Synthetic Methods and *In Vitro* Iron Binding Studies of the Novel 1-Alkyl-2-ethyl-3-hydroxypyrid-4-one Iron Chelators

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Abstract

Three novel iron chelators namely the 1-methyl-, 1-ethyl- and 1-propyl-2-ethyl-3-hydroxypyrid-4-ones were prepared in high yields from ethyl maltol and the related alkylamine in a one step reaction. These chelators formed 3 chelator:1 iron stable, coloured, neutral complexes at physiological pH and mobilise iron from transferrin, ferritin and haemosiderin. The rate of iron mobilisation from these proteins was of the order transferrin > haemosiderin > ferritin. The cheap synthesis and strong iron binding properties of the 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones at physiological pH requires the need for further investigation and development of these compounds and their homologues, for the treatment of iron overload and other diseases of iron imbalance and toxicity.

Introduction

The basic properties of an ideal iron chelator intended for clinical use are inexpensive synthesis, oral activity and low toxicity. The design of such chelators would save the lives of many iron loaded thalassaemia patients who would otherwise die untreated because of the high cost of desferrioxamine or due to non compliance with the 8–10 h long daily infusion treatment with this drug [1, 2].

Iron has also been implicated in the pathogenesis of several other diseases, where its catalytic formation of toxic oxygen activated products such as superoxide, hydrogen peroxide and hydroxyl radical may result in tissue damage [3, 4]. The design of site-specific chelators may be needed to deal with such diseases [5]. Other uses of chelators include the removal of other toxic metals such as Pu and Al [6, 7] which have similar metabolic pathways to iron and the design of lipophilic iron complexes for the treatment of iron deficiency anaemia.

The design of a new potent group of iron chelators, namely the α-ketohydroxypyridines by Kontoghiorghes [8–11] and the identification of the 1-alkyl-2-methyl-3-hydroxypyrid-4-ones as the most promising sub group in the mobilisation of iron *in vivo* [12–14] led us recently to undertake the first clinical trials in the treatment of iron overload using the oral chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1) [15] and the design of new 1,2-dialkyl-3-hydroxypyrid-4-ones.

In this paper we report the synthesis and characterisation of a novel group of iron chelators namely the 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones. In addition we have examined the iron complex formation and the iron mobilisation properties of these chelators from transferrin, ferritin and haemosiderin *in vitro*.

Experimental

1-Methyl-, 1-ethyl- and 1-propyl- 2-ethyl-3-hydroxypyrid-4-ones were prepared from ethyl maltol (Pfizer, U.K.) using the same method as that previously described for the preparation of 1-alkyl-3-hydroxy-2-methylpyrid-4-ones from maltol [16] as follows.

2-Ethyl-3-hydroxy-1-methylpyrid-4-one

This compound was prepared from ethyl maltol (30.0 g), 40% aqueous methylamine (55.4 ml, 3 equiv) and water (600 ml) by refluxing for 7 h. The mixture was then allowed to cool, rotary evaporated *in vacuo* forming a thick black paste which on three recrystallisations from methanol gave white crystals.

1,2-Diethyl-3-hydroxypyrid-4-one and 2-ethyl-3-hydroxy-1-propylpyrid-4-one

These compounds were similarly prepared from 70% aqueous ethylamine (3 equiv) and neat propylamine (3 equiv) respectively, using the same method as for 2-ethyl-3-hydroxy-1-methylpyrid-4-one giving in both cases, white crystals. Yields, % yields, melting point, infrared and proton NMR for each of these three compounds are shown in Tables I–III.

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Iron Binding Studies

pH titration curves of the chelator–iron complexes were obtained using a mixture of the chelators (25 ml, 2 mM in 0.1 M NaCl) and freshly prepared iron(III) chloride solution (25 ml, 0.5 mM in 0.1 M NaCl), and titrating with HCl (10 M) and NaOH (5 M) (Fig. 1). Job plots of the chelators (0–5 ml, 2 mM in 0.1 M NaCl) and iron (0–5 ml, 2 mM in 0.1 M NaCl) at selected molar ratios were carried out at pH 7.3 using 4 X PBS (phosphate buffered saline, 5 ml) as previously described [17]. These were compared to theoretical plots of 1:1, 2:1 and 3:1 chelator:iron complexes (Fig. 2). The n-octanol/water (PBS) partition coefficients (*K* par) of the chelators and their iron complexes were determined using a previously described method [8].

TABLE I. Yields of 1,2-Dialkyl-3-hydroxypyrid-4-ones Obtained from 2-Alkyl-3-hydroxypyr-4-ones and Alkylamines

3-Hydroxypyrid-4-one	Yield (g)/yield(%) (number of crystallisations) obtained from quantity of starting material 2-alkyl-3-hydroxypyr-4-one	
	30.00 g	80.00 g
2-Ethyl-1-methyl	4.64/14.2(3)	28.98/33.1(5)
1,2-Diethyl	1.93/5.4(3)	20.70/21.7(5)
2-Ethyl-1-propyl	2.00/5.2(2)	13.47/13.0(3)
1,2-Dimethyl	19.27/58.2(2)	54.92/62.2(2)
1-Ethyl-2-methyl	16.56/45.5(3)	33.78/37.9(3)[a]
2-Methyl-1-propyl	10.56/26.6(2)	21.72/32.8(2)[b]

[a]From 70.00 g maltol. [b]From 50.00 g maltol.

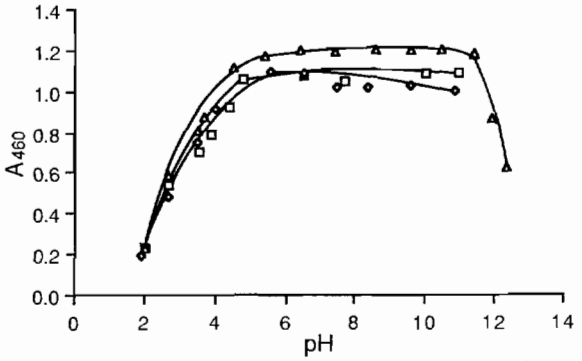

Fig. 1. pH titration of the chelator iron complexes. Chelators (2 mM in 0.1 M NaCl, 25 ml) and freshly prepared iron(III) chloride (0.5 mM in 0.1 M NaCl, 25 ml) were mixed and titrated with HCl (10 M) and NaOH (5 M). The absorbance of the chelator iron mixture at 460 nm was measured after each pH change. 1-Methyl- (△), 1-ethyl- (□) and 1-propyl- (◇) -2-ethyl-3-hydrodypyrid-4-ones.

TABLE II. Infrared, Mass Spectroscopic[a] and Melting Point Data for 1-Alkyl-2-ethyl-3-hydroxypyrid-4-ones

2-Ethyl-3-hydroxypyrid-4-one	ν_{max} (KBr, cm^{-1})	M^+ (relative abundance)	Melting point (°C)
1-Methyl	3480 (O–H stretch) 1627 (C=O stretch)	153 (100%)	199–202[b]
1-Ethyl	3480 (O–H stretch) 1621 (C=O stretch)	167 (68%)	179–182[b]
1-Propyl	3480 (O–H stretch) 1626 (C=O stretch)	181 (35%)	129–132

[a](I) (Found: 153.0781. $C_8H_{11}NO_2$ requires M^+ 153.0790); (II) (Found: 167.0953. $C_9H_{13}NO_2$ requires M^+ 167.0946); (III) (Found: 181.1103. $C_{10}H_{15}NO_2$ requires M^+ 181.1103). [b]With decomposition.

TABLE III. 250 MHz ^1H NMR Data for 1-Alkyl-2-ethyl-3-hydroxypyrid-4-ones Recorded in D_2O (ppm)[a]

2-Ethyl-3-hydroxypyrid-4-one	1-Alkyl	2-CH_2CH_3	H-5	H6
1-Methyl	3.68 (3H, s, N–CH_3)	1.05 (3H, t, *J* 8 Hz, –CH_3) 2.71 (2H, q, *J* 8 Hz, –CH_2)	6.35 (d, *J* 7 Hz)	7.46 (d, *J* 7 Hz)
1-Ethyl	1.25 (3H, t, *J* 7 Hz, –CH_2CH_3) 3.98 (2H, q, *J* 7 Hz, –CH_2CH_3)	1.06 (3H, t, *J* 8 Hz, –CH_3) 2.71 (2H, q, *J* 8Hz, –CH_2)	6.39 (d, *J* 7 Hz)	7.52 (d, *J* 7 Hz)
1-Propyl	0.78 (3H, t, *J* 7 Hz, –$CH_2CH_2CH_3$) 1.64 (2H, tq, *J* 7 Hz, –$CH_2CH_2CH_3$) 3.90 (2H, t, *J* 8 Hz, –$CH_2CH_2CH_3$)	1.06 (3H, t, *J* 8 Hz, –CH_3) 2.71 (2H, q, *J* 7 Hz, –CH_2)	6.38 (d, *J* 7 Hz)	7.51 (d, *J* 7 Hz)

[a]Multiplicities s, d, t and q denote singlet, doublet, triplet and quartet, respectively, reported from HOD at 4.65 ppm.

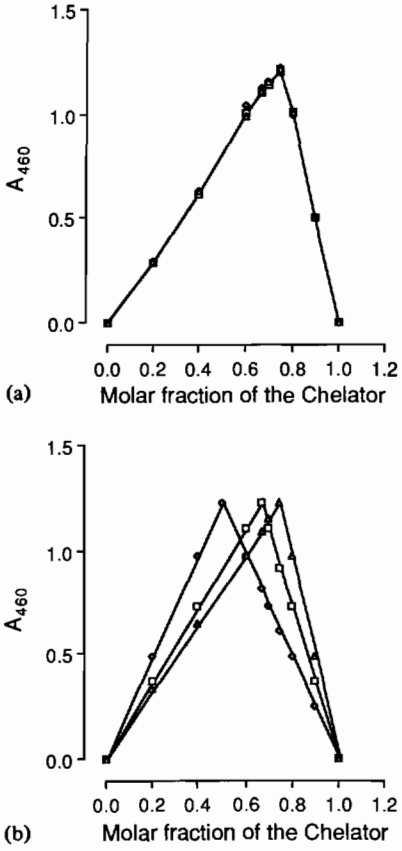

Fig. 2. (a) Job plots of the 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones with iron at pH 7.3. Chelators (2 mM in 0.1 mM NaCl 0–5 ml) and iron (2 mM in 0.1 M NaCl, 0–5 ml) were mixed at different molar ratios to a final volume of 5 ml. 4 X PBS (5 ml), pH 7.3 was then added and the absorbance at 460 nm measured. 1-Methyl- (△), 2-ethyl- (□), 1-propyl- (◇) 2-ethyl-3-hydroxypyrid-4-ones. (b) Theoretical plots of 1:1, 2:1 and 3:1 chelator:iron complexes. Theoretical Job plots for 1:1, 2:1, 3:1 chelator:iron complexes were obtained using the maximum absorbance values from the Job plots in (a): 1:1 (◇), 2:1 (□), 3:1 (△).

Iron Mobilisation from Transferrin, Ferritin and Haemosiderin

Human apotransferrin (Sigma) was radioactively labelled with ^{59}Fe and saturated with iron as previously described [18]. Samples (1.5 ml) of the ^{59}Fe transferrin (0.54 mg protein, 0.75 μg iron, 0.07 μCi ^{59}Fe) were enclosed in a dialysis bag and dialysed against a chelator solution (10 ml, 4 mM) by continuous stirring at 37 °C. Samples (1 ml) of the dialysate were removed at different time intervals, the ^{59}Fe activity measured and then returned back into the incubation mixture. Ferritin solution and haemosiderin solid were isolated from a spleen of a thalassaemia patient [19] and samples containing the same amount of iron (0.25 mg, 1.5 ml) were dialysed at 37 °C against a chelator solution (4 mM, 10 ml) over a 12 day period. The amount of iron mobilised was

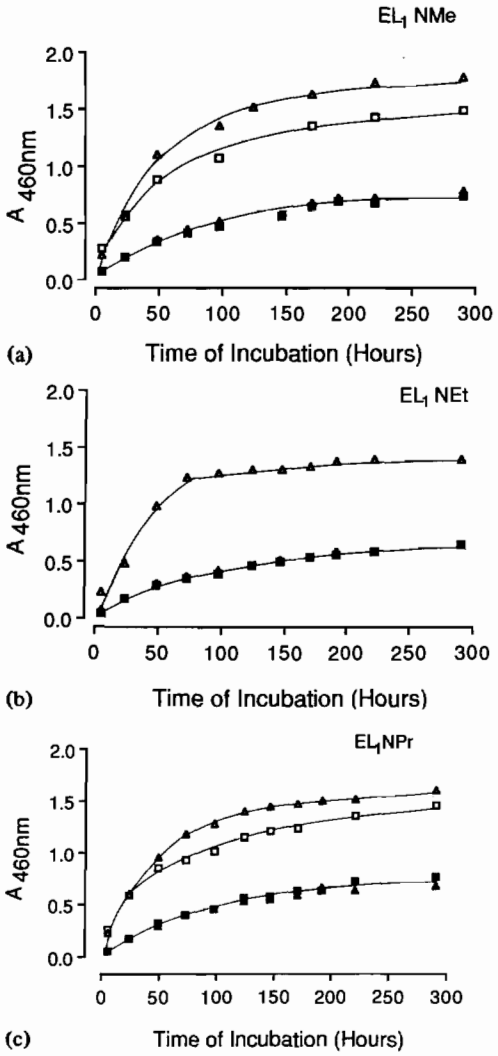

Fig. 3. Iron mobilisation from human ferritin and haemosiderin using 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones. Ferritin (0.25 mg iron, 10.7% *w/w* iron, 1.5 ml) and haemosiderin (0.25 mg iron, 16.5% *w/w* iron, 1.5 ml) were dialysed against a chelator solution (4×10^{-3} M, 10 ml) in 2 X PBS, pH 7.3, 37 °C. The progress of the reaction was followed by measuring the absorbance at 460 nm of the dialysate at different time intervals. EL_1NMe; 2-ethyl-3-hydroxy-1-methylpyrid-4-one; EL_1NEt; 1,2-diethyl-3-hydroxypyrid-4-one; EL_1NPr: 2-ethyl-3-hydroxy-2-propylpyrid-4-one; ferritin (▲■), haemosiderin (△□).

estimated spectrophotometrically by measuring the visible absorbance of the dialysate and by using the extinction coefficient of the chelator iron complex (Fig. 3, Table V). All the protein incubations with the chelators were carried out in 2 X PBS pH 7.3.

Results

All three 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones were easily prepared from ethyl maltol in large yields

using the single step reaction. These yields are comparable to those of 1-methyl-, 1-ethyl- and 1-propyl-2-methyl-3-hydroxypyrid-4-ones which are obtained under the same conditions from maltol (Table I). In most cases repeated recrystallisations were required to obtain pure, white crystals. The infrared, mass-spectroscopic, melting point and ^{1}H NMR data of the three new derivatives are shown in Tables II and III.

Coloured complexes were formed when mixing the 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones with iron which are characterised by similar pH titration curves (Fig. 1) containing a plateau region above and below the physiological pH. The Job plots at pH 7.4 revealed the formation of a tris chelator to one iron complex which predominates over this plateau region (Fig. 2). Lower than 3:1 chelator to iron ratio complexes are expected to be formed at acidic pHs similar to the 1-alkyl-3-hydroxy-2-methylpyrid-4-ones and 4-substituted-1-hydroxypyrid-2-ones [10, 17]. The pKs of all three 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones were very similar (Table IV). The pK of the hydroxyl group was sufficiently high (>9.0) for the formation of neutral molecules at physiological pH. In contrast, the n-octanol/water (PBS) partition coefficients of these chelators were found to increase with increasing the size of the 1-alkyl substitution (Table IV).

Iron mobilisation by the 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones from human transferrin progressively increased with time and was higher than 90% after 8 h of incubation (Table V). In contrast haemosiderin and ferritin iron release by these chelators was

TABLE IV. Physicochemical Properties of the 1,2-Dialkyl-3-hydroxypyrid-4-ones and their Iron Complexes

3-Hydroxypyrid-4-one	Extinction coefficient (M^{-1} cm^{-1})		pK_a(1)	pK_a(2)	K_{par}	
	chelator	iron complex			chelator	iron complex
2-Ethyl-1-methyl	13800 (282 nm)	4910 (470 nm)	3.7	9.8	0.4	0.02
1,2-Diethyl	14100 (282 nm)	5000 (470 nm)	3.7	10.1	1.4	0.28
2-Ethyl-1-propyl	14500 (283 nm)	5050 (470 nm)	3.4	10.1	4.2	9.68
1,2-Dimethyl	13300 (280 nm)	5040 (460 nm)	3.3	9.7	0.19	0.24
1-Ethyl-2-methyl	13800 (282 nm)	5140 (460 nm)	3.6	10.3	0.37	0.52
1-Propyl-2-methyl	14200 (282 nm)	5170 (460 nm)	3.7	10.2	3.16	4.12

TABLE V. Iron Mobilisation from Transferrin, Ferritin and Haemosiderin[a]

Percentage iron removal							
3-hydroxypyrid-4-one (4×10^{-3} M)	transferrin ^{59}Fe			haemosiderin		ferritin	
	1 h	2 h	8 h	24 h	12 d	24 h	12 d
1,2-Dimethyl	37.7	69.6	99.5				
1-Methyl-2-ethyl	36.0	56.6	100.0	26.7 25.2[b]	58.8 62.7[b]	9.4 9.9[b]	27.0 29.0[b]
1,2-Diethyl	24.9	51.8	96.6	23.1 22.7[b]	51.0 62.7[b]	8.1 8.5[b]	30.6 29.8[b]
1-Propyl-2-ethyl	23.2	50.3	97.6	27.8	74.5	8.5 8.3[b]	33.9 27.0[b]

[a]Haemosiderin (0.25 mg iron, 16.5% w/w iron, 1.5 ml). Ferritin (0.25 mg iron, 10.7% w/w iron, 1.5 ml) and transferrin (0.75 μg iron, 0.54 mg protein, 0.07 μCi ^{59}Fe, 1.5 ml) in dialysis bags were dialysed against the chelators (4×10^{-3} M, 10 ml) at 37 °C, pH 7.3 in 2 × PBS by continuous stirring. Iron mobilisation was estimated from samples of the dialysates taken at different time intervals as explained in 'Experimental'. [b]Duplicate samples.

much slower, and a smaller percentage of iron was released following several days of incubation (Fig. 3). Haemosiderin iron release was by comparison faster than ferritin as previously shown by other chelators [19].

Discussion

It was initially decided to proceed with the synthesis of new 1-alkyl substituted 2-ethyl-3-hydroxypyrid-4-ones because previously, 1-alkyl substituted 2-methyl-3-hydroxypyrid-4-ones were identified as more effective *in vivo* than other α-ketohydroxypyridines [14]. The quantitative synthesis of all three 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones from ethyl maltol in a one step reaction, which is similar to that of 1-alkyl-3-hydroxy-2-methylpyrid-4-ones from maltol, fulfills one of the basic criteria of iron chelators intended for clinical use, that is to be cheap as explained in the Introduction.

The 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones and their iron complexes were found to be highly stable in air and in solutions of variable pHs (Fig. 1, Table IV). Using a theoretical model the stoichiometry of the complex with iron was easily identified as neutral, with a 3:1 chelator:iron ratio at a wide range of pH (6–11), including the physiological pH (Fig. 2). This property ensures the maintenance of a buffer capacity by the neutral complex over a wide range of pH usually found in *in vivo* media. At physiological pH the neutral charge of the chelators and their iron complexes will also facilitate the permeability towards cell membranes, which, depending on the K_{par} of the molecules, could either result in an increase in iron donation (by lipophilic chelators) or iron withholding (by hydrophilic chelators) in cells [16, 20]. In addition, 2-ethyl-3-hydroxy-1-propylpyrid-4-one which is the most lipophilic of the three new derivatives is expected to be highly toxic *in vivo* as previously shown with the most lipophilic of the 1-alkyl-3-hydroxy-2-methylpyrid-4-ones, namely the 3-hydroxy-2-methyl-1-propylpyrid-4-one [16].

Substantial iron mobilisation from transferrin, ferritin and haemosiderin took place following their incubation with 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones. The ability of these chelators to mobilise transferrin iron makes them one of the most effective group of iron chelators known. Transferrin iron was more rapidly mobilised in comparison to that found as a polynuclear form in ferritin and haemosiderin. While almost all the iron was released from transferrin within 8 h (Table V), iron mobilisation from haemosiderin and ferritin was slow and only a fraction was mobilised following days of incubation (Fig. 3). Haemosiderin iron mobilisation was faster than ferritin which is in agreement with our previous observations [19]. It is expected therefore that *in vivo* iron would be available for chelation from all three iron pools. The comparative studies of iron mobilisation from transferrin, ferritin and haemosiderin, and also the iron binding affinities shown in the Job plot studies indicate that the three 1-alkyl-2-ethyl-3-hydroxypyrid-4-ones have similar kinetic and thermodynamic iron binding constants despite their differences in n-octanol/water partition. However, differences in K_{par} may result in differences in *in vivo* activity as previously suggested [14, 16]. Further work is planned for the *in vivo* evaluation of these three chelators and also the design and evaluation of other related derivatives from ethyl maltol.

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