) complexes display very similar UV–Vis spectra as the absorption is due to the ligand only. Therefore, UV–Vis data regarding Al(III) become less reliable when several species coexist in solution.

3.5. Al(III)/ligand complexes: ^{1}H NMR results

The ^{1}H NMR spectra of each free ligand show only singlets due to the absence of resolvable H–H coupling constants. For DQ6 at pD = 1.5, signals were observed at δ 2.56 (3H, $CH_3(6)$), 6.98 (1H, H(5)), and 8.70 (1H, H(2)). For pyridinic ring numbering see Fig. 1. For DQ726 at pD = 2.6, signals were at δ 2.48 (3H, $CH_3(6)$), 2.79 (3H, $CH_3(2)$), and 6.78 (1H, H(5)). Signals of DQ6 and DQ726 moved upfield by increasing the pD value due to the deprotonation of the pyridinic nitrogen at pD *ca.* 6 (Table 3): for example, at pD = 5.8 $CH_3(6)$, $CH_3(2)$, and H(5) protons of DQ726 resonate at δ 2.41, 2.53, and 6.51, respectively. The aromatic proton spectra for free DQ6 solutions at various pD are shown in Fig. 3a.

In the presence of Al(III), the resonances of the free ligands were still observed, together with new peaks due to the complex formation at all the investigated pD values. Fig. 3b displays the spectra (aromatic proton region only) of Al(III)/DQ6 solutions.

At pD = 1.5 one new set of narrow signals appeared, which belongs clearly to a single species. On the basis of the potentiometric data this complex is AlLH. The H(5) signal of AlLH is well resolved from the corresponding signal of the free ligand.

At pD = 2.8 new signals appear together with those of the free ligand and those of AlLH. The H(5) peaks are again the most interesting and they show the sharp peak of the free ligand (δ 6.93), that of AlLH (δ 6.85), a broad band centred at δ 6.77, and four small peaks at δ 6.64–6.72 (see inset in Fig. 3b). According to the

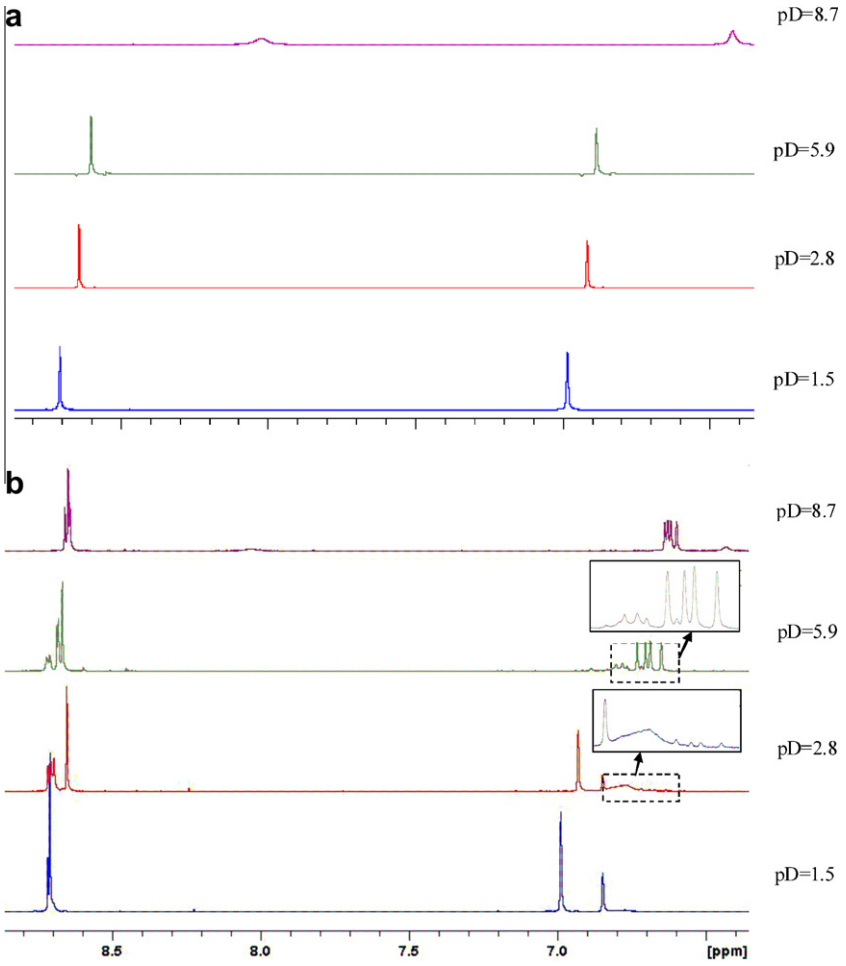


Fig. 3. ^{1}H NMR spectra (aromatic zone) at various pD values of D_2O solutions containing: (a) only DQ6 ($C_{DQ6} = 8.9 \times 10^{-3}$ m); (b) Al(III) ($C_{Al} = 3.0 \times 10^{-3}$ m) and DQ6 ($C_{DQ6} = 8.9 \times 10^{-3}$ m).

potentiometric results, AlL_2H_2 is the main species at this pD, so the broad band should be assigned to AlL_2H_2 and the four peaks to AlL_3H_3 (see below). The broadness of the AlL_2H_2 signal can be due both to slight differences in the chemical shifts of several isomers (up to eight diastereoisomers can exist in solution for this complex), and to the interconversions of the diastereoisomers at a rate comparable with the NMR time scale. Broad NMR signals for Al:L 1:2 species are commonly observed for Al(III)/HPs complexes [1,3,4], and slow kinetics of interconversion was confirmed [3,6].

At pD 5.9 the signals of AlLH and of the free ligand become very small. Together with the signals of AlL_2H_2, four new signals appear for H(5) at δ 6.65–6.73 (see inset in Fig. 3b), which can be assigned to AlL_3H_3. DQ6 is asymmetric, thus it may chelate the metal, forming AlL_3H_3, in two different spatial configurations. If the three ligands of AlL_3H_3 chelate the metal ion in the same configurations, a symmetrical diastereoisomer forms, and the three protons are chemically equivalent. If one ligand chelates differently from the other two, an unsymmetrical diastereoisomer forms, and the three protons should give rise to different signals in the NMR spectrum. As a consequence, four signal are expected (and observed in this case) in the spectrum. These signals show the same integral: this can be explained if the formation microconstant of the symmetric and of the unsymmetric diastereoisomer are very similar, so that their ratio in solution is 1:3. A similar ^{1}H NMR pattern was observed for other Al(III)/HP solutions [3,4,6].

At pD 8.7 the signal sets shift upfield, in agreement with the deprotonation detected for the complexes at this pD by potentiometric titrations.

The ^{1}H NMR spectra for Al(III)/DQ726 are shown in Fig. S3 (Supplementary material). At pD = 2.6 three sets of signals were observed: one can be assigned to the free ligand, the other two sets to AlLH (narrow, more intense) and AlL_2H_2 (broad, low intensity). At pD 4.2 the narrow signals of AlLH vanish, and a number of new broad signals appear. In the aromatic zone, for example, four separated signals were observed at δ 6.42, 6.50, 6.55, and 6.65 together with the peak of the free ligand. In agreement with previous findings, these new signals are due to different structural isomers of AlL_2H_2 and AlL_3H_3. The pattern changes when pD increases from 4.2 to 5.8, allowing the provisional assignment of some signals to AlL_2H_2 (which predominates at pD 4.2) and the others to AlL_3H_3 (which predominates at pD 5.8).

Semiquantitative data can be obtained from NMR spectra by calculating the relative integrals of the signals of Al(III)/DQ6 and Al(III)/DQ726 complexes and those of the free ligand. These values can be compared with those calculated on the basis of the speciation data reported in Tables 3 and 4 computed at the same Al(III) and ligand concentrations and at the same pH values. Table 5 summarises the results for Al(III)/DQ6, where the relative amounts of AlLH, AlL_2H_2 and AlL_3H_3 were computed at most pD values. Differences can be explained not only by the uncertainty of the NMR integration values and the broadness of some signals, but mainly by the presence of isotopic effects due to the use of D_2O as NMR solvent. The conditional stability constants of the complexes in D_2O are lower than in H_2O, because the deuterated ligand is less acidic than the protonated one (primary isotopic effect), *i.e.* D^+ is a better metal competitor for the ligand than H^+ [28]. A significant

Table 5
Relative amounts of free DQ6 and Al(III)/DQ6 complexes (with respect to total ligand concentration) at various pD values according to NMR (integration values of H(5)) and theoretical speciation. At pD 5.9, the notation "AlL_2H_x" denotes AlL_2H_2 + AlL_2H + AlL_2.

pD values	Relative amounts according to NMR data	Relative amounts according to theoretical speciation
1.5	Free ligand: 72%	Free ligand: 70%
	AlLH: 28%	AlLH: 21%
	AlL_2H_2: 0%	AlL_2H_2: 9%
2.8	Free ligand: 53%	Free ligand: 37%
	AlLH: 15%	AlLH: 8%
	AlL_2H_2: 29%	AlL_2H_2: 41%
	AlL_3H_3: 3%	AlL_3H_3: 14%
5.9	Free ligand: 8%	Free ligand: 2%
	AlLH: 4%	AlLH: 0%
	AlL_2H_x: 28%	AlL_2H_x: 7%
	AlL_3H_3: 60%	AlL_3H_3: 91%
8.7	Free ligand: 14%	Free ligand: 53%
	Complexed ligand: 86%	$3AlL_3H_3$ + $2AlL_2H_x$: 47%

larger amount of free ligand is expected in D_2O than in H_2O. For Al(III)/DQ726 complexes, only the relative amount of total free ligand was computed, and it was 72%, 45%, and 30% at pD = 2.6, 4.2, and 6.8, respectively. Corresponding theoretic values are 69%, 36%, and 21%. Here, too, the differences can be explained by the primary isotopic effect.

3.6. Metal/ligand complexes: discussion

In order to evaluate the effects of the 2- and 6-methyl substitutions to DQ0, pM versus pH (pM = −log[M]) was computed for DQ716, DQ726, DQ6, DQ2, and DQ0 (Fig. 4): higher pM values indicate stronger metal/ligand complexes. Computations were performed at metal and ligand stoichiometric concentrations [29] which are representative of physiologic conditions (10^{-6} m metal ion and 5×10^{-5} m ligand). The pFe and pAl plots for L1 at the same conditions (speciation data from Clarke and Martell [30]) are reported for comparison. Fig. 4 demonstrates that HPs are weaker Fe(III) and Al(III) chelators than L1. It is also important to note that Al(III)/DQ6 complexes are as strong as those formed by DQ716 (this derivative forms the most stable complexes among the HPs synthesised so far [6]). However, Fe(III)/DQ6 complexes are *ca.* 1 order of magnitude less stable than Fe(III)/DQ716 complexes. DQ726 and DQ2 have practically the same affinity, and they form the weakest Fe(III) and Al(III) complexes among all HPs.

The high stability of Al(III)/DQ6 complexes confirms that the 6-methyl substitution of the pyridinic ring increases the Lewis basicity of DQ0. This can be explained by the electron-donating effect of the methyl group, which has a positive effect on the HP's affinity towards hard metal ions. However, Fe(III)/DQ6 complexes are less stable than Fe(III)/DQ716 complexes, indicating that the 6-methyl effect is less important for Fe(III) than for Al(III). For Fe(III), the maximum positive effect on the metal stability of the ligands appears to be due to the 1-methyl substitution.

The stability decrease of the Fe(III) and Al(III) complexes due to the 2-methyl substitution on DQ0 was observed previously for DQ2 [6], and confirmed here for DQ726. The most probable reason is an ortho effect: the steric hindrance of the methyl group in the 2-position does not allow the bulky carboxyl group in 3 to be coplanar with the pyridine ring. This causes a distortion of the chelate ring and thus a decrease of the complex stability.

3.7. n-Octanol/water distribution and chelation efficacy

The octanol/water distribution coefficients ($D_{o/w}$) of free DQ6 and DQ726 were measured in both the Al and Fe distribution systems (each by four replicate trials with duplicate observations) at pH 7.4. The two sets for DQ6 data gave $D_{o/w} = 0.030 \pm 0.003$ and 0.021 ± 0.002, the two for DQ726 gave $D_{o/w} = 0.0024 \pm 0.0012$ and 0.0014 ± 0.0003. Both ligands are markedly hydrophilic at physiologic pH. Metal/ligand complexes retain hydrophilicity: $D_{o/w} = 0.022 \pm 0.002$ for Fe(III)/DQ6, 0.015 ± 0.001 for Al(III)/DQ6, 0.0032 ± 0.0011 for Fe(III)/DQ726, 0.0019 ± 0.006 for Al(III)/DQ726. In the presence of the Al ion, DQ6 is significantly more hydrophilic than in the absence of it (unpaired two-tailed *t*-test): this is a favourable property for metal chelators as they should enhance metal renal clearance.

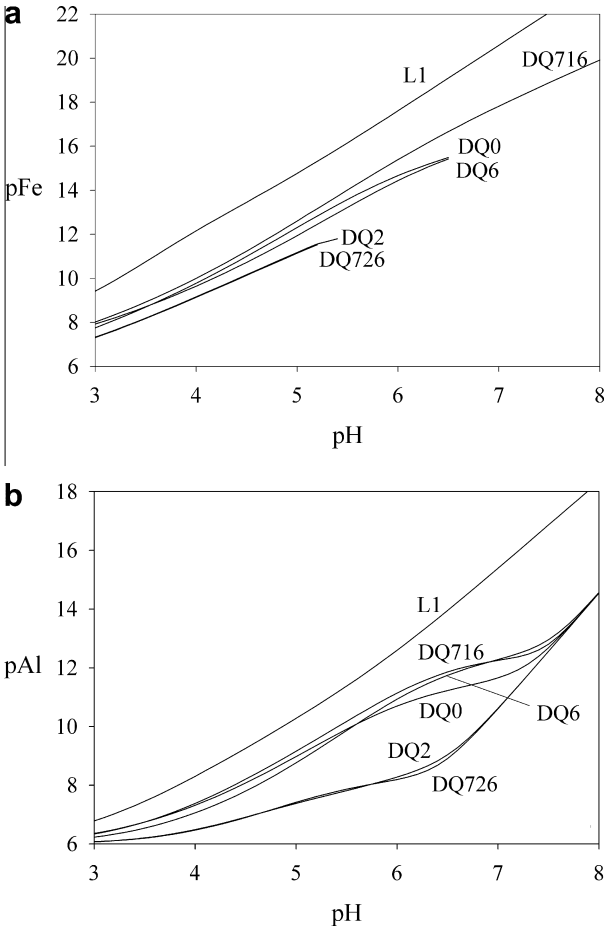


Fig. 4. pFe (a) and pAl (b) plots for several ligands; $C_{metal} = 10^{-6}$ m, $C_{ligand} = 5 \times 10^{-5}$ m. pFe plots stop at the theoretical starting pH for hydroxide precipitation.

The chelation efficiency measurements showed different behaviours of the two ligands. DQ6 chelated a significant amount of Fe(III) (20% ± 3%) and especially of Al(III) (47% ± 7%) at pH 7.4. The chelation efficiency of DQ726 was negligible for both metal ions (efficiency < 1%). In fact, $D_{o/w}$ values of DQ726 in the absence and in the presence of metal ion are statistically equivalent. pM values calculated from thermodynamic data (Fig. 4) are in agreement with these chelation efficiency results.

3.8. In vitro cytotoxic activity

Analyzing the percentage of living cells with respect to cells exposed following 3 days exposure, it was apparent that DQ6 or DQ726, when used at concentrations up to 0.1 mM, shows no cytotoxic activity on human cancer cell lines (OVCAR, OE33, A549, and HeLa) and also on primary keratinocytes and fibroblasts. Concentrations higher than 0.1 mM could not be investigated because of the toxic effects due to the pH variation in the culture medium

(produced by solvent additions). Therefore no IC_{50} value (concentration of chemical resulting in 50% inhibition of cell viability) could be calculated. The IC_{50} was larger than 0.1 mM for all the investigated compounds.

4. Conclusions

Both DQ6 and DQ726 form strong Fe(III) and Al(III) complexes in aqueous solution having 1:1, 1:2, and 1:3 metal:ligand stoichiometry. The free ligands and their metal complexes are hydrophilic at physiologic pH. None of the ligands was cytotoxic in the investigated concentration range ($IC_{50} > 0.1$ mM). Both ligands form weaker Fe(III) and Al(III) complexes than L1: this means, in principle, that a similar metal depletion efficiency can be obtained if higher doses of ligand are administered in the chelation therapy. This should not be a problem if the absence of toxicity *in vitro* displayed by all HPs will be confirmed *in vivo*.

The DQ726 complexes with Fe(III) and Al(III) are weaker than those formed by the other HPs, as confirmed by the very low chelation efficiencies measured for metal/DQ726 solutions at physiologic conditions simulated *in vitro*. The coordination strength is very similar to that of DQ2. The decrease of the metal ion binding ability is attributed to an ortho effect. It is likely that a similar effect would be observed with any functional group in position 2 other than the methyl. Therefore, any substitution of DQ0 at the position 2 should be avoided to let the ligand be effective as a metal ion chelator *in vivo*.

The Fe(III)/DQ6 complexes are more stable than those of other HPs, but less stable than those of DQ716. On the other hand, the Al(III)/DQ6 complexes have practically the same stability as those of DQ716. The substitution at position 1 appears to be much more important in enhancing the Fe(III) coordination strength, the substitution at 6 appears to be more important for Al(III). The 1-substituted derivatives of DQ0 are good candidates as chelating agents for Fe. The 6-substituted derivatives of DQ0 can represent a family of ligands which are relatively more effective for Al(III) than for Fe(III), *i.e.* these chelators would not deplete Fe during Al chelation therapy. DQ6 itself can be proposed as a chelating agent for Al.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ica.2011.04.009.

References

[1] V.B. Di Marco, R.A. Yokel, M.G. Ferlin, A. Tapparo, G.G. Bombi, Eur. J. Inorg. Chem. (2002) 2648.
[2] V.B. Di Marco, R.A. Yokel, H. Li, A. Tapparo, G.G. Bombi, Inorg. Chim. Acta 357 (2004) 3753.
[3] A. Dean, M.G. Ferlin, P. Brun, I. Castagliuolo, D. Badocco, P. Pastore, A. Venzo, G.G. Bombi, V.B. Di Marco, Dalton Trans. (2008) 1689.
[4] V.B. Di Marco, A. Dean, M.G. Ferlin, R.A. Yokel, H. Li, A. Venzo, G.G. Bombi, Eur. J. Inorg. Chem. (2006) 1284.
[5] V.B. Di Marco, A. Dean, R.A. Yokel, H. Li, G.G. Bombi, Polyhedron 26 (2007) 3227.
[6] A. Dean, M.G. Ferlin, P. Brun, I. Castagliuolo, R.A. Yokel, D. Badocco, P. Pastore, A. Venzo, G.G. Bombi, V.B. Di Marco, Dalton Trans. (2009) 1815.
[7] R.C. Hider, T. Zhou, Ann. NY Acad. Sci. 1054 (2005) 141.
[8] É. Sija, A. Dean, T. Jakusch, V.B. Di Marco, A. Venzo, T. Kiss, Monatsh. Chem. 142 (2011) 399.
[9] Z.D. Liu, R.C. Hider, Med. Res. Rev. 22 (2002) 26.
[10] V.B. Di Marco, A. Tapparo, A. Dolmella, G.G. Bombi, Inorg. Chim. Acta 357 (2004) 135.
[11] H. Tendeloo, F. Broekman, V. Stroer, J. Siemelink, Neth. Appl. 70 (1972) 11749.
[12] W.O. Foye, J.M. Kauffman, Chim. Ther. 2 (1967) 462.
[13] H.G. Kraft, L. Fiebig, R. Hotovy, Arzneimittelforschung (Drug Res.) 11 (1961) 922.
[14] H. Yamada, H. Tobiki, K. Jimpo, K. Gooda, Y. Takeuchi, S. Ueda, T. Komatsu, T. Okuda, H. Noguchi, K. Irie, T. Nakagome, J. Antibiot. 36 (1983) 532.
[15] M. Sato, H. Ogasawara, K. Kato, M. Sakai, T. Kato, Chem. Pharm. Bull. 31 (1983) 4300.
[16] V.B. Di Marco, A. Tapparo, G.G. Bombi, Ann. Chim. (Rome) 89 (1999) 397.
[17] V.B. Di Marco, A. Tapparo, G.G. Bombi, Ann. Chim. (Rome) 91 (2001) 595.
[18] G.J. Janz, Silver–silver halide electrodes, in: D.J.G. Ives, G.J. Janz (Eds.), Reference Electrodes: Theory and Practice, Academic Press, New York, London, 1961.
[19] G. Biedermann, L.G. Sillén, Ark. Kemi 5 (1953) 425.
[20] V.B. Di Marco, A. Tapparo, G.G. Bombi, Ann. Chim. (Rome) 89 (1999) 535.
[21] V.B. Di Marco, Ph.D. Thesis, University of Padova, 1998.
[22] L.G. Sillén, Acta Chem. Scand. 18 (1964) 1085.
[23] W.H. Press, B.P. Flannery, S.A. Teukolsky, W.T. Vetterling, Numerical Recipes: The Art of Scientific Computing, Cambridge University Press, Cambridge, 1986.
[24] C.M. Flynn Jr., Chem. Rev. 84 (1984) 31.
[25] L.O. Öhman, Inorg. Chem. 27 (1988) 2565.
[26] A.K. Covington, M. Paabo, R.A. Robinson, R.G. Bates, Anal. Chem. 40 (1968) 700.
[27] R.A. Yokel, H.B. Kostenbauder, Toxicol. Appl. Pharmacol. 91 (1987) 281.
[28] C.A. Bunton, V.J. Shiner Jr., J. Am. Chem. Soc. 83 (1961) 42.
[29] T. Kiss, E. Farkas, Perspect. Bioinorg. Chem. 3 (1996) 199.
[30] E.T. Clarke, A.E. Martell, Inorg. Chim. Acta 191 (1992) 57.