Comparative iron binding studies of bis- and tris(3-hydroxy-2-methylpyrid-4-ones) and desferrioxamine

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Abstract

The iron binding properties of three tetradentate and one hexadentate 3-hydroxy-2-methylpyrid-4-one chelators, intended for clinical use, were studied using visible spectrophotometry and compared to the iron binding of desferrioxamine at both acidic and neutral pH using Job plot estimations. The chelators bis(3-hydroxy-2-methylpyrid-4-one) (bis(L1)), nitrilotris[1-(2-ethyl)-3-hydroxy-2-methylpyrid-4-one] (tris(ethyl L1)), 1,4-bis(3-hydroxy-2-methylpyrid-4-one)butane (bis(ethyl L1)) and 1,6-bis(3-hydroxy-2-methylpyrid-4-one)hexane (bis(propyl L1)) appear to form a mixture of iron complexes at pH 2 of more than one of the following chelator:iron stoichiometries: 1:1, 1:2 and 2:3. In contrast, at pH 7.4, bis(L1) and tris(ethyl L1) appear to form a complex with a higher chelator:iron molar ratio of 3:2. Desferrioxamine differed from the oligomeric 3-hydroxy-2-methylpyrid-4-ones by forming a 1:1 chelator:iron complex both at pH 2 and 7.4. The differences in the stoichiometry of iron complexes between desferrioxamine and oligomeric 3-hydroxy-2-methylpyrid-4-one may have implications in the mode of action of these chelators *in vivo*. In particular the higher iron binding capacity of oligomeric 3-hydroxy-2-methylpyrid-4-ones at acidic pH may have application in the treatment of acute iron poisoning and other conditions of iron overload caused by increased gastrointestinal absorption.

Introduction

One of the most potent groups of chelators intended for the treatment of transfusional iron overload and other diseases of iron imbalance and toxicity is the α-ketohydroxypyridines [1]. Three subclasses of the α-ketohydroxypyridines have been designed and tested so far, namely, the 1-hydroxypyrid-2-ones, 1-substituted-3-hydroxypyrid-2-ones and the 1-substituted-2-alkyl-3-hydroxypyrid-4-ones [1–6]. Of these, some of the 1-substituted-2-alkyl-3-hydroxypyrid-4-ones have been shown earlier to be particularly promising candidates for use as orally active chelators in the treatment of transfusional iron overload [7].

Desferrioxamine (DF) which is currently the drug employed in cases of transfusional iron overload and acute iron poisoning has several drawbacks. It is very expensive, has to be administered parenterally and has side effects especially in children or patients with low iron stores [8–10].

Other chelators have also been tested for oral activity in animals and man including pyridoxal isonicotinoyl hydrazone, desferrithiocin, 2,3-dihydroxybenzoic acid, cholylhydroxamic acid and DTPA [11–13]. Such chelators, however, have not reached the same stage of development as the orally active 1,2-dimethyl-3-hydroxypyrid-4-one (L1), which was shown to be effective in iron removal from thalassaemia [14], rheumatoid arthritis [15] and other iron loaded patients [16]. L1 and other bidentate 1,2-dialkyl-3-hydroxypyrid-4-ones were previously shown to form 3:1 chelator:iron complexes at pH 7.4 and 1:1 complexes at pH 2 [1, 3, 17]. In contrast to bidentate chelators, tetradentate and hexadentate derivatives may have certain advantages, in particular a higher iron binding capacity and greater stability at low concentrations [1, 18].

In an attempt to investigate the prospect of designing more potent chelators than L1, we set out to synthesize oligomers built up of L1-type monomers as previously suggested [1, 16]. This present paper describes the iron-binding and other physicochemical properties of such oligomers and compares their iron binding properties to desferrioxamine. Details on the possible uses of specific chelators designed for particular diseases of iron imbalance and toxicity have been previously discussed [3, 19, 20].

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Experimental

Maltol (3-hydroxy-2-methylpyr-4-one) used in the synthetic part of this work was obtained from Forum Chemicals Ltd. (Redhill, U.K.). Phosphate buffer saline (PBS) tablets were obtained from Oxoid (Basington, U.K.). The bis- and tris(3-hydroxy-2-methylpyrid-4-ones) studied were: bis(3-hydroxy-2-methylpyrid-4-one) (bis(L1)); nitrilotris[1-(2-ethyl)-3-hydroxy-2-methylpyrid-4-one] (tris(ethyl L1)); 1,4-bis(3-hydroxy-2-methylpyrid-4-one)butane (bis(ethyl L1)) and 1,6-bis(3-hydroxy-2-methylpyrid-4-one)-hexane (bis(propyl (L1)) (Fig. 1). Melting points (uncorrected) were determined on an Electrothermal melting point apparatus. UV, IR (KBr disc), ^1H NMR (in d_6-DMSO with internal TMS) and mass spectra were determined on Unicam SP 1700, Unicam SP 1000, Brucker WM 250 and VG Analytical ZAB SE instruments, respectively (Table 1).

Bis(3-hydroxy-2-methylpyrid-4-one) (bisL1))

Maltol (30.0 g, 0.238 mol) and hydrazine monohydrate (11.5 ml, 1.0 equiv.) were refluxed in water (200 ml) for 21 h. Evaporation *in vacuo*, washing (methanol, then dichloromethane (3×100 ml)) and recrystallization twice from boiling methanol and decolorizing charcoal gave pale brown granules of bis(L1) (1.27 g, 4%).

Nitrilotris[1-(2-ethyl)-3-hydroxy-2-methylpyrid-4-one] (tris(ethyl L1))

Benzyl maltol (30.0 g, 0.139 mol) [1] and tris(2-aminoethyl)amine (7.40 ml, 0.357 equiv.) were left to stand in ethanolic aqueous sodium hydroxide (H_2O (1250 ml):NaOH (12.5 g, 2.25 equiv.):EtOH (625 ml)) at room temperature for 2 weeks. The pH was adjusted to pH 4 using 11 M hydrochloric acid (34 ml), decolorizing charcoal added, placed in a hot water-bath for 30 min, filtered and evaporated to *c.* 70 ml. Deprotection using 100 ml of 11 M hydrochloric acid over a steam-bath for 1–2 h, followed by neutralization with ammonia solution to pH 8 (15 M, 74 ml) and evaporation gave a brown solid. Ammonium chloride was removed by repeated dissolution in hot methanol, partial evaporation and filtration, leaving a light brown solid together with a brown syrup. Recrystallization of both components (hot water) gave a light brown powder (0.59 g) and fluffy white needles (0.46 g), respectively, of tris(ethyl L1) (1.05 g total, 5%).

1,4-Bis(3-hydroxy-2-methylpyrid-4-one)butane (bis(ethyl L1))

Benzyl maltol (32.4 g, 0.15 mol) and 1,4-diaminobutane (6.93 g, 0.525 equiv.) were left to stand in ethanolic sodium hydroxide (H_2O (1350 ml):NaOH (13.5 g, 2.25 equiv.):EtOH (675 ml)) at room temperature in the dark for 5 days. Suction-filtration gave cream needles of dibenzyl bis(ethyl L1) (14.49 g, 40%). Deprotection using 5.5 M hydrochloric acid (800 ml) over a steam-bath for 3 h, followed by filtration and partial evaporation gave light brown granules (8.72 g, 19%). Part (1.00 g) was recrystallized in 20 ml of hot water and decolorizing charcoal to give grey granules of bis(ethyl L1) (0.40 g).

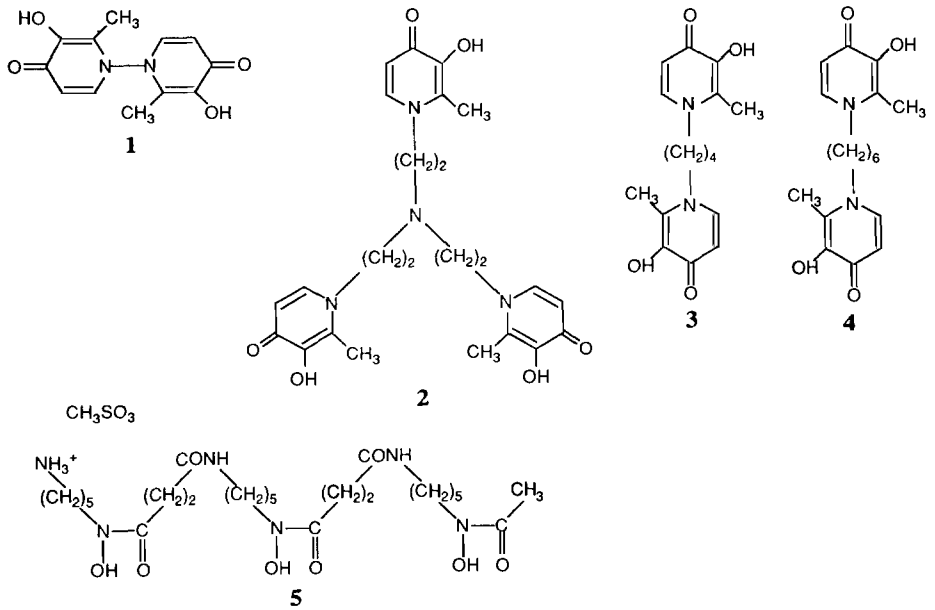

Fig. 1. Structure of bis(L1) (**1**), tris(ethyl L1) (**2**), bis(ethyl L1) (**3**), bis(propyl L1) (**4**) and desferrioxamine (**5**).

TABLE 1. Physicochemical parameters of bis- and tris(3-hydroxypyrid-4-ones)

Chelator	Bis(L1)	Tris(ethyl L1)	Bis(ethyl L1)	Bis(propyl L1)
Melting point (°C)	266–277 (blackens)	271–275.5 (decomp.)	280–281 (decomp.)	264–268 (decomp.)
Infrared				
C=O	1649	1630	1637	1634
(KBr; cm^{-1})	1623			1632
^1H NMR[a]				
Ring CH_3	2.17 (6 H, s)	2.27 (9 H, s)	2.54 (6 H, s)	2.53 (6 H, s)
Ring H-5	6.08 (2 H, d)	6.08 (3 H, d)	7.31 (2 H, d)	7.29 (2 H, d)
Ring H-6	7.39 (2 H, d)	7.36 (3 H, d)	8.24 (2 H, d)	8.24 (2 H, d)
Mass spectra	249	471	305	333
m/e (FAB)[b]	[(*M*+H), 0]	[(*M*+H), 10]	[(*M*+H), 100]	[(*M*+H), 100]
[Abund. %]	126 [(*M*/2+2H), 100]			
Ultraviolet[c]				
λ_{max} (nm)	274	287	284	283
ϵ (M^{-1} cm^{-1})	25600	40800	21500	22200
solvent	H_2O	CH_3OH	CH_3OH	CH_3OH

[a]250 MHz ^1H NMR spectra are quoted in ppm and were obtained in d_6-DMSO with internal TMS. All doublets have *J* values of 7 Hz. [b]FAB = fast atom bombardment. [c]Desferrioxamine had λ_{max} and ϵ values of 203 nm and 22800 in methanol, with 201 nm and 35200 in phosphate buffered saline.

1,6-Bis(3-hydroxy-2-methylpyrid-4-one)hexane (bis(propyl L1))

Benzyl maltol (10.80 g, 0.05 mol) and 1,6-diaminohexane (3.05 g, 0.525 equiv.) were left to stand in ethanolic aqueous sodium hydroxide (H_2O (450 ml):NaOH (4.50 g, 2.25 equiv.):EtOH (225 ml)) at room temperature in the dark for 1 week. Suction-filtration gave orange crystals of dibenzyl bis(propyl L1) (2.73 g, 11%). Deprotection of part (1.00 g) using 100 ml of 2 M hydrochloric acid over a steam-bath for 2 h, followed by filtration and evaporation *in vacuo* gave a brown solid, bis(propyl L1) (0.49 g, 76%).

pK_a Determination of bis(L1) and tris(ethyl L1)

Bis(L1) (1 ml; 2 mM in 0.1 M NaCl) was diluted (49 ml 0.1 M NaCl) and titrated with capillary tube aliquots of acid (11 M HCl) and alkali (5 M NaOH) to effect pH changes of *c.* 0.5 over a pH range 1.5–12.0. A returnable aliquot was withdrawn at each pH and the UV spectrum run at 210–350 nm. Spectra were superimposed. One or more wavelengths were then selected from the spectra (e.g. 275 and 305 nm) such that a plot of absorbance against pH gave an inflection [1]. Maximum gradient gave pK_a (1) 3.62, pK_a (2) 9.77 (A_{275} plot) and pK_a (2) 9.77 (A_{305} plot). Tris(ethyl L1) (2 ml; 1 mM in 0.1 M NaCl) diluted in 48 ml 0.1 M NaCl was investigated similarly, to give pK_a (1) 2.92 (A_{288}), pK_a (1) 2.88 (A_{292}) and pK_a (2) 9.76 (A_{310}).

pH titration curves for the iron(III) complexes of desferrioxamine, bis(L1) and tris(ethyl L1)

Freshly prepared iron(III) chloride (25 ml, 0.5 mM in 0.1 M NaCl) was added to the chelator (25 ml; 2 mM in 0.1 M NaCl) and the complex titrated at pH intervals of 0.5 over a pH range of *c.* 1.0–12.5 using 11 M HCl and 5 M NaOH. An aliquot was withdrawn at each pH and a visible spectrum run (350–750 nm), before the aliquot was replaced. Spectra were superimposed. A plot was made of absorption at λ_{max} against pH.

Preparation of chelator iron complexes

The stoichiometry of the chelator:iron complexes was estimated using Job plots [21]. Aqueous solutions of chelator (e.g. DF (4 mM) or bis(ethyl L1) (1 mM)) and freshly prepared equimolar iron(III) chloride solutions, both made up in 0.1 M NaCl, were mixed in duplicate (A and B) in the following ratios (chelator (ml):iron (ml)): (1) 5:0; (2) 4.5:0.5; (3) 4:1; (4) 3.75:1.25; (5) 3.5:1.5; (6) 3.33:1.67; (7) 3:2; (8) 2.75: 2.25; (9) 2.5: 2.5; (10) 2.25:2.75; (11) 2:3; (12) 1.5:3.5; (13) 1:4; (14) 0.5:4.5; and (15) 0:5, followed

by addition of acid (5 ml; *c.* 20 mM HCl) to sample A (pH 2) and 4×PBS (5 ml) phosphate buffered saline to sample B (pH 7.4). Complexes of tris(ethyl L1) (2 mM) and bis(propyl L1) (1 mM) were similarly prepared using half volumes. Spectra were obtained between 350–750 nm, and plots made of absorbance at a fixed wavelength (λ_{max} for most peaks) against mole fraction of chelator.

Results and discussion

Chelator pK_as and chelator–iron pH titration curves

The pK_as of bis(L1) and tris(ethyl L1) determined from their pH titration curves using UV spectroscopy, were found to be similar to corresponding values for L1 as shown in Table 2 [17]. This indicates that L1 and related 3-hydroxy-2-methylpyrid-4-one oligomers will be neutral at physiological pH and positively charged at acidic conditions of pH 1–2. In contrast DF will be positively charged under the same conditions at pH 1–8 because of the positive charge of its protonated terminal amino group (Fig. 1).

Chelator–iron pH titration curves obtained from visible spectroscopy for bis(L1), tris(ethyl L1) and DF are shown in Fig. 2. Each curve consists of a steep incline, a plateau and a tail. The incline at acidic pH, can be explained in terms of a mixture of different coloured complexes of low chelator:iron ratios. Colours ranged from violet for bis(L1), purple for tris(ethyl L1) and deep-orange for DF at the foot of the incline to orange (in all cases) at the top. The plateau region, by contrast, shows constant absorbance and hence constant chelator:iron stoichiometric complexes of orange colour. A slight reduction in absorbance at the tail (basic pH) shows the presence of less complex which is due to the strongly alkaline conditions where hydroxide ions can compete effectively with the chelator for iron, resulting in a decrease in the chelator–iron complex formation. Solutions producing such tails were orange for DF and tris(ethyl L1) and orange–brown for bis(L1). The stability of the main chelator iron complexes, i.e. 1:1 for DF and 3 chelator:2 iron for bis(L1) and tris(ethyl L1) both at acidic and basic pH regions, was variable. The main bis(L1) iron

TABLE 2. pK_a Values for bis(L1), tris(ethyl L1) and L1

Chelator	pK_a (1)	pK_a (2)
Bis(L1)	3.6	9.8
Tris(ethyl L1)	2.9	9.8
L1	3.3	9.7

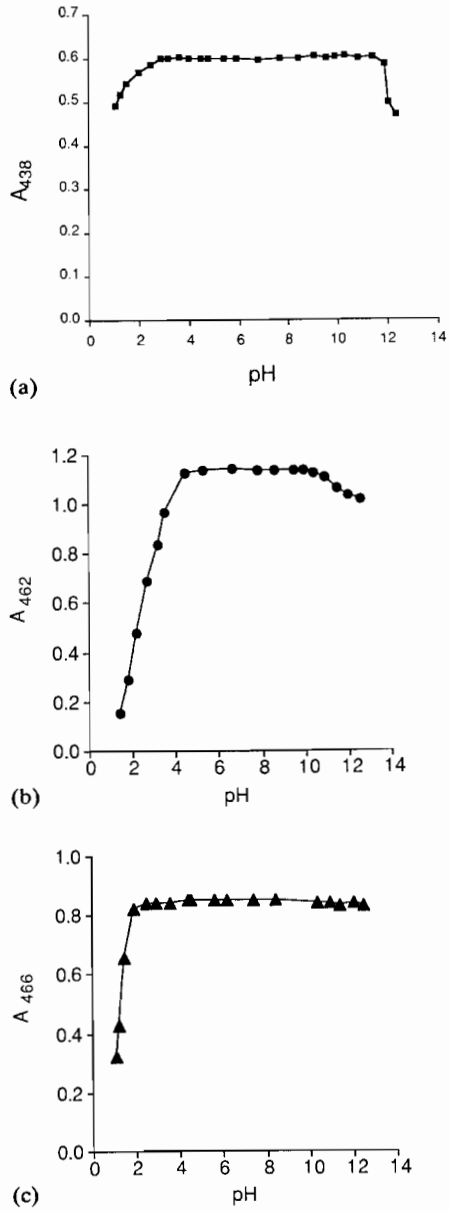

Fig. 2. pH Titration curves for bis(L1) [●], tris(ethyl L1) [▲] and desferrioxamine [■].

complex was stable between pH 4.5–11.0, the DF–iron complex between pH 3–11.5 and the tris(ethyl L1) between pH 2.5–12.5. Overall the tris(ethyl L1) iron complex appears to be the most stable with respect to both acidic and basic dissociation conditions.

Chelator–iron stoichiometry by Job plots

Chelator–iron stoichiometries of the iron(III) chloride complexes of bis(L1), tris(ethyl L1) and DF were estimated by the Job plots shown in Fig. 3. Some are single curves (Fig. 3(b), 3(d) and 3(h)) whilst others are made up of 2 curves (Fig. 3(a),

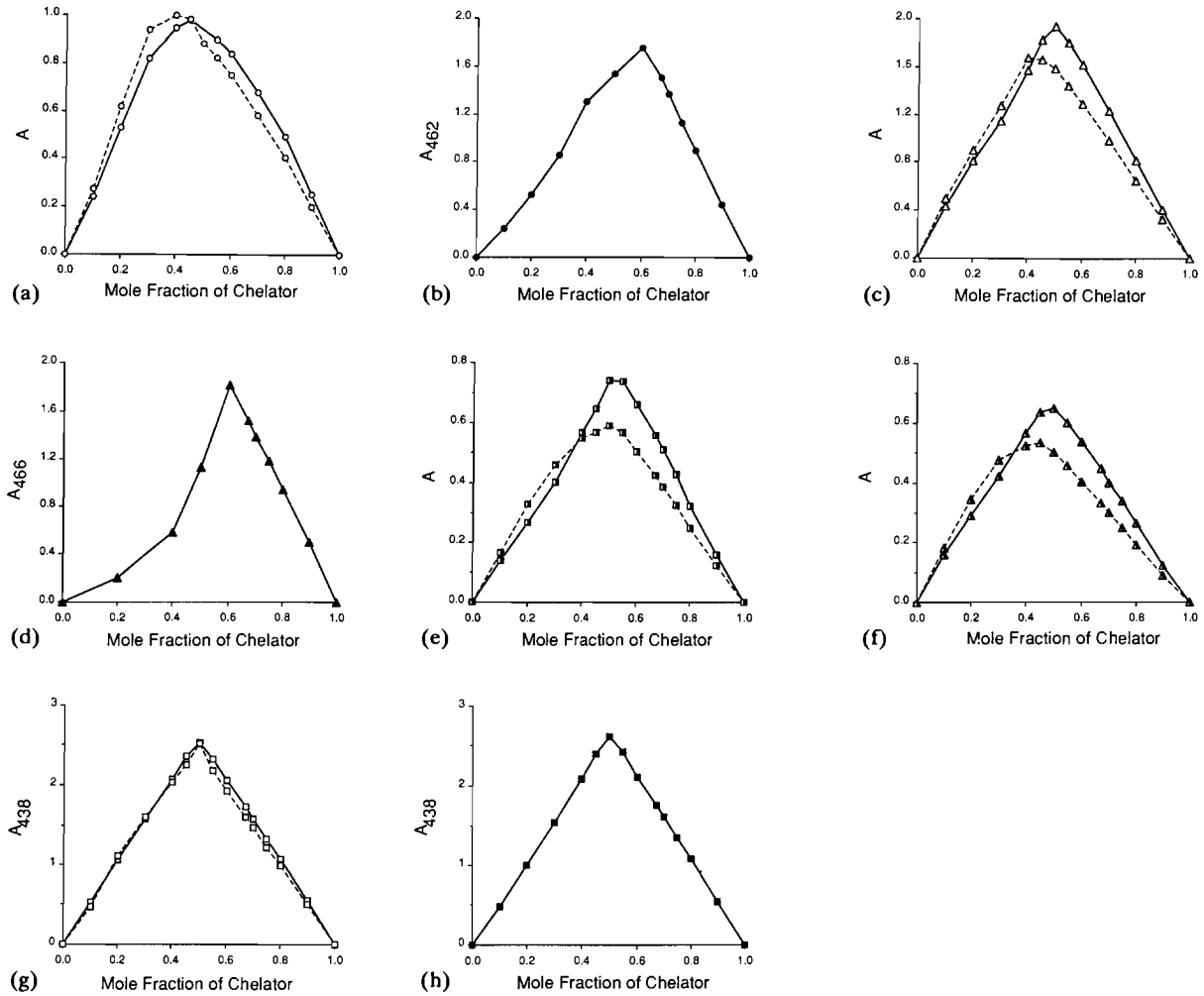

Fig. 3. Job plots of the iron(III) complexes of: (a) bis(L1) at pH 2 at 524 (solid line) and 568 (broken line) nm; (b) bis(L1) at pH 7.4 at 462 nm; (c) tris(ethyl L1) at pH 2 at 466 (solid line) and 506 (broken line) nm; (d) tris(ethyl L1) at pH 7.4 at 466 nm; (e) bis(ethyl L1) at pH 2 at 517 (solid line) and 569 (broken line) nm; (f) bis(propyl L1) at pH 2 at 520 (solid line) and 577 (broken line) nm; (g) DF at pH 2 at 438 (solid line) and 464 (broken line) nm; (h) DF at pH 7.4 at 438 nm.

3(c), 3(e), 3(f) and 3(g)). The single curves were obtained at pH 7.4 from the visible spectrum which had a constant λ_{max}. In contrast visible spectra at pH 2 showed that λ_{max} was variable. Highest and lowest λ_{max} values were therefore selected to take into consideration the different complexes present. This resulted in two Job plots at pH 2, one at each λ_{max}.

Theoretical curves, with which the Job plots were compared, comprised plots of absorbance (proportional to complex concentration) against chelator mole fraction which peaked linearly at a chelator:iron ratio equal to the chelator:iron stoichiometry of the complex [5].

Application of this analysis to Fig. 3(a) (bis(L1), pH 2) suggested a chelator:iron stoichiometry of 1:1 (524 nm) and 2:3 (568 nm). This contrasted with a chelator:iron stoichiometry of 3:2 for bis(L1) at pH 7.4 (Fig. 3(b)). The chelator:iron stoichiometry of the tris(ethyl L1) appeared to be similarly affected by pH, resulting in a mixture of 1:1 and 2:3 (Fig. 3(c)) complexes at pH 2.0 and a 3:2 complex at pH 7.4 (Fig. 3(d)). A proposed structure for the 3:2 tris(ethyl L1):iron complex at pH 7.4, based on Dreiding-type models is shown in Fig. 4, where iron is situated in an octahedral arrangement of six oxygens from three pyridine rings. Bis(ethyl L1) and bis(propyl L1) at pH 2 also gave mixtures of 1:1 and 2:3 chelator:iron complexes (Fig. 3(e) and 3(f)), although a complex of 1 bis(propyl L1):2 iron could not be ruled out (Fig. 3(f)). The remaining Job plots refer

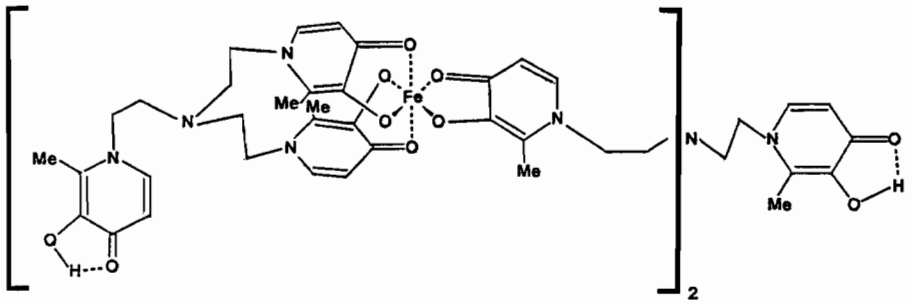

Fig. 4. Proposed structure of the tris(ethyl L1):iron(III) (3:2) complex formed at pH 7.4 where it is assumed that two of three 3-methyl-3-hydroxypyrid-4-one rings of tris(ethyl L1) are involved in iron binding. The unit shown in brackets is repeated.

to DF at pH 2 (Fig. 3(g)) and 7.4 (Fig. 3(h)). In both cases the chelator:iron ratio was 1:1. Presumably at both pHs, DF acts as a multidentate chelator in which the six hydroxamate oxygens, arranged octahedrally, bind to iron [22]. It should be emphasized that the above suggested speciation of the chelator iron complexes obtained from Job plots at different pHs may be an oversimplification of a more diverse mixture of iron complexes. Confirmation for the presence of such iron complexes in solution could be obtained from the analysis of solid material isolated from such solutions by X-ray crystallography and other means.

These results indicate that all the oligomeric 2-methyl-3-hydroxypyrid-4-ones have higher iron binding capacity for iron than DF at pH 2.0. This property of the oligomeric 3-hydroxy-2-methylpyrid-4-ones may have a possible use in conditions of iron overload caused by increased gastrointestinal iron absorption or acute iron poisoning. In the former condition soluble forms of iron found in food are absorbed at a higher rate than in normal individuals. The solubility of iron from accidental oral iron poisoning increases at the acidic conditions of the stomach (pH 1–2), which subsequently results in increased iron absorption mainly from the intestine followed by iron loading and toxicity to the tissues. Administration of oligomeric 3-hydroxy-2-methylpyrid-4-ones could in principle block increased iron absorption by binding soluble iron in the stomach and later in the intestine. It can be envisaged that because of their higher binding capacity for iron at acidic pH these oligomeric chelators will be more effective than DF or bidentate α-ketohydroxypyridines on a mole:mole basis.

In conclusion bis- and tris(3-hydroxy-2-methylpyrid-4-ones) appear to be neutral at physiological conditions (pH 7.4) but charged at the acidic environment of the stomach (pH 1–2), similar to L1. Bis- and tris(3-hydroxy-2-methylpyrid-4-ones) form low chelator:iron stoichiometries of 2:3, 1:1 and 1:2 at pH 2 but a higher chelator:iron stoichiometry of 3:2 at pH 7.4. In contrast DF forms 1:1 chelator:iron complexes both at acidic and neutral pHs. The high capacity for iron by the oligomeric 2-methyl-3-hydroxypyrid-4-ones at acidic pH similar to that of the stomach may have a use in the prevention of increased gastrointestinal iron absorption and the treatment of acute iron poisoning.

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References

1 G. J. Kontoghiorghes, *Ph.D. Thesis*, Essex University, 1982, British Library Microfilm D66194/86.
2 G. J. Kontoghiorghes, *Lancet, ii* (1985) 454.
3 G. J. Kontoghiorghes, *Inorg. Chim. Acta, 135* (1987) 145.
4 G. J. Kontoghiorghes and L. N. Sheppard, *Inorg. Chim. Acta, 136* (1987) L11.
5 G. J. Kontoghiorghes, L. N. Sheppard and J. Barr, *Inorg. Chim. Acta, 152* (1988) 195.
6 R. C. Scarrow and K. N. Raymond, *Inorg. Chem., 27* (1988) 4140.
7 G. J. Kontoghiorghes, M. A. Aldouri, A. V. Hoffbrand, J. M. Barr, B. Wonke, T. Kourouclaris and L. N. Sheppard, *Br. Med. J., 295* (1987) 1509.
8 *WHO Bull., 61* (1983) 63.
9 N. F. Olivieri, R. J. Buncic, E. Chew, T. Gallant, R. V. Harrison, N. Keenan, W. Logan, D. Mitchell, G. Ricci, B. Skarf, M. Taylor and M. Freedman, *N. Engl. J. Med., 314* (1986) 869.
10 S. De Virgillis, M. Congia, F. Frau, F. Argiolu, D. Guiseppe, F. Cucca, A. Varsi, G. Sanna, G. Podda, M. Fodde, G. F. Piratsu and A. Cao, *J. Paediatr., 113* (1988) 661.

11 G. J. Kontoghiorghes and A. V. Hoffbrand, *Recent Adv. Haematol., 5* (1988) 75.
12 R. W. Grady, J. H. Graziano, G. P. White, A. Jacobs and A. Cerami, *J. Pharmacol. Exp. Ther., 205* (1988) 757.
13 M. J. Pippard, M. J. Jackson, K. Hoffman, M. Petrou and C. B. Modell, *Scand. J. Haematol, 36* (1986) 466.
14 G. J. Kontoghiorghes, A. N. Bartlett, A. V. Hoffbrand, J. G. Goddard, L. N. Sheppard, J. Barr and P. Nortey, *Br. J. Haematol., 76* (1990) 301.
15 G. Vreugdenhil, A. J. G. Swaak, G. J. Kontoghiorghes and H. G. Van Eijk, *Lancet, ii* (1989) 1398.
16 G. J. Kontoghiorghes, *Ann. N. Y. Acad. Sci., 612* (1990) 339.
17 G. J. Kontoghiorghes, L. N. Sheppard and S. Chambers, *Arzneim.-Forsch./Drug Res., 37* (1987) 1099.
18 J. Schubert, in F. Gross (ed.), *Iron Metabolism*, Ciba Symposium, Springer, Basel, Switzerland, 1984, p. 466.
19 G. J. Kontoghiorghes, in C. R. Evans (ed.), *Free Radicals Oxidant Stress and Drug Action*, Richelieu Press, London, 1987, p. 277.
20 G. J. Kontoghiorghes, *Arzneim.-Forsch./Drug Res., 40* (1990) 1332.
21 P. Job, *Ann. Chim., (Paris), 9* (1928) 113.
22 R. Van der Helm and M. Poling, *J. Am. Chem. Soc., 98* (1976) 82.