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Synthesis and Evaluation of Iron Chelators with Masked Hydrophilic Moieties

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Survival of virtually all living organisms depends on the uptake of iron from the environment. Iron is crucial for a wide variety of vital cell functions, ranging from oxygen metabolism and electron-transfer processes to DNA and RNA synthesis.[1−3] A surplus of iron, however, induces oxidative damage, rendering the intracellular scavenging of iron a major therapeutic target.[4] Current chelation therapies for iron overload are based on the removal of this excess iron by desferrioxamine (DFO). Although DFO is a highly effective iron chelator, its slow onset of acting, short plasma half-life, and prolonged parental administration of extensive dosages have triggered the search for improved efficient Fe chelators.[5] It occurred to us that we could contribute to this field of research and achieve progress on the basis of rapid cellular uptake and delayed exit of iron chelators, thereby possibly enhancing deactivation of toxic iron. Synthetic iron chelators based on the natural siderophore ferrichrome have been shown to bind Fe(III) with high affinity ($pK_f > 27$) and have shown no toxicity to mammalian cell cultures in vitro.[6]

In this communication, we present a new class of lipophilic ferrichrome analogues carrying acetoxymethyl (AM) ester moieties. We will demonstrate that these molecules turn highly hydrophilic upon esterase mediated hydrolysis of the lipophilic termini in lab experiments and in cells (Figure 1). The intracellular retention was visualized by labeling these analogues with a fluorescent probe. In addition, we provide evidence that these ferrichrome analogues are highly effective as complexing agents against free iron and are capable of reducing oxidative damage. An *O*-benzyl protected ferrichrome analogue containing ethyl ester groups (**1a**) was synthesized following a protocol previously described.[6] From this intermediate, the prohydrophilic analogues were constructed (Figure 2). Hydrolysis of the esters, coupling with bromomethyl acetate, and deprotection of the hydroxamate moieties gave trishydroxamate **3**. The stability of the AM-esters was found to be much greater under aqueous conditions than analogously synthesized pivaloyloxymethyl derivatives. The half-life of **3** in water (with 10% DMSO) was determined to be approximately 3 h. An enzymatic assay using pig liver esterase was performed in D_2O and was monitored by ^1H NMR spectroscopy, showing rapid and complete hydrolysis of the AM-ester groups. To enable visualization of the permeation process by fluorescence spectroscopy, a reporter group based on naphthalic diimide was introduced. Bromination of naphthalic anhydride, followed by condensation with butylamine and coupling with piperazine, yielded a bright yellow probe, which served as a label to produce fluorescent analogues of **2** and **3** as outlined in Figure 2.

Experiments using murine erythroleukemia (MEL) cells revealed a clear difference in the entry and in the exit rates between the two

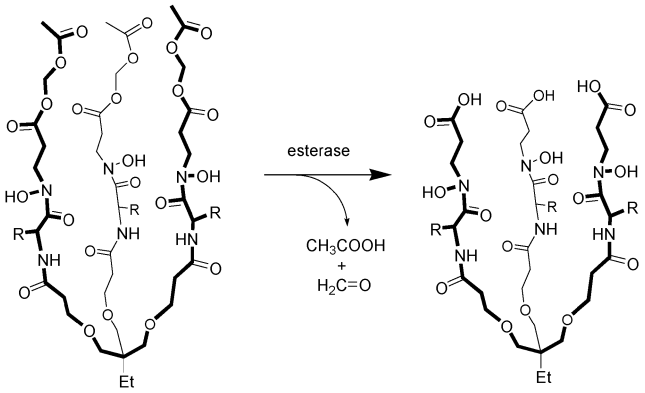

Figure 1. Transformation of a lipophilic ferrichrome analogue to a highly hydrophilic analogue.

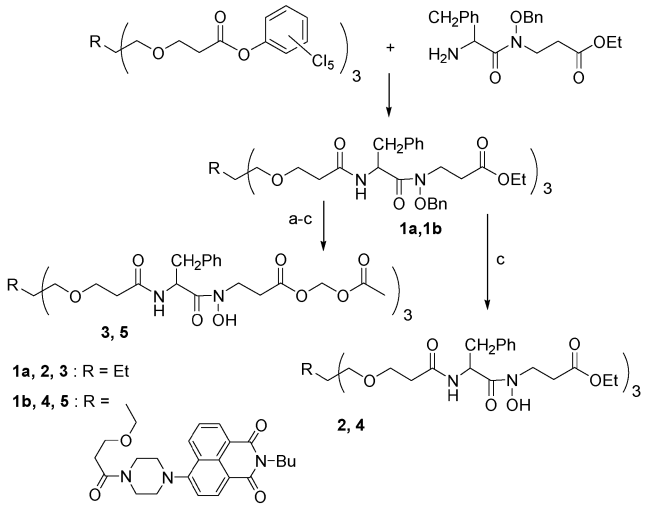

Figure 2. Synthesis of trishydroxamates containing acetoxymethyl and ethyl esters, and their fluorescent analogues: (a) $NaOH/H_2O$, (b) bromomethyl acetate/DIPEA, (c) $H_2/Pd-C/EtOH$.

compounds **4** and **5**. It was observed that cells incubated with the fluorescent compound **4** lost all fluorescence after equilibration with fresh buffer for 60 min, whereas a significant amount of fluorescence, indicative of the trishydroxamate **5**, was retained within the cells after the same period of time.[7] A more direct method for tracking the movement of the compounds in and out of the cells was used. According to this technique, immobilized cells were studied using fluorescence microscopal imaging. Solutions (100 μM each) of fluorescent compounds **4** and **5** in PBS (pH 7.4) were added to hepatoma cells, which were immobilized on glass plates. After 10 min at 37 °C, the solutions were replaced with fresh PBS, and their fluorescence images were captured every 30 s, while continuously refreshing the buffer solution. Fluorescence emerging

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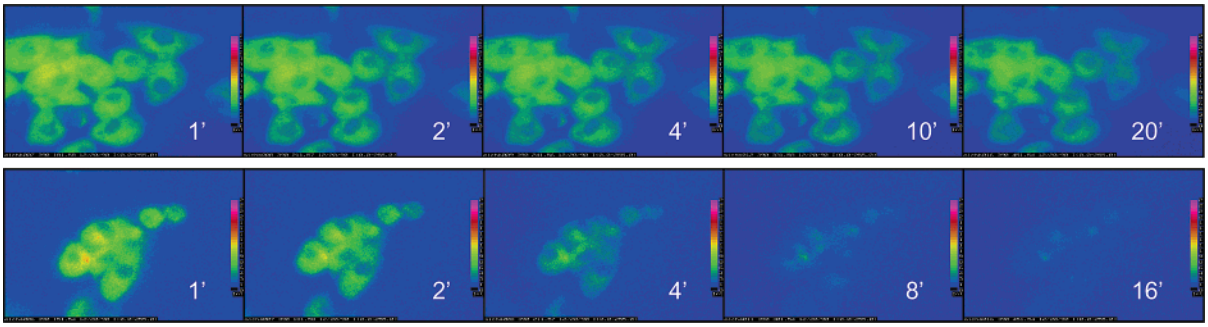

Figure 3. The time-dependent diffusion of fluorescent iron chelators out of mammalian cells (HepG2). Top row: retention of chelators bearing labile ester groups (**5**) that undergo intracellular hydrolysis. Bottom row: fast egress of chelators containing stable ethyl ester groups (**4**).

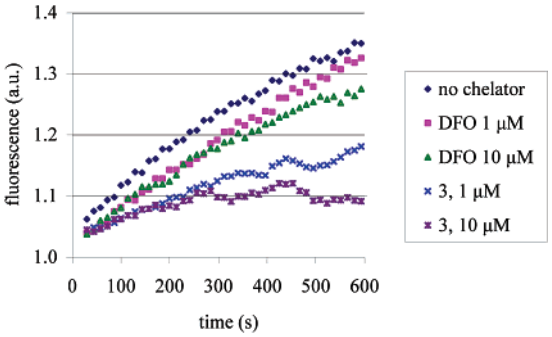

Figure 4. Formation of ROS was determined in MEL cells (10^6/mL) loaded with 10 μM CDCDHF-AM (6′-carboxy-2′,7′-dichlorodihydro-fluorescein acetoxymethyl ester) for 20 min at 37 °C. The initial rate of the H_2O_2 (5 μM)-induced conversion of the nonfluorescent 2′-7′-CDCF to the fluorescent 2′-7′-oCDCF was measured fluorometrically (exc-em: 488−517 nm, *y*-axis in relative au), and the inhibiting effect of DFO and **3** at two different concentrations was measured.

from compound **4** dropped rapidly, whereas compound **5** appeared to be largely retained within the cells (Figure 3). After 2 min, the fluorescence of **4** dropped to less than 20% of its original value (the initial intracellular concentrations of **4** and **5** were determined to be 0.65 and 0.80 mM, respectively), indicating that almost all of compound **4** had left the cells. With compound **5**, however, over 70% of the initial intracellular fluorescence intensity was measured after 20 min. Consecutive measurements showed no significant further reduction in fluorescence intensity. These results suggest that ferrichrome analogues carrying labile acetoxymethyl esters might be highly effective as protective agents against free-iron induced oxidative damage. To check this hypothesis, we loaded MEL cells with a nonfluorescent fluorescein derivative that emits fluorescence only upon interaction with reactive oxygen species (ROS). This way we determined that iron chelator **3** is much more effective than DFO in inhibiting the intracellular metal-catalyzed formation of ROS (Figure 4).

The ability to trap intracellular iron might also reflect itself in arresting parasite growth, based on deprivation of intracellular iron from invading microorganisms. Preliminary experiments with the malaria parasite *Plasmodium falciparum* indeed show compound **3** to be a potent inhibitor of parasitemia (IC_{50}: 10 μM). Additional experiments aimed at probing the stage and site of antimalarial action of prohydrophilic ferrichrome analogues are currently in progress.

We have shown that novel ferrichrome analogues turn highly hydrophilic upon cell entrance by way of esterase mediated hydrolysis of the lipophilic termini, causing a highly retarded egress. Further in vivo experiments on iron overload as well as parasite infection are planned, along with additional mechanistic studies using esterase depleted cell models.

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Supporting Information Available: Spectral data for compounds **1**−**5** and experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Murine erythroleukemia cells were incubated with compounds **4** and **5** (100 μM) for 60 min at 37 °C in PBS (pH 7.4) and centrifuged at 3000 rpm for 2 min. After being washed in PBS, the cells were centrifuged again and resuspended in PBS, and the fluorescence (excitation and emission at 400 and 530 nm, respectively) was measured. After another 60 min, the cells were centrifuged and resuspended in PBS, and the fluorescence was measured again.

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