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Novel iron-specific fluorescent probes

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Abstract—A series of novel iron-specific fluorescent probes is reported where the chelator function unusually forms part of the fluorescent moiety. The ability of this range of molecules to permeate human erythrocyte ghost membranes was investigated.

3-Hydroxypyridin-4-ones (HPOs) are currently one of the main candidate groups for the development of orally active iron chelators.[1] Indeed, the 1,2-dimethyl derivative CP20 (Deferiprone) is in clinical use (marketed by Apotex Inc., Toronto, Canada, as Ferriprox™).[2] As a variety of diseases such as thalassaemia, haemochromatosis and Parkinson's disease are associated with elevated levels of iron, there is an increasing demand for reliable methods for the measurement of intracellular iron pools. Such an analysis can act as a guide to the efficiency and site of action of iron-chelating pharmaceuticals. Numerous methods such as high-pressure liquid chromatography (HPLC),[3] spectrophotometry,[4] electron spin resonance (ESR)[4,5] and atomic absorption spectroscopy (AAS)[6] have been developed for such measurements. However, there are many disadvantages with these methods and this has led to the development of fluorescence-based methods.[7,8] Recently, we reported a series of iron-specific fluorescent probes, some of which are suitable for this type of measurement.[9,10] Until now, all iron-specific fluorescent probes have been composed of two covalently linked moieties, the fluorescent and chelating functions.[7–13] The introduction of fluorescent moieties (particularly the larger ones, for instance, fluorescein) appreciably influences the overall size of the probe molecule, and hence its biological properties. In the present paper, we report the synthesis of novel iron-specific fluorescent probes where the fluorescent moiety forms part of the chelating moiety and therefore have molecular weights lower than those of typical fluorescent probes. Such molecules have the potential for providing information on the intracellular distribution of related chelating agents.

The synthetic procedure utilized to prepare the parent compound **6** is summarized in Scheme 1. The 3-hydroxyl group of commercially available kojic acid **1** was protected using the benzyl group. The primary alcohol of the resulting protected kojic acid was oxidized to a carboxylic acid by Jones reagent. A conventional route to 4-pyridones is condensation of the corresponding 4-pyrone with ammonia or a primary amine in an EtOH–H_2O solvent.[14] We found that by increasing the proportion of EtOH, the reaction mixture turned darker and the yield of the expected pyridone **4** was low. For this reason, the acidic pyranone **3** was treated with ethylenediamine in a pure-water solvent at 60 °C for 4 h. This resulted in an improved yield, of over 95%, of the corresponding bicyclic lactam **5**. In contrast, if the reaction was undertaken at room temperature, the major product, which was not fluorescent, could be noncyclic pyridone **4**,[15] which produced a fluorescent product on heating (Scheme 1). The protecting benzyl group was removed by catalytic hydrogenation to yield the corresponding fluorescent chelator **6**.[16]

The fluorescence emission spectrum of **6** was found to be metal sensitive and marked fluorescence quenching was observed in the presence of ferrous ammonium sulfate (Fig. 1). This iron(II) was immediately oxidized after combining with the probe to form the corresponding iron(III) complex, even in the presence of a large amount of ascorbic acid. When the metal-to-ligand ratio was less than 1:3, a linear relationship between the fluorescence intensity of **6** and the iron concentration was observed (Fig. 1). The progressive quenching eventually

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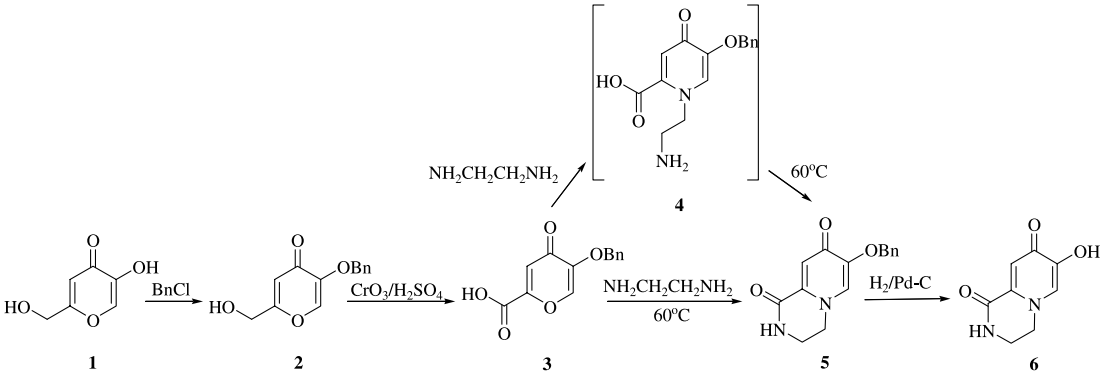


Scheme 1.

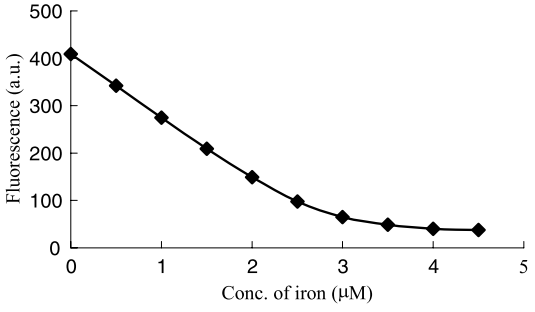
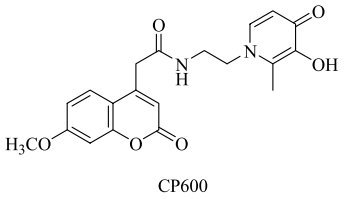


Figure 1. Fluorescence (λ_{ex} = 323 nm, λ_{em} = 464 nm) of **6** (10 μM) was quenched by iron(II) in imidazole buffer (10 mM) at pH 7.4.

leveled off when the metal-to-ligand ratio was greater than 1:3, suggesting that no additional free ligand was available to be quenched. The preliminary data indicate that compound **6** is behaving as a bidentate ligand.

The physiochemical properties of **6** and those of the closely related CP600[9] (Scheme 2) are compared in Table 1. Although the excitation wavelengths of **6** and CP600 are similar, the emission value of **6** is higher (464 nm) than that of CP600 (380 nm). This is an important difference since the influence of background autofluorescence in biological materials becomes markedly stronger at lower wavelengths. Two factors determine the fluorescence intensity, namely extinction coefficient and quantum yield. Although the extinction coefficient of **6** is less than that of CP600, the quantum yield of **6** was found to be much larger. Thus, when the same concentrations of **6** and CP600 are measured at identical instrument settings, the fluorescence intensity of **6** is slightly higher than that of CP600 (Fig. 2). Comparison of the iron(II)-induced quenching of both compounds demonstrated that the fluorescence of **6** (10 μM) was almost completely quenched in the presence of 10 μM iron(II) (Table 1 and Fig. 2).

To confirm the prediction that **6** will permeate cell membranes,[17] the permeability of **6** across red blood cell ghost membranes was investigated. Red cell ghosts were resealed in the presence of iron(II) (100 μM) and ascorbic acid (1 mM). After the removal of non-trapped iron, the ghost suspension was supplemented with 10 μM **6** with and without the impermeable non-fluorescent iron chelator, diethylenetriamine–pentaacetic acid (DTPA), and the resulting fluorescence of the suspension was followed with time (Fig. 3). The fluorescence was quenched to a greater extent in the absence of DTPA (Fig. 3-6A) because not only did the probe permeate into the re-



Scheme 2.

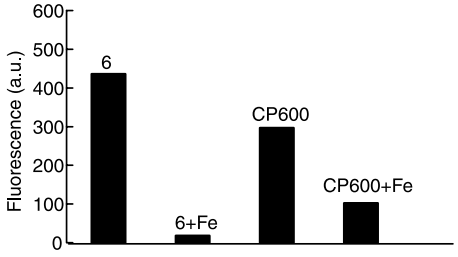


Figure 2. Comparison of the fluorescence intensity of **6** and CP600 (10 μM) before and after quenching by iron(II) (10 μM) in imidazole buffer (10 mM) at pH 7.4.

Table 1. Physiochemical properties of **6** and **CP600**

	Excitation wavelength (nm)	Emission wavelength (nm)	Extinction coefficient (M^{-1} cm^{-1})	Quantum yield	Quenching ratio (%)[a]	Molecular weight	*C*log *P*
6	323	464	4700	0.28	96	180	−2.55
CP600	325	380	12200	0.04	66	384	−0.48

[a] Quenching of fluorescence intensity (%) (metal-to-ligand molar ratio 1:3).

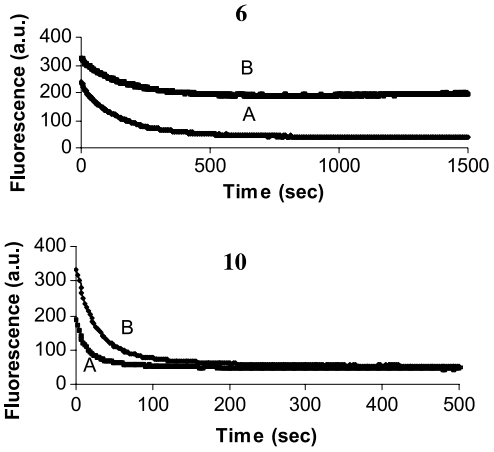


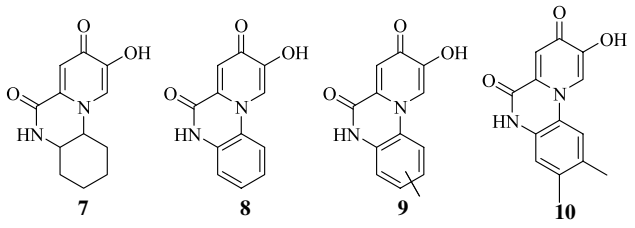
Figure 3. Fluorescence intensity of 10 μM **6** (λ_{ex} 324 nm; λ_{em} 464 nm) and 6 μM **10** (λ_{ex} 383 nm; λ_{em} 447 nm) recorded during incubation at 20 °C in the presence of suspension of washed human erythrocyte ghosts resealed in the presence of 100 μM iron(II) and 1 mM ascorbic acid. The suspension medium A also contained 25 mM MOBS and 1 mM ascorbic acid, and medium B contained 25 mM MOBS and 1 mM ascorbic acid plus 0.1 mM DTPA.

sealed ghosts to chelate iron but also the iron leaked from the ghosts to combine with the probe in the supernatant. The two processes are consistent with a fit of the respective data to a double exponential decay function, as shown in Table 2. However, in the presence of DTPA (Fig. 3-6B), the effluxed iron was preferentially bound to the high-affinity ligand, DTPA, and led to a single-exponential decay function, representing permeation of the fluorescent chelator into the resealed ghosts (Fig. 3 and Table 2).

Previous experiments on human erythrocyte ghosts indicate that enhanced lipophilicity of iron fluorescent probes led to enhanced membrane penetration.[10] To increase the lipophilicity of fluorescent probe **6**, more hydrophobic diamine derivatives were reacted with the acidic pyranone **3** to produce the corresponding bicyclic lactam derivatives **7**, **8**, **9** and **10** (Scheme 3). *C*log *P* values (calculated log *P* values; the logarithm of partition coefficients were calculated using Chemoffice 6.0 from Cambridgesoft Corporation, Cambridge, UK) for the molecules are −1.16, −1.28, −0.85 and −0.40, respectively. Compared with **6**, the most hydrophobic probe

Table 2. Permeability parameters for **6** and **10** across human erythrocyte ghost membranes

	−DTPA	+DTPA
6		
A_1 (a.u.)	127	117
t_1 (s)	108	127
A_2 (a.u.)	65	—
t_2 (s)	283	—
10		
A_1 (a.u.)	127	252
t_1 (s)	15	24
A_2 (a.u.)	14	—
t_2 (s)	207	—



Scheme 3.

10 penetrated the cell membranes much more quickly (Fig. 3 and Table 2) and therefore has greater potential as an intracellular iron probe. Studies are currently in progress with these probes in both hepatocytes and lymphocytes.

Acknowledgments

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