Methoxylation of Desazadesferrithiocin Analogues: Enhanced Iron Clearing Efficiency

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The impact of altering the octanol−water partition properties (log *P*) of analogues of desazadesferrithiocin, (*S*)-4,5-dihydro-2-(2-hydroxyphenyl)-4-methyl-4-thiazolecarboxylic acid, on the ligands' iron clearing properties is described. Increasing chelator lipophilicity can both substantially augment iron clearing efficiency in *Cebus apella* primates as well as alter the mode of iron excretion, favoring fecal over urinary output. The complications of iron overload are often associated with the metal's interaction with hydrogen peroxide, generating hydroxyl radicals (Fenton chemistry) and, ultimately, other related deleterious species. In fact, some iron chelators actually promote this chemistry. All of the compounds synthesized and tested in the current study are shown to be both inhibitors of the iron-mediated oxidation of ascorbate, thus removing the metal from the Fenton cycle, and effective radical scavengers.

Introduction

Humans manage iron quite efficiently; they absorb and excrete only small quantities of this transition metal.[1,2] It is essential to any number of biological redox systems, including mitochondrial cytochromes[3] and ribonucleotide reductase.[4] Except for perhaps the lactobacilli, life, even in its most primitive forms, is unknown without iron.

Owing to the absence in humans of any mechanism for this metal's effective excretion,[5−7] trouble can unfold once excess iron is introduced into the closed metabolic loop. For example, individuals with β-thalassemia, a hereditary anemia, require continued transfusions that increase their body iron by 200−250 mg/unit of blood. Unless these patients receive chelation therapy, they frequently die in their third decade from iron overload. The origin of the problem with iron overload is often associated with the transition metal's reaction with hydrogen peroxide (H_2O_2) or other peroxides to yield hydroxyl or peroxyl free radicals.[8] Whereas H_2O_2 can be produced by several amine oxidases, it usually derives from superoxide anion.[9] Many oxidoreductases (e.g., NADPH reductase[9] and xanthine oxidase[10]) operate on oxygen to produce superoxide anion ($O_2^{\bullet-}$), which is converted either spontaneously or via superoxide dismutase to H_2O_2. Under normal circumstances, H_2O_2 is managed by housekeeping enzymes, such as catalase and glutathione peroxidase, converting H_2O_2 either to H_2O and O_2 or to H_2O, respectively. However, if unbound iron, particularly iron in the ferrous [Fe(II)] state, is available to react with excess H_2O_2, the Fenton reaction unfolds,[8,11−13] in which hydroxyl radical ($HO^{\bullet}$) and hydroxide anion (HO^-) are produced (eq 1):

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^{\bullet} + HO^- \quad (1)$$

$$Fe(III) + O_2^{\bullet-} \rightarrow O_2 + Fe(II) \quad (2)$$

Hydroxyl radicals can react with lipids, destroying membranes, can generate nicks in DNA, or can facilitate the production of carcinogens.[8,13,14] In and of itself, this sequence of events is problematic; worse, it is amplified by the reduction of Fe(III) back to Fe(II) via a number of physiological reductants, such as superoxide anion (eq 2), glutathione, and ascorbate;[8] thus, the Fenton reaction becomes cyclic. Although the precise mechanism of iron-mediated cellular, tissue, and organ damage is still under investigation, it is clear that removal of excess metal ameliorates the problem.[8] Unfortunately, some iron chelators, such as ethylenediaminetetraacetic acid (EDTA)[15] and nitrilotriacetic acid (NTA),[11] can actually exacerbate the Fenton reaction. The issue then becomes the identification of a ligand that will bind to iron and facilitate its excretion in humans without promoting the Fenton reaction.

In an effort to identify such ligands, we have predicated our search and design concepts on natural product iron chelators produced by microorganisms. These low-molecular-weight, virtually Fe(III)-specific molecules, siderophores, were the microoorganisms' response to an iron accessibility problem. This difficulty arose when the blue-green algae generated molecular oxygen, oxidizing soluble Fe(II) in the biosphere to insoluble ($K_{sp} = 2 \times 10^{-39}$)[16] Fe(III) hydroxide. When secreted into the extracellular milieu, these chelators sequester the metal and render it utilizable by the microorganism. Many siderophores, including desferrioxamine B (DFO), containing three hydroxamate functionalities, and enterobactin, which has three catecholamide moieties (Chart 1), are hexacoordinate ligands, forming 1:1 complexes

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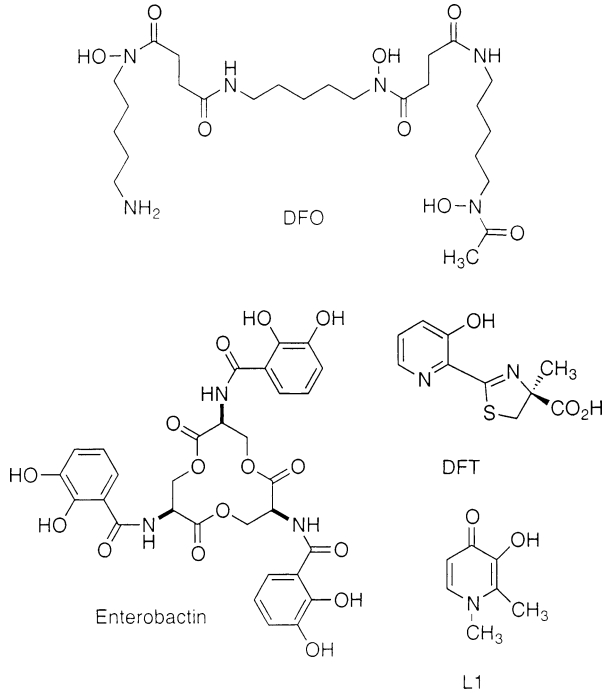
Chart 1. Natural Product Iron Chelators (Siderophores) Desferrioxamine B (DFO), Enterobactin, and Desferrithiocin (DFT); the Synthetic Ligand 1,2-Dimethyl-3-hydroxypyridin-4-one (L1).



with Fe(III). The hydroxamate Fe(III) chelates have formation constants on the order of 10^{30} M^{-1}, whereas the catecholamides, having stability constants of > 10^{42} M^{-1}, form stronger complexes with Fe(III).[17−21]

In fact, although many synthetic iron chelators have been assembled in hopes of identifying an alternative, DFO, which is produced by *Streptomyces pilosus*,[22] remains the ligand of choice for the treatment of iron overload in the clinic.[23,24] This is the case despite many shortcomings, the most profound of which is its efficiency, as defined below. The drug is generally administered by subcutaneous (sc) infusion 8−12 h daily, 4−6 days a week.[23,25] Further, intravenous administration of DFO must be done very cautiously, as serious hypotensive reactions can occur.[26] Frequently, the drug causes significant and often painful reactions at the site of injection,[27−30] can affect hearing,[31] and can promote *Yersinia* infections.[32,33] The combination of these side effects has led to marked patient compliance problems.[23,24]

Iron clearing efficiency is a measure of how much iron excretion is promoted by a chelator. It is a comparison of actual iron excretion vs theoretical iron clearance; this number is given as a percentage. For example, because DFO forms a very tight 1:1 complex with Fe(III), one millimole of DFO (656 mg as its mesylate salt) given to a patient should cause the excretion of one millimole of Fe(III) (56 mg). Unfortunately, the efficiency of DFO is only between 5 and 7%.[25,34] If this chelator were considerably more efficient, the dosing regimen would be very different; many of the compliance problems could be circumvented. Alternatively, an orally active iron chelator would also significantly reduce patient compliance problems.

Desferrithiocin (DFT, Chart 1) is a tridentate siderophore isolated from *Streptomyces antibioticus*.[35] Unlike enterobactin or DFO, it is neither a catecholamide nor a hydroxamate. Further, it forms a 2:1, rather than a 1:1, complex with Fe(III); the cumulative formation constant is 4×10^{29} M^{-1}.[36,37] The donor groups include a phenolic oxygen, a thiazoline nitrogen, and a carboxyl.

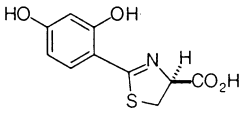
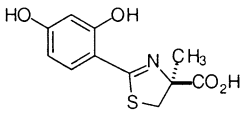
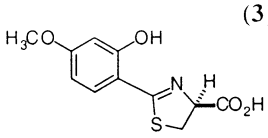
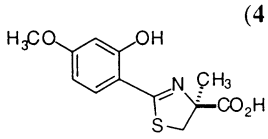
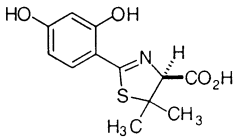
Desferrithiocin was one of the first iron chelators that was shown to be orally active in both the bile duct-cannulated rodent model, at an efficiency of 5.5%,[38] and the iron-overloaded *Cebus apella* primate model, at an efficiency of 16%.[39,40] This is three times the efficiency of DFO given sc in the primate model; the drawback is that DFT elicits severe nephrotoxicity.[40] Nevertheless, because of this remarkable oral activity, DFT was chosen as a pharmacophore from which to launch structure−activity studies, which were intended to identify orally active DFT analogues without the toxicity of the parent molecule.[38,40−44] These initial studies were focused on identifying the minimal platform compatible with iron clearance when oral administration is employed.

Removal of the thiazoline methyl of DFT yielded (*S*)-4,5-dihydro-2-(3-hydroxy-2-pyridinyl)-4-thiazolecarboxylic acid [(*S*)-DMDFT], a molecule with diminished iron clearing efficiency, 8% in the primate model at a dose of 300 μmol/kg.[40] Abstraction of DFT's aromatic nitrogen provided (*S*)-4,5-dihydro-2-(2-hydroxyphenyl)-4-methyl-4-thiazolecarboxylic acid [(*S*)-DADFT]; its efficiency was 13% in *C. apella*, significantly higher than that of (*S*)-DMDFT at the same dose.[44] Finally, removing both the aromatic nitrogen and the thiazoline methyl afforded (*S*)-4,5-dihydro-2-(2-hydroxyphenyl)-4-thiazolecarboxylic acid [(*S*)-DADMDFT], which was still effective, 12% efficiency at the equivalent dose in primates.[40,44] Accordingly, this very simple compound served as the platform from which further structure−activity studies were carried out.

The subsequent structural changes to (*S*)-DADMDFT revealed that few alterations were compatible with iron clearance. The distances between the ligating centers are subject to strict requirements and cannot be modified without loss of efficacy.[43] The thiazoline ring must remain intact.[41,43] Benz-fusions, which were designed to improve the ligands' tissue residence time and possibly iron-clearing efficiency, are ineffective.[42,43] The maintenance of an (*S*)-configured C-4 carbon is optimal in the design of desferrithiocin-based iron chelators.[42,44]

Once the structural changes compatible with iron clearance were delineated, structure−activity studies were undertaken to minimize toxicity.[43,44] At this point, the most effective systems, (*S*)-DADFT and (*S*)-DADMDFT, were still quite toxic. This structure−activity analysis assessed the effect of changing the potential metabolic profile of the compounds by introducing various substituents into the aromatic ring. We found that addition of aromatic hydroxyl groups, as in the systems (*S*)-2-(2,4-dihydroxyphenyl)-4,5-dihydro-4-thiazolecarboxylic acid [(*S*)-4′-(HO)-DADMDFT, **1**] and (*S*)-2-(2,4-dihydroxyphenyl)-4,5-dihydro-4-methyl-4-thiazolecarboxylic acid [(*S*)-4′-(HO)-DADFT, **2**] (Table 1), profoundly reduced ligand toxicity. In both instances, the hydroxylated compounds appear to be less active than their corresponding parent drugs, 5.3% efficiency for **1** (vs 12.4% for (*S*)-DADMDFT when compared at a 300 μmol/kg dose) and 17% efficiency for **2** (vs 21% for

Table 1. Desferrithiocin Analogues' Iron Clearing Activity When Administered Orally to *Cebus apella* Primates and the Partition Coefficients of the Compounds

desferrithiocin analogue (compd. no.)	iron clearing efficiency (%)[a] [% stool/% urine]	log P[d]
(**1**) 	4.2 ± 1.4[b] [70/30]	−1.33
(**2**) 	13.4 ± 5.8[c] [86/14]	−1.05
(**3**) 	16.2 ± 3.2 [81/19]	−0.89
(**4**) 	24.4 ± 10.8 [91/9]	−0.70
(**5**) 	12.3 ± 2.7 [64/36]	−0.91

[a] In the monkeys (n = 4, except for compound **4**, n = 7), the dose was 150 μmol/kg. The efficiency of each compound was calculated by averaging the iron output for 4 days before the administration of the drug, subtracting these numbers from the 2-day iron clearance after the administration of the drug, and then dividing by the theoretical output; the result is expressed as a percent. [b] From ref 43. [c] From ref 44. [d] Data are expressed as the log of the fraction in the octanol layer; measurements were done in TRIS buffer, pH 7.4, using a "shake flask" direct method.

(*S*)-DADFT when compared at a 75 μmol/kg dose).[43,44] One of these studies also suggested, not unexpectedly, that hydroxylation changes the partition between octanol and water, favoring the aqueous phase.[43] This compelled us to consider methoxylation in place of hydroxylation at the 4′-position of these desferrithiocins. Methoxylation could potentially increase lipophilicity, altering the octanol−water partition values, and improve iron clearing efficiency.

The current work assesses methoxylated DFT analogues as deferrating agents in the primate model, compares the efficiency of these ligands to octanol−water partition coefficients, and evaluates the capacity of the chelators to prevent Fenton chemistry and to act as free radical scavengers.

Results and Discussion

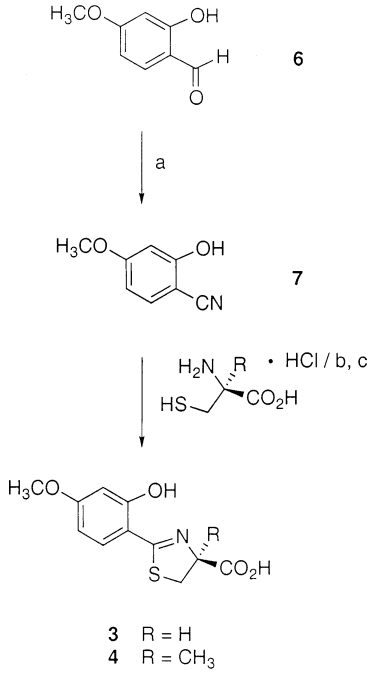
Design Concept and Synthesis. Comparisons of octanol−water partition coefficients of selected desferrithiocin analogues[43] do not show any correlations with iron clearing efficiency. For example, although DFT and (*S*)-DMDFT had similar partition values (log P = −1.77 and −1.87, respectively), their iron clearing efficiencies in primates were quite different at a po dose of 150 μmol/kg (16.1 ± 8.5 and 4.8 ± 2.7%, respectively). However, when DADMDFT (log P = −0.93) and (*S*)-4′-(HO)-DADMDFT (**1**, log P = −1.2) are compared, the results suggest that the more octanol-soluble ligand, DADMDFT, is more efficient than the less octanol-soluble ligand, **1** (12.4 ± 7.6% vs 5.3 ± 1.7% at 300 μmol/kg po). This finding encouraged us to further explore the effect of altering the lipophilicity of the (*S*)-2-(2,4-dihydroxyphenyl)-4,5-dihydro-4-thiazolecarboxylic acids on their iron clearing efficiency. The differences in iron clearing efficiency and even in toxicity might be accounted for by the disposition of the drug in a particular cellular or subcellular compartment. Accordingly, we have synthesized three new analogues, employing two modifications to alter lipophilicity. In one case, alkyl groups were added to the oxygen of the 4′-hydroxyl of **1** and **2**, thus removing a hydrogen bond donor while maintaining the molecular hydrogen bond acceptor capacity, providing (*S*)-4,5-dihydro-2-(2-hydroxy-4-methoxyphenyl)-4-thiazolecarboxylic acid [(*S*)-4′-(CH_3O)-DADMDFT, **3**] and (*S*)-4,5-dihydro-2-(2-hydroxy-4-methoxyphenyl)-4-methyl-4-thiazolecarboxylic acid [(*S*)-4′-(CH_3O)-DADFT, **4**], respectively. In the other, alkyl groups were added to the thiazoline ring of **1**, producing (*S*)-2-(2,4-dihydroxyphenyl)-4,5-dihydro-5,5-dimethyl-4-thiazolecarboxylic acid (DM, **5**) (Table 1).

Synthetic approaches to ligands **3**−**5** drew on our previous methodologies.[38,41,43,44] Heating 2-hydroxy-4-methoxybenzaldehyde (**6**) with nitroethane and sodium acetate in acetic acid[45] furnished 2-hydroxy-4-methoxybenzonitrile **7** in 70% yield. Condensation of nitrile **7** with D-cysteine·HCl or D-α-methylcysteine·HCl in aqueous CH_3OH maintained at pH 6 generated (*S*)-4′-(CH_3O)-DADMDFT (**3**) (71% yield) or (*S*)-4′-(CH_3O)-DADFT (**4**) (46% yield), respectively (Scheme 1). Hydrogen chloride-promoted addition of EtOH across the triple bond of 2,4-dihydroxybenzonitrile (**8**) generated ethyl 2,4-dihydroxybenzimidate·HCl (**9**) (69% yield), which was previously made from resorcinol.[46] Cyclization of D-penicillamine with imino ester **9** in refluxing EtOH provided DM (**5**), a *gem*-dimethyl DFT analogue, in 43% yield (Scheme 2).

Iron Clearance. The iron-overloaded *C. apella* monkey model was used to evaluate the iron-clearing properties of the compounds. The procedures employed in this model allow us to measure the total amount of iron cleared and the proportion of iron excreted in the stool and urine. Because of the many similarities of the iron-overloaded *C. apella* monkey to humans,[39,47] it serves as a screen for evaluating iron chelators before human studies.

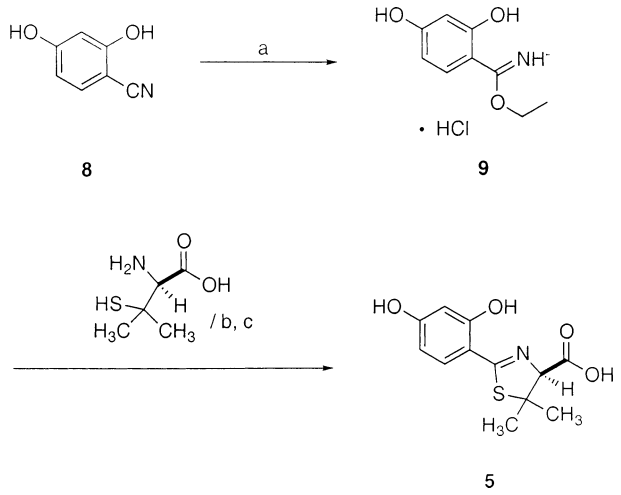
In this model, (*S*)-4′-(HO)-DADMDFT (**1**) performs moderately well when given orally (Table 1).[43,44] At a po dose of 150 μmol/kg, the efficiency was 4.2 ± 1.4%; the stool/urine ratio was 70:30. Introduction of a methyl group into the thiazoline ring of **1** [(*S*)-4′-(HO)-DADFT, **2**] led to a substantial increase in iron clearing efficiency, to 13.4 ± 5.8% (P < 0.05 vs **1**); 86% of the iron excretion was fecal.[44] Methylation of the 4′-hydroxyl of

Scheme 1. Synthesis of (*S*)-4,5-Dihydro-2-(2-hydroxy-4-methoxyphenyl)-4-thiazolecarboxylic Acid [(*S*)-4′-(CH_3O)-DADMDFT, **3**] and (*S*)-4,5-Dihydro-2-(2-hydroxy-4-methoxyphenyl)-4-methyl-4-thiazolecarboxylic Acid [(*S*)-4′-(CH_3O)-DADFT, 4][a]



[a] Reagents: (a) $CH_3CH_2NO_2$, NaOAc, HOAc, reflux, 70%; (b) $NaHCO_3$, CH_3OH, phosphate buffer, pH 6; (c) HCl (aq), 71% (**3**); 46% (**4**).

Scheme 2. Synthesis of (*S*)-2-(2,4-Dihydroxyphenyl)-4,5-dihydro-5,5-dimethyl-4-thiazolecarboxylic Acid (DM, **5**)[a]



[a] Reagents: (a) HCl (g), CH_3CH_2OH, 69%; (b) CH_3CH_2OH, $NaHCO_3$, reflux; (c) citric acid (aq), 43%.

1 to afford **3** more than tripled the iron clearing efficiency to 16.2 ± 3.2% ($P < 0.005$ vs **1**); 81% of the iron excretion was fecal, and 19% was urinary. The same modification to **2**, affording (*S*)-4′-(CH_3O)-DADFT (**4**), further augmented the deferration efficacy, to 24.4 ± 10.8%. This efficiency is superior to that of **1** ($P < 0.005$), although the differences in efficiency between **4** and either **2** or **3** were not statistically significant. In the case of analogue **4**, slightly more of the iron excretion, 91%, was via the stool; only 9% was via the urine. Finally, addition of two methyl groups to the

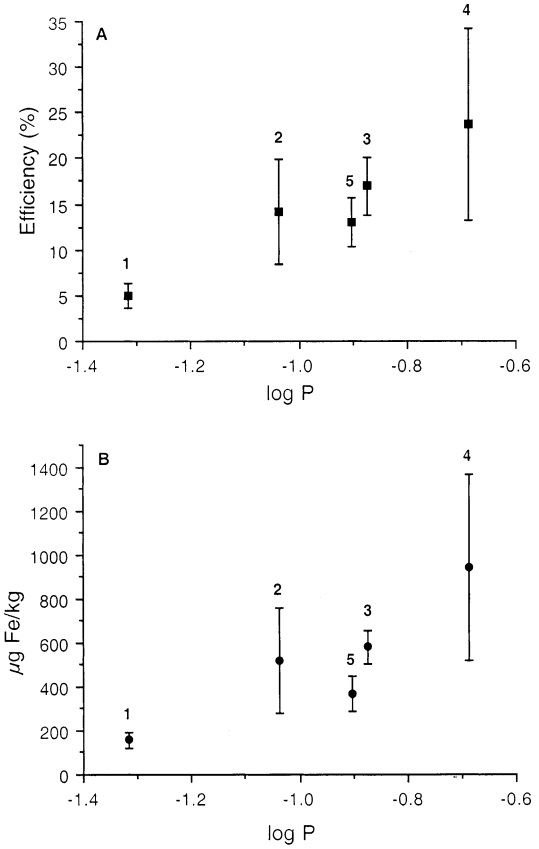


Figure 1. Iron clearing efficiency (percent, panel A) and fecal iron clearance (μg of stool Fe/kg of body weight, panel B) of ligands **1**−**5** plotted vs the respective partition coefficients of the compounds. The r^2 values for efficiency vs log *P* were 0.707 and 0.770, respectively, when **5** was encompassed and omitted. The r^2 value for fecal iron clearance vs log *P* was 0.653 when **5** was included and increased to 0.794 when **5** was excluded.

5-position of the thiazoline ring of **1** (DM, **5**) had an obvious impact on iron clearing efficacy; the efficiency was 12.3 ± 2.7% ($P < 0.005$ vs **1**). When **5** is compared with **3** and **4**, considerably less of the iron was excreted in the feces, 64%, and 36% of the iron was in the urine.

Partition Coefficients vs Iron Clearing Efficiency and Mode of Excretion. The partition coefficients of ligands **1**−**5** were measured in buffered octanol−water. The log *P* values (Table 1) ranged from −0.70 for **4** to −1.33 for **1**. It is not surprising, and clear from Table 1, that attaching alkyl groups to a carbon increases a compound's lipophilicity, but interestingly, when the iron clearing efficiency is plotted vs partition coefficient (Figure 1A), it appears that among these ligands, increasing the lipophilicity also augments the iron clearing efficiency. A similar relationship can be observed when fecal iron clearance is plotted vs log *P* (Figure 1B). To assess the significance of the apparent linear trends consistent with these observations, weighted regression analyses were carried out both with and without ligand **5**. The results indicate that there was sufficient evidence to conclude a significant ($P < 0.001$) relationship between efficiency and log *P* regardless of whether **5** was included; however, the strength of the slope (and therefore the relationship) is greater when **5** was omitted from the analysis. These findings indicate that methylation of the 4′-hydroxyl, as in compound **3**, seems to have a more dramatic effect on lipophilicity, iron clearing efficiency, and fecal iron clearance than

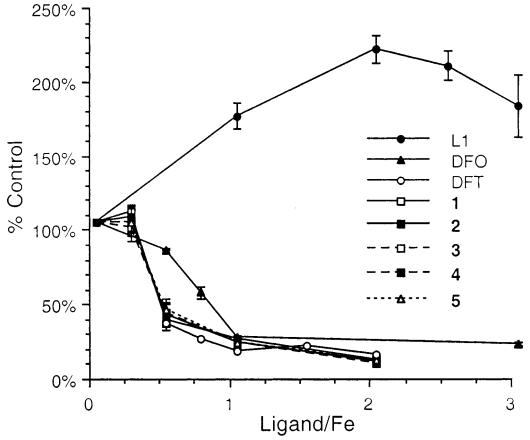


Figure 2. Effect of various chelators on the iron-mediated oxidation of ascorbate (Fenton chemistry) (percent of control, *y*-axis): 1,2-dimethyl-3-hydroxypyridin-4-one (L1, $n = 3$), desferrioxamine (DFO, $n = 3$), DFT ($n = 1$), and DFT analogues **1**−**5** ($n = 4$) at several ligand/metal ratios (*x*-axis). Typically, each assay contained three controls with only ascorbate and $FeCl_3$; these varied less than 5% (± SD). Each assay also usually included a "negative control" containing ascorbate, $FeCl_3$, and L1 at a ligand/iron ratio of 2:1; this value was 213% ± 12%. The curves for L1, DFO, and compound **1** are from ref 50; the curves for DFT and compound **2** are from ref 49.

does appending a methyl to the 4-position of the thiazoline ring, as in **2**, or attaching two methyls to the 5-position of the thiazoline ring, as in compound **5**. These results have encouraged us to begin preclinical toxicity trials with (*S*)-4′-(CH_3O)-DADFT (**4**), a compound that is both orally active and easy to synthesize.

Fenton Chemistry. A major concern in the design of iron chelators for therapeutic use is related to their effect on the reduction of Fe(III) to Fe(II). In the latter oxidation state, iron plays a critical role in the Fenton reaction (eq 1). Thus, a situation in which the reduction of Fe(III) to Fe(II) (eq 2) is facilitated would worsen any Fenton chemistry-mediated cell damage. Since some iron chelators promote this reduction,[11,15] assessments of whether a ligand promotes, prevents, or has no effect on Fe(III) reduction are critical in moving deferrating agents closer to a clinical setting. Of the known physiological reductants, ascorbate is a frequently used model; its oxidation is easy to follow in a qualitative manner by its disappearance spectrophotometrically.[15,48] The data in Figure 2 are plotted as the change in ascorbate concentration as a function of the ligand/metal ratio; a value less than 100% of control indicates diminished Fe(III)-mediated ascorbate oxidation. The equilibria that are occurring in this assay are quite complicated; one must be careful not to overinterpret these results. The question addressed in these experiments is, simply, in the presence of a test compound, is the rate of ascorbate oxidation/iron reduction increased, decreased, or the same relative to controls containing iron(III) and ascorbate without the ligand? The reference chelator DFO serves as a positive control, as it decreases ascorbate loss;[15] conversely, 1,2-dimethyl-3-hydroxypyridin-4-one (L1, Chart 1), which increases ascorbate disappearance, functions as a negative control. Recall that the desferrithiocin analogues form 2:1 complexes with Fe(III); accordingly, the ligand-to-metal ratios from 0.5 to 3 are measured for ligands **1**−**5** (Figure 2). Both the 4′-hydroxy (**1**, **2**, and **5**) and 4′-methoxy (**3** and **4**) analogues slow Fe(III) reduction considerably, even at a 0.5:1 ratio, as did the parent compound DFT.[49,50] The results with the parent ligand are consistent with those reported in this same assay[15,48] and in a cultured cell system.[48] This suggests that whatever the origin of the toxicity of some of the desferrithiocins is, it is not likely to be derived from any enhancement of Fenton chemistry.

Table 2. ABTS Radical Cation Quenching Activity of Selected Compounds

compound	slope × 10^3 OD units/μM[a]
DFT	−0.9[b]
3	−33
4	−36
Trolox	−37[b]
5	−70
1	−102[b]
2	−106[b]

[a] The slope was derived from A_{734} vs concentration data after a 6-min reaction period between the chelator of interest and the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$), which was formed from the reaction between ABTS and persulfate. A negative slope represents a decrease in the amount of highly colored radical cation over the time interval from an initial A_{734} of about 0.900. Trolox, an analogue of vitamin E, served as a positive control. [b] From ref 49.

Free Radical Scavenging. The issue of Fenton chemistry and chelator design has a dimension beyond the prevention of the reduction of Fe(III). In the Fenton reaction, the liberated $HO^{\bullet}$ molecules are very short-lived, reacting with most surrounding molecules at a diffusion-controlled rate. Ultimately, less active, more selective radicals, which can initiate a radical chain process, are produced and can cause significant cell damage.[9] Radical traps can help to attenuate the already ongoing Fenton chemistry-induced, free radical-mediated damage; thus, the radical scavenging properties of a particular ligand are of importance. Two hexacoordinate iron chelators, *N*,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid (HBED)[51] and DFO,[49,52] are excellent radical traps.

We have evaluated the methoxylated analogues (**3** and **4**), their parent molecules (**1** and **2**), and the 5,5-dimethyl analogue (**5**) in a free radical scavenging assay. The ability of each of these ligands to function as a one-electron donor to the preformed radical monocation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was compared with that of Trolox, an analogue of vitamin E.[53] Table 2 shows the calculated slopes of the decrease in A_{734} vs ligand concentration line for each compound; a more negative slope indicates a more effective radical scavenger. It was not unexpected that the 4′-methoxylated compounds were less effective radical scavengers than were the corresponding 4′-hydroxylated molecules; nevertheless, both **3** and **4** were as effective as Trolox at trapping free radicals and much better than the parent DFT. Analogue **5**, on the other hand, was about twice as active as Trolox.

Conclusion

The increase in iron clearing efficiency observed when (*S*)-4′-(HO)-DADMDFT (**1**) and (*S*)-4′-(HO)-DADFT (**2**) are converted to the corresponding methoxy compounds

(**3** and **4**, respectively) suggests that this modification may be useful in augmenting the iron clearing efficiency of other DFT analogues, probably by increasing their lipophilicity and their bioavailability. Furthermore, the correlation between log *P* and fecal iron clearance is also useful in predicting fecal vs urinary excretion, which may be valuable information if nephrotoxicity is an issue. Methoxylation did not diminish the capacity of the ligands to prevent ascorbate-mediated Fe(III) reduction; these compounds are still effective at preventing Fenton chemistry. Although methoxylation did alter the ability of the ligands to serve as free radical scavengers when compared to the hydroxylated compounds, the former compounds were still as effective one-electron donors as the vitamin E analogue, Trolox. Experiments with positional isomers (i.e., 3-hydroxy and -methoxy compounds) are in progress to assess whether there is a difference in the sensitivity of these compounds to shifts in lipophilicity and/or changes in iron clearing efficiency and fecal iron clearance vs their 4-hydroxy and -methoxy counterparts. After this, what remains is to establish the general utility of methoxylation with other desferrithiocin analogues utilizing both single and multiple methoxylations to the aromatic ring and to define the impact of methoxylation on toxicity.

Experimental Section

D-α-Methylcysteine hydrochloride and **8** were obtained from DSM Fine Chemicals (Linz, Austria). Other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used without further purification. Fisher Optima-grade solvents were routinely used, and reactions were run under nitrogen unless otherwise specified. Distilled solvents and glassware that had been presoaked in 3 N HCl for 15 min were employed for reactions involving chelators. Sodium phosphate buffer at a pH of 6 was made up to a concentration of 0.1 M.[54] Organic extracts were dried with anhydrous sodium sulfate. Silica gel 32−63 from Selecto Scientific, Inc. (Suwanee, GA) was used for flash column chromatography, and melting points are uncorrected. NMR spectra were obtained at 300 MHz (^{1}H) or 75 MHz (^{13}C) on a Varian Unity 300 in DMSO-d_6, unless otherwise indicated. Chemical shifts (δ) for ^{1}H spectra are given in parts per million downfield from tetramethylsilane for DMSO-d_6 or sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 for D_2O. Chemical shifts (δ) for ^{13}C spectra are given in parts per million referenced to 1,4-dioxane (δ 67.19) in D_2O or to the residual solvent resonance in DMSO-d_6 (δ 39.50) or CD_3OD (δ 49.0). Coupling constants (J) are in hertz. Optical rotations were measured at 589 nm (sodium D line), and c is expressed as grams of compound per 100 mL of solution. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA).

Desferrioxamine B in the form of the methanesulfonate salt, Desferal (Novartis Pharma AG, Basel, Switzerland), was obtained from a hospital pharmacy. 1,2-Dimethyl-3-hydroxypyridin-4-one (L1) was a generous gift from Dr. H. H. Peter (Ciba-Geigy, Basel). Compounds **1** and **2** were accessed by methods published from this laboratory.[43,44]

C. apella monkeys were obtained from World Wide Primates (Miami, FL). Ultrapure salts were obtained from Johnson Matthey Electronics (Royston, UK). All hematological and biochemical studies[40] were performed by Antech Diagnostics (Tampa, FL). Atomic absorption (AA) measurements were made on a Perkin-Elmer model 5100 PC (Norwalk, CT).

Spectrophotometric readings (A_λ) for the ascorbate and radical cation assays were taken on a Perkin-Elmer Lambda 3B spectrophotometer.

(*S*)-4,5-Dihydro-2-(2-hydroxy-4-methoxyphenyl)-4-thiazolecarboxylic Acid (3). Sodium bicarbonate (0.54 g, 6.43 mmol) was added to **7** (0.583 g, 3.91 mmol) and D-cysteine hydrochloride monohydrate (1.13 g, 6.43 mmol) in phosphate buffer (20 mL) and CH_3OH (30 mL). The mixture was degassed under vacuum and then stirred under argon at 70 °C for 2 days. The mixture was allowed to cool to room temperature, concentrated under reduced pressure, and saturated $NaHCO_3$ (80 mL) was added. The resulting solution was washed with EtOAc (3 × 30 mL), acidified to pH 2 with concentrated HCl, and extracted with EtOAc (3 × 50 mL). The latter extracts were concentrated under reduced pressure. Column chromatography on Sephadex LH-20 (4% EtOH in toluene) gave **3** (0.706 g, 71%) as a yellow solid, mp 133−134 °C: $[\alpha]_D^{25}$ +37.9° (*c* 1.00, CH_3OH); ^{1}H NMR δ 3.60 (dd, 1 H, J = 7.3, 11.3), 3.68 (dd, 1 H, J = 9.4, 11.3), 3.80 (s, 3 H), 5.42 (dd, 1 H, J = 7.3, 9.4), 6.52−6.56 (m, 2 H), 7.35 (dd, 1 H, J = 2.3, 6.9), 12.76 (s, 1 H), 13.18 (br s, 1 H); ^{13}C NMR δ 33.2, 55.5, 75.9, 100.8, 106.9, 109.1, 131.8, 160.5, 163.5, 171.5, 172.1. Anal. ($C_{11}H_{11}NO_4S$): C, H, N.

(*S*)-4,5-Dihydro-2-(2-hydroxy-4-methoxyphenyl)-4-methyl-4-thiazolecarboxylic Acid (4). A mixture of **7** (6.09 g, 40.8 mmol), D-α-methylcysteine hydrochloride (7.01 g, 40.8 mmol), and $NaHCO_3$ (3.65 g, 43.4 mmol) in degassed phosphate buffer (150 mL) and degassed CH_3OH (210 mL) was stirred at 48−50 °C for 3 days. The mixture was concentrated under reduced pressure and acidified to pH 2 by dropwise addition of 1 N HCl. The suspension was extracted with EtOAc (6 × 250 mL), which was removed under reduced pressure. Column chromatography on Sephadex LH-20 (6% EtOH in toluene) gave **4** (5.02 g, 46%) as a yellow foam, mp 77−79 °C: $[\alpha]_D^{23}$ +53.0° (*c* 1.00, CH_3OH); ^{1}H NMR δ 1.58 (s, 3 H), 3.36 (d, 1 H, J = 11.5), 3.79 (s, 3 H), 3.79 (d, 1 H, J = 11.5), 6.51−6.56 (m, 2 H), 7.33 (m, 1 H), 12.79 (s, 1 H), 13.22 (br s, 1 H); ^{13}C NMR (CD_3OD) δ 24.7, 40.5, 55.9, 84.2, 101.8, 107.6, 110.8, 132.8, 162.3, 165.5, 172.3, 176.0; HRMS *m/z* calcd for $C_{12}H_{14}NO_4S$ 268.0643 (M + H), found 268.0654. Anal. ($C_{12}H_{13}NO_4S$): C, H, N, S.

(*S*)-2-(2,4-Dihydroxyphenyl)-4,5-dihydro-5,5-dimethyl-4-thiazolecarboxylic Acid (5). Sodium bicarbonate (0.807 g, 9.60 mmol) was added to D-penicillamine (8.61 g, 57.7 mmol) and **9** (9.98 g, 45.8 mmol) in degassed EtOH (400 mL), and the mixture was heated at reflux for 1 day. The bulk of solvent was removed by rotary evaporation. Citric acid (0.5 M, 100 mL) was added to the residue, and the mixture was stirred for 1 h with ice cooling. Solid was filtered and was dissolved in boiling EtOH (1.8 L) and H_2O (3 mL), and the solution was hot filtered. After solvent was removed in vacuo, CH_2Cl_2 (250 mL) was added, and the mixture was heated at reflux for 5 h followed by filtration. The solid was taken up in H_2O (90 mL), heated at reflux for 10 min, filtered by suction while hot, and washed with H_2O (2 × 25 mL). Drying under high vacuum resulted in **5** (5.29 g, 43%) as a pale tan solid, mp 238.5−239 °C (dec): $[\alpha]_D^{21}$ +6.9° (*c* 1.04, DMF). The relatively low magnitude of the specific rotation may indicate epimerization at C-4.[55] ^{1}H NMR δ 1.42 (s, 3 H), 1.72 (s, 3 H), 4.93 (s, 1 H), 6.29 (d, 1 H, J = 2.2), 6.36 (dd, 1 H, J = 8.6, 2.2), 7.15 (d, 1 H, J = 8.6), 10.2 (s, 1 H), 12.8 (s, 1 H), 13.1 (br s, 1 H); ^{13}C NMR δ 25.5, 27.9, 58.8, 83.4, 102.4, 107.7, 108.3, 131.9, 160.7, 162.2, 169.9, 171.4. Anal. ($C_{12}H_{13}NO_4S$): C, H, N.

2-Hydroxy-4-methoxybenzonitrile (7). Sodium acetate (9.34 g, 114 mmol) and **6** (8.67 g, 57.0 mmol) were added to a solution of nitroethane (8.54 g, 114 mmol) in glacial HOAc (25 mL). The mixture was refluxed for 3 h, allowed to cool to room temperature overnight, poured over ice (50 g), and extracted with ether (5 × 200 mL). The combined extracts were washed with saturated $NaHCO_3$ (200 mL) and brine (100 mL). Solvents were removed under reduced pressure; flash chromatography of the residue [2:1 petroleum ether/EtOAc (2.5 L), then 1:1 petroleum ether/EtOAc (3 L)] gave 3.57 g of **7**. Additional **7** (2.41 g) was obtained by combining the impure fractions from the chromatographic separation, concentrating under reduced pressure, dissolving the residue in EtOAc (50 mL), and precipitating by addition of petroleum ether (400 mL); a total of 5.98 g of **7** (70%) was generated, mp 178−179 °C: ^{1}H NMR δ 3.77 (s, 3 H), 6.47−6.56 (m, 2 H), 7.51 (d, 1 H, J = 8.4), 11.08 (br s, 1 H); ^{13}C NMR δ 55.5, 91.2, 101.0, 106.7, 117.4, 134.4,

161.9, 164.0; HRMS *m/z* calcd for $C_8H_7NO_2$ 149.0477 (M), Found 149.0474. Anal. ($C_8H_7NO_2$): C, H, N.

Ethyl 2,4-Dihydroxybenzimidate Hydrochloride (9). A solution of **8** (50.0 g, 0.370 mol) in EtOH (2.2 L) in a 5-L three-necked flask, equipped with a pipet through a serum cap and a $CaCl_2$ drying tube, was cooled in ice water, and HCl gas ($CaCl_2$ drying tube, 873 g, 23.9 mol) was bubbled in over 3 h. After 18 days at 6 °C, filtration and washing the solid with ether gave 24.57 g of **9** as a red solid. The combined filtrate was sealed at ≤ 6 °C. Four more lots of **9** were filtered, recharging the flask with HCl gas (197 g, 5.4 mol), for a total of 55.9 g of **9** (69%) over 6 months, mp 173.5−174.5 °C. ^{1}H NMR (D_2O) δ 1.54 (t, 3 H, J = 7.0), 4.50 (q, 2 H, J = 7.0), 6.45 (d, 1 H, J = 2.2), 6.54 (dd, 1 H, J = 9.0, 2.4), 7.85 (d, 1 H, J = 9.0); ^{13}C NMR (D_2O) δ 13.5, 68.7, 102.9, 109.8, 133.4, 161.3, 165.0, 169.3. Anal. ($C_9H_{12}ClNO_3$): C, H, N.

Iron Loading of *C. apella* Monkeys. The monkeys were iron overloaded with iv iron dextran as accounted in earlier publications to provide about 500 mg of iron per kg of body weight;[56] the serum transferrin iron saturation rose to between 70 and 80%. At least 20 half-lives, 60 days,[57] elapsed before any of the animals were used in experiments evaluating iron-chelating agents.

Primate Fecal and Urine Samples. Fecal and urine samples were collected at 24-h intervals and processed as described previously.[39,40,58] Briefly, the collections began 4 days prior to the administration of the test drug and continued for an additional 5 days after the drug was given. Iron concentrations were determined by flame atomic absorption spectroscopy as given in detail in other publications.[39,42]

Drug Preparation and Administration. The desferrithiocin analogues were solubilized in 40% Cremophor RH-40/water (v/v) and given po to the monkeys at a dose of 150 μmol/kg.

Calculation of Iron Chelator Efficiency. The theoretical outputs of the chelators were generated on the basis of a 2:1 ligand/iron complex. The efficiencies in the monkeys were calculated as set forth elsewhere.[44,59] Data are presented as the mean ± the standard error of the mean; *P*-values were generated via a two-tailed Student's *t*-test, in which the inequality of variances was assumed.

Determination of Partition Coefficients. The octanol−H_2O partition data are expressed as distribution coefficients uncorrected for partial ionization of the acids and were all measured at pH 7.4 (50 mM TRIS buffer) using UV spectrometry. The measurements were done using a "shake flask" direct measurement.[60] Quadruplicate samples were vigorously agitated with 1-octanol (HPLC grade 1, Sigma-Aldrich, St. Louis, MO) overnight in a Parr shaker, and the layers were allowed to settle for 1−2 h prior to separation. The experiments were conducted at room temperature (22−24 °C) using a Shimadzu UV-265 spectrophotometer (Columbia, MD).

Regression Analysis of Relationship between Partition Coefficients and Iron Clearing Efficiency. The relationship between the log *P* and the two response variables, iron clearing efficiency and induced Fe in the stool, for all of the drug groups and for just four of the dose groups (compound **5** omitted), was examined. The data consisted of the log *P* for each of the five drug groups and the two response variables; no outlier testing was done. A preliminary inspection of the descriptive statistics indicated that the variability was not homogeneous across the different groups for both response variables (i.e., there was residual fanning out), and an ordinary least squares estimation could not be applied to test for the relationship between iron chelator efficiency (or stool Fe clearance) and log *P*. Therefore, a weighted regression analysis was performed on the data using the SAS program (Version 8, SAS Institute, Cary, NC) on a Dell Pentium IV (1.5 GHz) PC running Windows 2000 Professional. To eliminate the fanning out, the weights were the reciprocal of the standard deviation of each group (chelators **1**−**5**). The slope of the relationship between iron clearing efficiency (or stool clearance) and log *P* was tested to determine whether this relationship was equal to 0 under the null hypothesis (i.e., H_0: β_1 = 0, that there was no relationship between iron clearing efficiency (or stool clearance) and log *P*, vs H_1: $\beta_1 \neq 0$) by a standard *Z*-test.

Prevention of Iron-Mediated Oxidation of Ascorbate. The iron chelators were tested for their ability to decrease the iron-mediated oxidation of ascorbate by a literature method.[15] In brief, a solution of freshly prepared ascorbate (100 μM) in sodium phosphate buffer (5 mM, pH 7.4) was incubated in the presence of $FeCl_3$ (30 μM) and chelator (ligand/Fe ratios varied from 0 to 3) for 40 min. The A_{265} was read at 10 and 40 min; the ΔA_{265} in the presence of ligand was compared to that in its absence. It should be noted that under the conditions of the assay the maximum contribution by any of the desferrithiocin analogue-iron complexes was less than 25% of the total A_{265}. Also, any ascorbate oxidation (reduction of complex) would be expected to artificially accentuate the apparent rate of oxidation. This is further consistent with protection from Fenton chemistry-mediated damage.

Quenching of the ABTS Radical Cation. The capacity of the iron chelators to quench the radical cation formed from 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was measured by a published procedure.[53] Briefly, a stock solution of ABTS radical cation was generated by mixing ABTS (10 mM, 2.10 mL) with $K_2S_2O_8$ (8.17 mM, 0.90 mL) in H_2O and allowing the solution to sit in the dark at room temperature for 18 h. This stock solution of deep blue-green ABTS radical cation was diluted in sufficient sodium phosphate (10 mM, pH 7.4) to give an A_{734} of about 0.900. Test compounds were added to a final concentration ranging from 1.25 to 15 μM, and the decrease in A_{734} was read after 1, 2, 4, and 6 min. Assays were performed in triplicate at each concentration. The reaction was largely complete by 1 min, but the data presented are based on a 6-min reaction time.

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