An Investigation of Desferrithiocin Metabolism

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The hydrolyses of (*S*)-desferrithiocin (DFT, **1**), (*R*)-desmethyl-DFT (**2**), and (*R*)-desazadesmethyl-DFT (**3**) were studied at pH 2.5 and 7.2 in order to access the stability of the thiazolines at the pH of the stomach and the serum. At 37 °C and pH 2.5, DFT (**1**) ($t_{1/2}$ = 18.6 h), desmethyl-DFT (**2**) ($t_{1/2}$ = 8.74 h), and desazadesmethyl-DFT (**3**) ($t_{1/2}$ = 31.7 h) were shown to open principally to the thiol amides with trace amounts of the corresponding thioesters, ≤2%. The thiazolines were resistant to hydrolysis at pH 7.2. Iron(III) stabilized significantly the thiazolines in the complexes **16a/b** of **3** in regard to hydrolysis at pH 2.5 ($t_{1/2}$ > 20 days). The iron(III) complexes **16a/b** were shown to be stable at pH 7.2. While the thiol amides **13** and **14** of **1** and **2** were isolated from the hydrolysis of the parent desferrithiocins, the thioester **4** and the thiol amide **5** of **3** were synthesized and their stability in aqueous solution, iron-clearance properties, and toxicity were evaluated. Thioester **4** was shown to rearrange to thiol amide **5** at pH 2.5 and 37 °C with a half-life of 4.18 h and instantaneously at pH 7.2. Thiol amide **5** is in equilibrium with **4** (**5**/**4** = 49:1) at pH 2.5 and was shown to be stable at pH 7.2. Thioester **4** and thiol amide **5** demonstrated neither iron-clearance activity in iron-overloaded rats nor toxic side effects in mice. Hydrolysis products of the drug, which might be generated in the stomach, seem unlikely to be the source of the drug's toxicity or iron-clearing properties.

Introduction

Since there is no mechanism for the excretion of iron, patients with chronic hemolytic anemias ultimately develop toxic iron overload. For example, patients with β-Thalassemia, a hereditary anemia, require continued transfusions, thus increasing their body iron by 200–250 mg/unit of blood. Unless these individuals are subjected to chelation therapy, they frequently die in their third decade from iron overload.[1] The hydroxamate desferrioxamine B,[2] produced in large-scale fermentation by a strain of *Streptomyces pilosus*, exhibits a high selectivity for iron and is the drug of choice for the treatment of transfusional iron overload. However, because this hydroxamate is not orally active and has a short half-life in the body, it must be administered by continuous subcutaneous infusion. This is sufficiently unpleasant for patients undergoing long-term treatment that compliance soon becomes the main problem in dealing with Thalassemia.[3] Thus, considerable interest has developed in a variety of disciplines in the search for an orally active iron chelator.

Siderophores,[4] microbial iron chelators responsible for the transport of iron into microorganisms, have served as models in the development of orally active iron chelators. Desferrithiocin (DFT, **1**)[5] (Figure 1), a metabolite of *Streptomyces antibioticus*, belongs to a relatively new class of siderophores which contains a thiazoline ring.[6] It forms 2 to 1 complexes with iron(III) using the thiazoline nitrogen, the phenol oxygen, and a carboxylate oxygen[5b,c] as donor sites. Retrosynthetically it can be viewed as arising from the cyclodehydration condensation of D-α-methylcysteine with 3-hydroxypicolinic acid.

While DFT is a very efficient, orally effective iron chelator,[7] it presents with some toxic side effects.[8] Although it is possible to alter DFT (Figure 1, e.g., **2** and **3**) and substantially reduce these deleterious effects, even the least toxic of the DFT analogues is not completely acceptable. Thus it is of some importance to identify the origin of the toxicity in these systems. With this information in hand, it may well be possible to alter the structure of the molecule in order to eliminate the deleterious side effects, while still maintaining the ligand's iron-clearing properties. Previous studies suggested that there is no simple correlation between the side effects of the desferrithiocins and their ability to clear iron from whole animals.[8] However, this is not to say that the capacity of the ligands to selectively remove iron from certain subcellular storage sites or iron-dependent enzymes, e.g., ribonucleotide reductase, could not explain their toxicity.

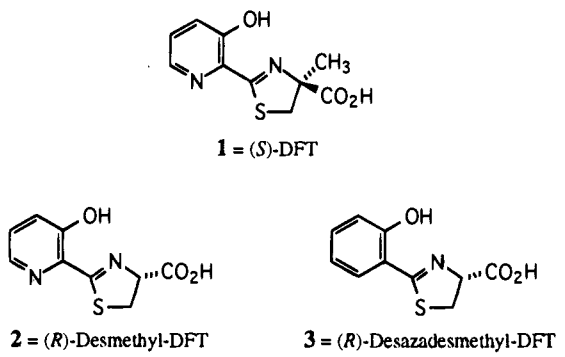

Figure 1. (*S*)-Desferrithiocin (1), (*R*)-desmethyldesferrithiocin (**2**), and (*R*)-desazadesmethyldesferrithiocin (**3**).

Results and Discussion

Two issues were addressed in this study, the metabolic significance of desferrithiocins' hydrolysis products with regards to iron clearance and toxicity as well as the toxicity of the iron chelates themselves.[9]

The first and most pressing questions regarding the metabolism of the desferrithiocins (Scheme 1) were

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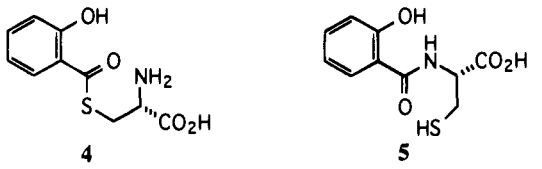

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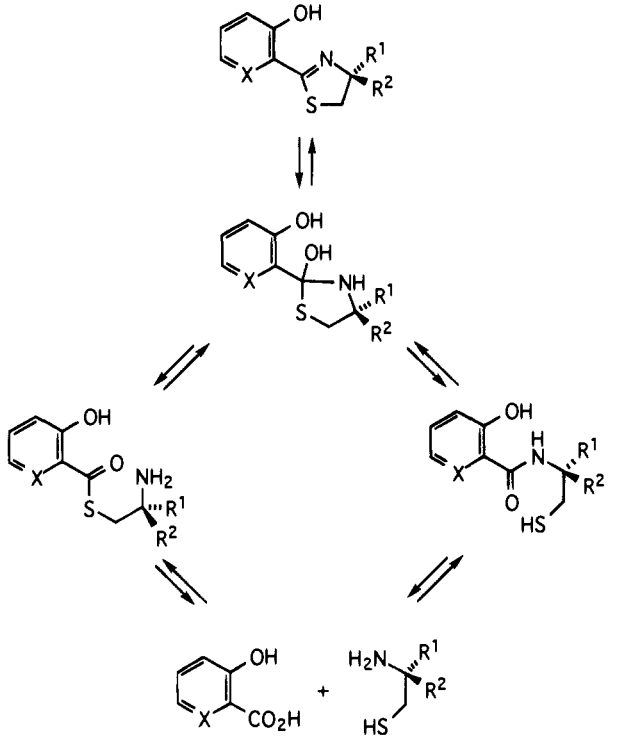
Figure 2. *S*-(2′-Hydroxybenzoyl)-L-cysteine (**4**) and *N*-(2′-hydroxybenzoyl)-L-cysteine (**5**).

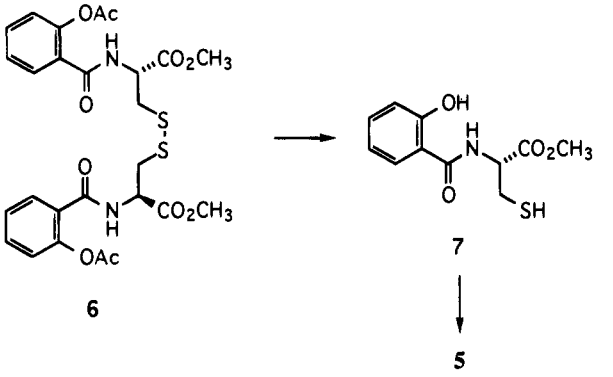
Scheme 1. Metabolism of Desferrithiocins



associated with two earlier studies of thiazoline hydrolysis in aqueous acid[10] and liver homogenates.[11] The acid hydrolyses of 2-phenyl-Δ^2-thiazoline-4-carboxylic acids were shown to occur with an optimum pH of 2.5, a pH essentially identical to that seen in the stomach of fasted rats. While the products were never identified, the authors suggested the presence of both a thiol amide and a thioester.[10] The second observation is that L-thiazolidine-4-carboxylic acid, when incubated with rat liver homogenate, was oxidized to the corresponding Δ^2-thiazoline-4-carboxylic acid.[11] Continued incubation resulted in hydrolysis of the thiazoline to *N,N′*-diformylcystine. Both of these experiments demanded not only an investigation of the hydrolysis rates of the desferrithiocins at relevant pH's, 2.5 and 7.2, but also an evaluation of the toxicity of the hydrolysis products and their iron-clearing properties.

Three ligands[7a] were chosen for study, (*S*)-DFT (**1**), (*R*)-desmethyl-DFT (**2**), and (*R*)-desazadesmethyl-DFT (**3**), as they represent a dichotomy in terms of toxicity. Orally administered **1** and **3** kill rodents in 6 days at a dose at which **2** does not present with any deleterious effects.[8] The hydrolysis products of (*R*)-desazadesmethyl-DFT (**3**), thioester **4**, and thiol amide **5** were synthesized and characterized in order to evaluate the general reactivity as well as spectral and chromatographic properties of these types of compounds (Figure 2). This made it possible to identify the hydrolysis products of DFT and desmethyl-DFT with greater assurance. Synthesis of thioester **4** and thiol amide **5** also provided enough material for an evaluation of their iron-clearing and toxic properties.

Scheme 2. Synthesis of *N*-(2′-Hydroxybenzoyl)-L-cysteine (**5**)

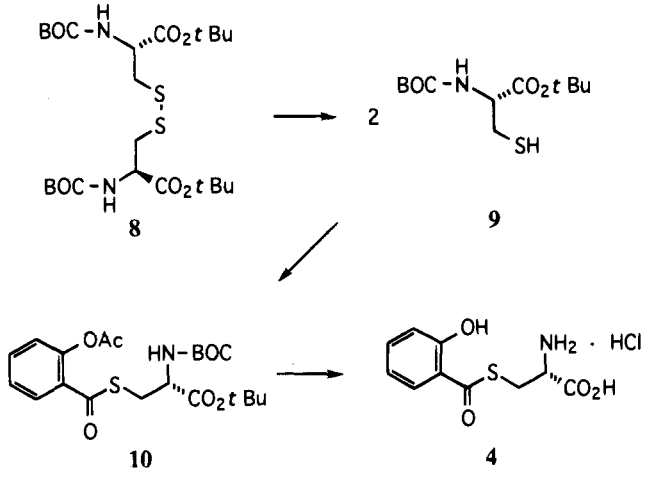


The hydrolysis studies were designed to address six questions. (1) What is the impact of removing the DFT methyl group or both the methyl group and the aromatic nitrogen on the hydrolytic stability of the thiazoline ring? (2) Does the Lewis acid iron(III) increase the hydrolysis rate of the thiazoline ring? (3) Can differences in hydrolysis rate be correlated with toxicity? (4) Are the hydrolysis products responsible for toxicity? Thioesters might act as acylation agents while the thiol group of the thiol amide could oxidatively couple to other thiols forming disulfides. (5) Do the thioesters or thiol amides of **1**–**3** hydrolyze to 3-hydroxypicolinic acid or salicylic acid and D-α-methylcysteine or L-cysteine? (6) Is it possible that the hydrolysis products clear iron? This could explain the previous observation[7] that oxazoline compounds show no iron-clearance activity; their hydrolysis products would contain a hydroxyl instead of a thiol function.

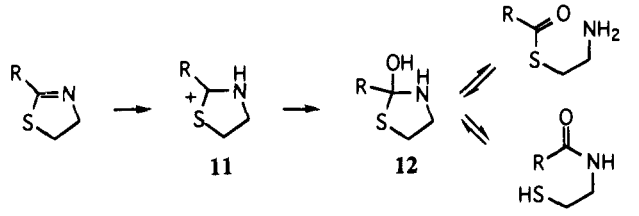
Synthesis. The syntheses of the thioester **4** and the amide **5** (Figure 2) are divided in two steps: (i) a reaction between acetylsalicyloyl chloride and a protected L-amino acid and (ii) deprotection. The starting material for the synthesis of thiol amide **5** was cystine dimethyl ester (Scheme 2). Joining with acetylsalicyloyl chloride in methylene chloride/pyridine provided the diamide in moderate yield (**6**, 31%). While the common method of reduction of cystine compounds with Zn/HCl[12] failed, probably because of the low solubility of **6** in methanol, the treatment with 1,4-DL-dithiothreitol[13] (ethanol, room temperature, 1 h) was successful. The basic conditions during this reaction (pH 8, 2 h) caused the hydrolysis of the acetate so that 2-hydroxybenzoyl amide **7** (75%) was isolated. Saponification of the methyl ester with sodium hydroxide under nitrogen (methanol/water, 5 h) provided **5** in 93% yield.

Because of the expected instability of the thioester **4** in neutral and basic aqueous medium, protecting groups were chosen which could be cleaved in an acidic solution (Scheme 3). The synthesis of the thioester **4** started with the N-protection of cystine di-*tert*-butyl ester[14] by treatment with di-*tert*-butyl dicarbonate (methanol, reflux, 5 min, 70% of **8**). The cystine **8** was reduced with 1,4-DL-dithiothreitol furnishing **9** (methanol, room temperature, 1 h, 78%). Coupling **9** with acetylsalicyloyl chloride gave **10** in a yield of 71% (pyridine, methylene chloride, room temperature, 2.5 h). Unreacted **9** (17%) was recovered by chromatography. Even with longer

Scheme 3. Synthesis of *S*-(2′-Hydroxybenzoyl)-L-cysteine (**4**)



Scheme 4. Mechanism of Thiazoline Hydrolysis



times, the reaction was not driven to completion. Treatment of **10** with TFA cleaved the *tert*-butoxycarbonyl (BOC) group and the *tert*-butyl ester (room temperature, 23 h). The hydrolysis of the acetate was accomplished by treatment with 1 N hydrochloric acid at room temperature for 72 h. Thioester **4** was isolated as a hydrochloride in 82% yield. Attempted hydrolysis at 70 °C generated a large amount of the thiazoline **3** (ca. 30%).

Kinetic Measurements. The optimum pH for the hydrolysis of 2-substituted-Δ^2-thiazolines is around 2, depending on the substitution.[10] The hydrolyses of **1**–**5** in aqueous solutions at pH 2.5 and 7.2 at 37 °C were followed by HPLC. In the case of desazadesmethyl-DFT (**3**), the hydrolysis products had already been synthesized and their HPLC properties determined, thus facilitating the kinetic studies. With DFT (**1**) and desmethyl-DFT (**2**), the hydrolysis products were isolated by chromatography on LH-20 and identified by ^{1}H NMR and mass spectra.

The previously proposed mechanism[10] of thiazoline hydrolysis proceeds via an attack of water on the protonated thiazoline species **11** providing the 2-hydroxythiazolidine **12** (Scheme 4). This can rearrange to either a thioester or a thiol amide. The rate-determining step is the attack of water on **11**. Hence the more stable **11** is, the slower the hydrolysis.

The main hydrolysis products of the thiazolines **1** and **2** at pH 2.5 are the corresponding oxygen-sensitive (→ disulfides) thiol amides **13** and **14**, which were isolated and characterized by NMR and mass spectra (Figure 3). The half-lives of **1** and **2** are 18.6 and 8.74 h, respectively. Hydrolysis to 3-hydroxypicolinic acid or salicylic acid was not observed. In the case of DFT (**1**), a second very small HPLC peak appeared. On the basis of the comparison with the retention time and spectral property of the thioester **4**, this was very likely the

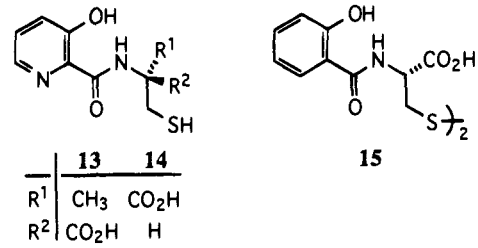


Figure 3. Structures of hydrolysis products: *N*-(3′-hydroxypyridin-2′-oyl)-D-α-methylcysteine (**13**), *N*-(3′-hydroxypyridin-2′-oyl)-L-cysteine (**14**), and *N*,*N*′-bis(2′-hydroxybenzoyl)-L-cystine (**15**).

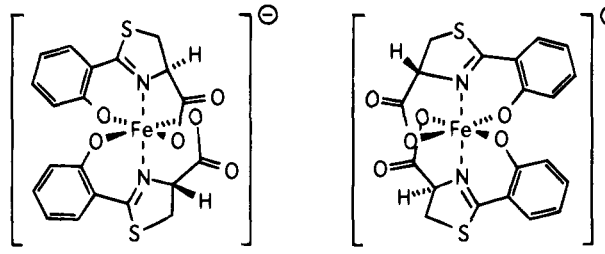


Figure 4. Proposed structures of diastereomeric (*R*)-desazadesmethyl-DFT 2 to 1 complexes **16a/b** with iron(III).

corresponding DFT thioester (≤2%). At pH 7.2, both of the thiazolines are stable.

The hydrolysis of desazadesmethyl-DFT (**3**) at pH 2.5 had a half-life of 31.7 h. In addition to thiol amide **5**, small amounts of thioester **4** (≤2%) and disulfide **15** (<2%) were observed. No salicyclic acid was formed. Thiazoline **3** is stable at pH 7.2 for at least 3 days. In the presence of iron(III), thiazoline **3** probably forms two diastereomeric complexes,[5c] **16a/b** (Figure 4). The thiazolines in **16a/b** are significantly stabilized in regard to hydrolysis at pH 2.5; the half-life is larger than 20 days. At pH 7.2, no hydrolysis was observed during a measured time period of 3 days.

The thioester **4** rearranges to the thiol amide **5** at pH 2.5 with a half-life of 4.18 h. In order to determine the half-time at pH 7.2, the pH of an acidic solution was adjusted to pH 7.2 and then again to 2.5 (<1 min). Only **5** was observed by HPLC. Hence, as expected,[15] the half-life is very short, <1 min. An intramolecular acyl transfer via **12** is likely for the reaction both in acidic and in neutral solutions (Scheme 4).[15]

The thiol amide is stable at pH 2.5 and did not hydrolyze to salicylic acid and L-cysteine. However, an equilibrium of 49:1 with the thioester is established after 48 h. The small amount of **4** corresponds to that which was observed during the hydrolysis of thiazoline **3**. While **5** is stable at pH 7.2 in a nitrogen atmosphere, in the presence of oxygen, it is slowly oxidized to the disulfide **15** that was characterized by ^{1}H NMR and mass spectrum (Figure 3).

The kinetic data of the compounds, studied at pH 2.5 and 7.2, are summarized in Table 1.

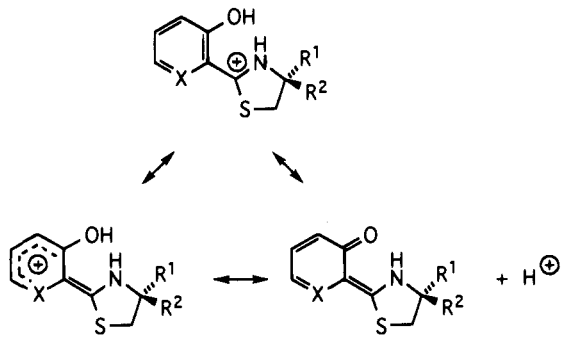
The observation that desazadesmethyl-DFT (**3**) has the longest half-life, 31.7 h, is in keeping with the more effective electron-donating ability of the hydroxyphenyl substituent in comparison with the hydroxypyridyl substituent; the electron-deficient pyridine ring contributes less to the resonance stabilization of the thiazolinium cation (Scheme 5). The methyl substituent in DFT (**1**) hinders the attack of water and causes its larger

Table 1. Kinetic Data of 1–5 and 16a/b Determined by HPLC Measurements

	$t_{1/2}$ (h)	
	pH 2.5	pH 7.2
1 = (*S*)-DFT	18.6 ± 0.80	stable
2 = (*R*)-desmethyl-DFT	8.74 ± 0.35	stable
3 = (*R*)-desazadesmethyl-DFT	31.7 ± 0.40	stable
4 = thioester of 3	4.18 ± 0.23	<1 min
5 = thiol amide of 3	stable[a]	stable[b]
16 = iron complexes of 3	>20 days	stable

[a] An equilibrium of 5/4 = 49:1 is established after 48 h. [b] In the presence of oxygen, thiol 5 is slowly oxidized to disulfide 15.

Scheme 5. Resonance Stabilization of the Thiazolinium Cation



stability compared to desmethyl-DFT (2). The half-lives are 18.6 and 8.74 h, respectively. The kinetic results suggest that thiazoline compounds with specific stability might be designed with different substitution patterns of the aromatic ring.

In order to evaluate the probability of reactions between thiazolines or thioesters with amino functions of proteins, amines were added to acidic and neutral solutions of desazadesmethyl-DFT (3) and the thioester 4, respectively. *m*-Nitroaniline and 2-[(2-aminoethyl)-amino]-5-nitropyridine were chosen for the study because of their good solubility in water and their suitable extinction coefficients for the UV detection during the HPLC. However, no reaction was observed. In the case of 4, the intramolecular amidation is obviously superior and prevents intermolecular reactions. This is in agreement with the finding[15] that *S*-acetylcysteamine undergoes intramolecular amidation even in an ammonium hydroxide solution.

Biological Studies. Only the hydrolysis products of desazadesmethyl-DFT (3), thioester 4, and thiol amide 5 were evaluated for their iron-clearing and toxic properties. The iron-clearing properties of the hydrolysis products were carried out in a non-iron-overloaded bile duct-cannulated rat model, while the toxicity studies were performed in CD-1 female mice.

The iron-clearing measurements were made at a dose of 150 μmol/kg, and both oral and subcutaneous administration of thioester 4 and thiol amide 5 were evaluated. The parent compound desazadesmethyl-DFT (3) served as a positive control. Neither the thioester nor the thiol amide promoted the clearance of iron.

The toxic properties of 4 and 5 were compared with those of the parent desazadesmethyl-DFT (3) and its iron(III) complexes **16a/b**. Because of the limited amount of material available, these studies were carried out in mice. The compounds were administered intraperitoneally at a dose of 384 μmol/kg. Previous studies[8] were done in rats at the same dose; however, the drugs were given by gavage. In the rat experiments, desazadesmethyl-DFT (3) was shown to be a severe gastrointestinal irritant; all rats were dead by the sixth day of treatment. In the current study, the mice were given thioester 4 or thiol amide 5 at 384 μmol/kg once daily for 10 days. The animals were monitored for weight loss, general appearance, food consumption, acute changes at the sight of injection, and changes in urine parameters including ketone bodies, glucose, bilirubin, specific gravity, blood, pH, protein, nitrites, and leukocytes. Necropsies were performed on the animals on day 11 of the study. The only change observed in any of the animals treated with either 4 or 5 was a slight but reversible increase in ketone bodies. However, mice treated with either parent chelator 3 or its 2 to 1 ligand to iron chelate sustained notable toxic effects. The animals given the chelate at a dose of 384 μmol/kg were all dead on day 2, while the mice given the free ligand intraperitoneally at 384 μmol/kg were dead by day 5.

Thus, the hydrolysis products of desazadesmethyl-DFT (3), thioester 4 and thiol amide 5, exhibit neither toxic effects nor iron-clearance activity. As L-cysteine, D-α-methylcysteine, 3-hydroxypicolinic acid, and salicylic acid are not toxic, hydrolysis products can be excluded as the cause of the toxicity of DFT and its analogues. Interestingly, desazadesmethyl-DFT and its iron complex presented with similar toxic properties; a finding that suggests that the role of Fenton chemistry[16] (e.g., the damage of cell membranes by the generation of hydroxy radicals) in the toxicity of these chelates should be investigated.

Conclusion

While the thiazolines studied were shown to sustain some hydrolysis at pH 2.5 at 37 °C, they were very stable at pH 7.2 at the same temperature. However, because of the relatively long half-lives of the desferrithiocins studied at pH 2.5 and the transit time of the stomach contents, it is unlikely that any significant hydrolytic decomposition of the drugs takes place. On the basis of the transit time of materials in a rat stomach and the half-life of desmethyl-DFT (2), 9 h at stomach pH, approximately 5% hydrolysis would be predicted for this chelator. Recall that this had the shortest half-life of all of the drugs at pH 2.5. In addition, the hydrolysis rates of the thiazolines 1–3 were no indicator for the toxicity.

In the case of desazadesmethyl-DFT (3), the hydrolysis products were not toxic and did not promote the clearance of iron from rodents. Inasmuch as these findings can be extrapolated to the hydrolysis products of the other two desferrithiocins, it seems likely that the corresponding thioesters and thiol amides should be equally inactive. The main products of all of the ligands exposed to aqueous acid were the thiol amides. Further hydrolysis did not occur. Although the thioesters are relatively stable in acidic solutions, at or above neutral pH, they quickly rearrange to the corresponding thiol amides. The intramolecular rearrangement was fast enough that there was no competition with external nucleophiles, e.g., primary or aromatic amines. In addition, it was clear that the iron complex stabilized the thiazolines in regard to reaction with nucleophiles.

It now seems likely, on the basis of these studies and the earlier work of Baker et al,[9] that the source of the

desferrithiocins' toxicity is related to the chelates causing Fenton chemistry and not to their hydrolytic products.

Experimental Section

Reagents were obtained from Aldrich, Sigma, or Fluka. Magnesium sulfate was employed as a drying reagent. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were run in methanol (c = 1 g/100 mL) at 589 nm (sodium D line) at 25 °C. Anal. TLC: Whatman silica gel 60A plates with F_{254} indicator were used; spots were observed in UV light and/or by treatment with a 5% ethanolic solution of phosphomolybdic acid. Column chromatography: silica gel 60 (70–230 mesh) was purchased from EM Science, Darmstadt, Germany; lipophilic sephadex LH-20 was obtained from Sigma. HPLC: the ET 125/8/4 Nucleosil 100-5 C_{18} column was purchased from Macherey-Nagel, Düren, Germany. ^{1}H NMR: spectra were recorded at 300 MHz using a General Electric QE 300 spectrometer. Chemical shifts are given in ppm relative to TMS (δ = 0 ppm). Mass spectra were carried out on a Fennigan 4516 instrument. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. Results are within 0.4% of theoretical values. Cremophor RH-40 was obtained from BASF, Parsippany, NJ. Sprague–Dawley rats were purchased from Charles River, Wilmington, MA. Nalgene metabolic cages, rat jackets, and fluid swivels were purchased from Harvard Bioscience, South Natick, MA. Intramediac polyethylene tubing PE 50 was obtained from Fisher Scientific, Pittsburgh, PA.

2-(2′-Hydroxyphenyl)-Δ^2-thiazoline-4(R)-carboxylic acid (3): UV (0.4 M phosphate buffer, pH 2.56) λ_{max} 206 (14 200), 274 (14 960), 341 nm (5480); ϵ_{250} 5220; ϵ_{320} 4550.

***N,N′*-Bis(2′-Acetoxybenzoyl)-L-cystine Dimethyl Ester (6).** To a mixture of L-cystine dimethyl ester dihydrochloride (11.2 g, 32.8 mmol) and pyridine (20.7 g, 21.2 mL, 262 mmol) in dry methylene chloride (140 mL) was added a solution of acetylsalicyloyl chloride (13.0 g, 65.6 mmol) in methylene chloride (30 mL) at 0 °C. The mixture was stirred at room temperature for 90 min and then diluted with methylene chloride (60 mL) and washed with water (200 mL), 0.5 N hydrochloric acid (200 mL), and water (2 × 200 mL). The organic layer was dried and concentrated in vacuo. Crystallization from ethyl acetate provided colorless crystals (6.10 g, 31%): mp 150–151 °C; ^{1}H NMR (DMSO-d_6) δ 8.76 (d, 2 NH, J = 8 Hz), 7.60 (m, 2 arom H), 7.54 (m, 2 arom H), 7.35 (m, 2 arom H), 7.22 (m, 2 arom H), 4.74 (ddd, 2 CH), 3.68 (s, 2 CO_2CH_3), 3.27 (dd, 2/2 CH_2, J_{gem} = 13 Hz, J_{vic} = 5 Hz), 3.10 (dd, 2/2 CH_2, J_{vic} = 8.5 Hz), 2.24 (s, 2 OAc). Anal. ($C_{26}H_{28}N_2O_{10}S_2$) C, H, N, S.

***N*-(2′-Hydroxybenzoyl)-L-cysteine Methyl Ester (7).** The pH of an ethanol mixture (200 mL) containing **6** (6.10 g, 10.3 mmol) was adjusted with 1 N ammonium hydroxide solution to 8. 1,4-DL-dithiothreitol (4.71 g, 30.9 mmol) was added under nitrogen, and the mixture was stirred at room temperature for 2 h. Hydrochloric acid (1 N, 10 mL) was added, and the solution was concentrated to about 50 mL. Water (125 mL) was added and the mixture extracted with ethyl acetate (3 × 100 mL). The organic layer was dried and concentrated. Repeated (2×) chromatography (90/3 cm, cyclohexane/ethyl acetate 1:1, R_f = 0.79) provided colorless crystals (3.95 g, 75%): mp 98–99 °C; ^{1}H NMR (DMSO-d_6) δ 11.92 (s, 1 OH), 9.14 (d, 1 NH, J = 6 Hz), 7.94 (m, 1 arom H), 7.44 (m, 1 arom H), 7.01–6.92 (m, 2 arom H), 4.75 (m, 1 CH), 3.71 (s, 1 CO_2CH_3), 3.06–2.98 (m, 1 CH_2), 2.61 (dd, 1 SH, J = 8 Hz); MS (CI, NH_3) m/z (%) 256 [M^+ + 1] (100), 224 [M^+ − OCH_3] (6), 139 (19), 121 (26).

***N*-(2′-Hydroxybenzoyl-L-cysteine (5).** To a solution of **7** (1.84 g, 7.21 mmol) in methanol (73 mL) was added 1 N sodium hydroxide solution (73 mL). The solution was stirred under nitrogen at room temperature for 5 h (TLC: ethanol/ethyl acetate 1:2, R_f = 0.67). Hydrochloric acid (1 N, 130 mL) was added, and the solution (pH 2) was concentrated to 150 mL. After extraction with ethyl acetate (3 × 100 mL), the organic layer was dried and concentrated providing colorless crystals (1.62 g, 93%): mp 200–201 °C; optical rotation (methanol) $[\alpha]^{25}_D$ = +9.2°; UV (0.4 M phosphate buffer, pH 2.56) λ_{max} 206 (18 100), 237 (9470), 296 nm (3550); ϵ_{250} 5220; ϵ_{292} 3430; ϵ_{299} 3430; ^{1}H NMR (DMSO-d_6) δ 13.09 (s, 1 CO_2H), 11.95 (s, 1 OH), 9.08 (d, 1 NH, J = 8 Hz), 7.96 (d, 1 arom H), 7.43 (dd, 1 arom H), 6.97–6.91 (m, 2 arom H), 4.68 (m, 1 CH, J = 5 Hz), 3.12–2.92 (m, 1 CH_2), 2.48 (dd, 1 SH, J = 8 Hz); MS (EI, 70 eV) m/z (%) 241 [M^+] (29), 223 (16), 208 [M^+ − SH] (1), 196 [M^+ − CO_2H] (2), 121 (100); FABMS calcd 241.0409, found 241.0393. Anal. ($C_{10}H_{11}NO_4S$) C, H, N, S.

***N,N′*-Bis(BOC)-L-cystine Di-*tert*-butyl Ester (8).** To a solution of L-cystine di-*tert*-butyl ester[14] (3.76 g, 10.7 mmol) in triethylamine (12 mL) and methanol (108 mL) was added a solution (7.5 mL) of di-*tert*-butyl dicarbonate (13.7 g, 62.7 mmol) in methanol. The mixture was heated under reflux for 5 min. The solvent was evaporated under reduced pressure, and the residue taken up in ice-cold 1 N hydrochloric acid (100 mL). After extraction with ethyl acetate (3 × 75 mL), the organic layer was dried and concentrated in vacuo. Chromatography (40/3 cm, cyclohexane/ethyl acetate 1:1, R_f = 0.75) provided a colorless oil. After addition of ether/cyclohexane 1:1 (0 °C, 12 h), colorless crystals (4.15 g, 70%) were obtained: mp 89–90 °C; ^{1}H NMR (DMSO-d_6) δ 7.26 (d, 2 NH, J = 8 Hz), 4.11 (ddd, 2 CH), 3.07 (dd, 2/2 CH_2, J_{gem} = 13 Hz, J_{vic} = 4.5 Hz), 2.89 (dd, 2/2 CH_2, J_{vic} = 8.5 Hz), 1.41 (s, 6 CH_3), 1.38 (s, 6 CH_3); MS (EI, 70 eV) m/z (%) 553 [M^+] (40), 497 (7), 441 (10), 385 (25), 329 (68), 285 (100). Anal. ($C_{24}H_{44}N_2O_8S_2$) C, H, N, S.

***N*-BOC-L-cysteine *tert*-Butyl Ester (9).** The pH of a nitrogen-saturated solution of **8** (4.15 g, 7.51 mmol) in ethanol (65 mL) was adjusted to 8 with ammonium hydroxide solution. 1,4-DL-dithiothreitol (2.31 g, 15.0 mmol) was added, and the solution was stirred at room temperature for 60 min (TLC: cyclohexane/ethyl acetate 2:1, R_f(**9**) = 0.73, R_f(**8**) = 0.71). Then, the solution was acidified with 1 N hydrochloric acid until pH 1 was obtained. After concentration to dryness, the residue was taken up in ether (100 mL) and 0.25 N hydrochloric acid (100 mL). The organic layer was washed with 0.25 N hydrochloric acid (4 × 100 mL) and water (100 mL), dried, and concentrated to provide colorless crystals (3.24 g, 78%): mp 46–47 °C; ^{1}H NMR ($CDCl_3$) δ 5.40 (br s, 1 NH), 4.47 (m, 1 CH), 2.97 (m, 1 CH_2), 1.50 (s, 3 CH_3), 1.45 (s, 3 CH_3). Anal. ($C_{12}H_{23}NO_4S$) C, H, N, S.

***N*-BOC-*S*-(2′-Acetoxybenzoyl)-L-cysteine *tert*-Butyl Ester (10).** To a solution of **9** (3.23 g, 11.6 mmol) in pyridine (2.75 g, 34.8 mmol) and methylene chloride (22 mL) was added a solution of acetylsalicyloyl chloride (2.30 g, 11.6 mmol) in methylene chloride (22 mL). After stirring at room temperature for 2.5 h, the mixture was diluted with methylene chloride (60 mL) and washed with 0.5 N hydrochloric acid (2 × 100 mL). The organic layer was dried and concentrated. Chromatography (87/3 cm, cyclohexane/ethyl acetate 2:1, R_f(**9**) = 0.73, R_f(**10**) = 0.58) provided **9** (0.56 g, 17%) and **10** (3.63, 71%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.92 (m, 1 arom H), 7.56 (m, 1 arom H), 7.32 (m, 1 arom H), 7.13 (m, 1 arom H), 5.29 (d, 1 NH, J = 8 Hz), 4.50 (m, 1 CH), 3.52 (m, 1 CH_2), 1.47 (s, 3 CH_3), 1.44 (s, 1 CH_3), 1.43 (s, 2 CH_3); FABMS [M^+ + 1] calcd 440.1743, found 440.1677. Anal. ($C_{21}H_{29}NO_7S$) C, H, N, S.

***S*-(2′-Hydroxybenzoyl)-L-cysteine Hydrochloride (4).** A solution of **10** (1.02 g, 2.32 mmol) in methylene chloride (8 mL) and TFA (16 mL) was stirred at room temperature for 23 h. The solution was concentrated to dryness. The residue was taken up in 1 N hydrochloric acid (10 mL) and stirred at room temperature for 72 h. The solution was diluted with 1 N hydrochloric acid (5 mL) and extracted with methylene chloride (3 × 20 mL). The aqueous layer was concentrated to dryness providing colorless crystals (530 mg, 82%): mp 157–158 °C; UV (0.4 M phosphate buffer, pH 2.56) λ_{max} 208 (18 400), 245 (6230), 270 (8720), 320 nm (4510); ϵ_{250} 5250; ϵ_{292} 3690; ϵ_{299} 3450; ^{1}H NMR (DMSO-d_6) δ 10.98 (s, 1 OH), 8.58 (s, 1 NH_3^+), 7.76 (m, 1 arom H), 7.49 (m, 1 arom H), 7.11 (m, 1 arom H), 6.95 (m, 1 arom H), 4.22 (m, 1 CH), 3.58 (dd, 1/2 CH_2, J_{gem} = 13 Hz, J_{vic} = 5 Hz), 3.46 (dd, 1/2 CH_2, J_{vic} = 6 Hz); FABMS [M^+ + 1 − HCl] calcd 242.0487, found 242.0506. Anal. ($C_{10}H_{12}ClNO_4S$) C, H, Cl, N, S.

***N*-(3′-Hydroxypyridin-2′-oyl)-D-α-methylcysteine (13).** The pH of a solution of the sodium salt of 1 (100 mg, 0.408 mmol) in water (50 mL) was adjusted with hydrochloric acid to 2.5. The solution was saturated with nitrogen and maintained at 37 °C for 6 days. The solution was concentrated to dryness, and the residue was chromatographed on LH-20 (5 g, 25/1 cm, chloroform/methanol 1:1) providing a colorless oil (55 mg, 53%): ^{1}H NMR (methanol-d_4) δ 8.14 (dd, 1 arom H), 7.46 (dd, 1 arom H), 7.34 (dd, 1 arom H), 3.55 (d, 1/2 CH_2, J = 14 Hz), 3.16 (d, 1/2 CH_2), 1.75 (s, 1 CH_3); FABMS [M^+ + 1] calcd 257.0596, found 257.0579.

***N*-(3′-Hydroxypyridin-2′-oyl)-L-cysteine (14).** The pH of a solution of **2** (100 mg, 0.446 mmol) in water (50 mL) was adjusted with hydrochloric acid to 2.5. The solution was saturated with nitrogen and maintained at 37 °C for 70 h. The solution was concentrated to dryness, and the residue was chromatographed on LH-20 (5 g, 25/1 cm, chloroform/methanol 1:1) providing colorless crystals (70 mg, 65%): mp 104–105 °C; ^{1}H NMR (DMSO-d_6) δ 9.18 (d, 1 NH, J = 8 Hz), 8.23 (dd, 1 arom H), 7.60 (dd, 1 arom H), 7.51 (dd, 1 arom H), 4.70 (m, 1 CH), 3.15–3.02 (m, 1 CH_2), 2.56 (dd, 1 SH, J = 8 Hz); MS (CI) m/z (%) 243 [M^+ + 1] (100), 225 [M^+ − OH] (27), 197 [M^+ − CO_2H] (14); FABMS [M^+ + 1] calcd 243.0439, found 243.0496. Anal. ($C_9H_{10}N_2O_4S$) C, H, N, S.

***N*,*N*′-Bis(2′-hydroxybenzoyl)-L-cystine (15).** The pH of a solution of **5** (24 mg, 0.10 mmol) in water (50 mL) was adjusted with NaOH solution to 7.2. The solution was stirred at 60 °C for 116 h. Hydrochloric acid (1 N) was added until pH 2 was obtained. The solution was extracted with ethyl acetate (3 × 30 mL). The organic layer was dried and concentrated providing colorless crystals (20 mg, 83%): mp 96–97 °C; ^{1}H NMR (DMSO-d_6) δ 12.10 (s, 2 OH), 9.18 (d, 2 NH, J = 6 Hz), 7.93 (d, 2 arom H), 7.41 (dd, 2 arom H), 6.95 (d, 2 arom H), 6.91 (dd, 2 arom H), 4.68 (ddd, 2 CH), 3.34 (dd, 2/2 CH_2, J_{gem} = 14 Hz, J_{vic} = 5 Hz), 3.23 (dd, 2/2 CH_2, J_{vic} = 9 Hz); FABMS [M^+ + 1] calcd 481.0739, found 481.0739.

Incubation Solutions for Kinetic Measurements. The pH of 2 mM solutions (15 mL) of 1–**5** in the mobile phase B (see HPLC Measurements), containing 2,6-lutidine as a standard (4 mM), was adjusted with hydrochloric acid or KOH solution to 2.5 and 7.2, respectively. The solutions were maintained at 37 °C, and the samples were analyzed by HPLC during a time period of up to 22 days. The iron(III)-containing solutions were made by the addition of $Fe(NO_3)_3$ (1 mM). In order to control the stability of **3** in the presence of iron(III) not only by HPLC but also by NMR, the iron(III)-containing solutions (15 mL) were mixed with 1 N NaOH solution (15 mL) and $Fe(OH)_3$ was separated by filtration. The filtrate was acidified with 1 N hydrochloric acid (20 mL). The solution was extracted with ethyl acetate (3 × 20 mL), dried, and concentrated. The residue was taken up in DMSO-d_6 and analyzed by ^{1}H NMR.

HPLC Measurements. The mobile phase was an acidic phosphate buffer (pH 3.3)/acetonitrile gradient. Mobile phase A: acetonitrile and mobile phase B were mixed in a 4:1 ratio. Mobile phase B: to a solution of KH_2PO_4 (6.00 g, 44.1 mmol) and o-phosphoric acid (85%, 1.00 g, 8.67 mmol) in water (200 mL) was added o-phosphoric acid until pH 2.5 was obtained; 30 mL was diluted with water to 3000 mL (pH 3.3). Two different methods were used (Table 2). Method 1: flow rate 1.5 mL/min, detector wavelength 250 nm, time 0.0 min (90% B), 15.0 (50), 18.0 (20), 20.0 (90), 23.0 (90). Method 2: flow rate 1.0 mL/min, detector wavelength 250 nm, time 0.0 min (90% B), 17.0 (20), 19.0 (90), 21.00 (90).

Bile Duct Cannulation: Male Sprague–Dawley rats averaging 400 g were housed in Nalgene plastic metabolic cages and given free access to water. The animals were anesthetized with sodium pentobarbital (50 mg/kg), given ip. The bile duct was cannulated with 22 gauge PE 50 tubing which was inserted ca. 2 cm into the duct and tied firmly in place.

A skin-tunneling needle was inserted from the shoulder area around to the abdominal incision. The cannula was threaded through the needle until it emerged from the shoulder opening. The cannula was then passed from the animal to the swivel inside a metal torque-transmitting tether which was attached to a rodent jacket. The cannula was directed from the animal to a Gilson microfraction collector by a fluid swivel mounted above the metabolic cage. This system allowed the animal to move freely in the cage while continuous bile samples were being collected. Bile samples were collected in plastic disposable tubes at 3 h intervals for 24 h. Urine samples were collected in plastic disposable tubes for 24 h.

Table 2. HPLC Data of 1–**5**, **13**–**15**, **16a/b**, and Standards

	method	retention time (min)
1	1	9.9–10.1
13	1	11.2–11.3
2	1	7.0–7.3
14	1	7.7–7.9
3	2	11.8–12.0
4	2	9.4–9.6
5	2	5.7–5.8
15	2	12.5–12.6
16a/b	2	10.7–10.8 and 12.1–12.2
2,6-lutidine	1	1.2
2,6-lutidine	2	1.7–1.8
3-hydroxypicolinic acid	1	1.0
salicylic acid	2	8.2–8.5

Atomic Absorption Iron Determination. Samples were analyzed on a Perkin-Elmer 5100 PC atomic absorption spectrophotometer fitted with a Model AS-51 autosampler using a quartz sampling probe. The urine and bile samples were analyzed at 248.3 nm (slit width 0.20 nm) with appropriate linear calibration of 0–5 ppm. Representative samples were checked by the method of addition. Rat urine (2.5 mL) was diluted to 7.5 mL with 6% low-iron nitric acid, heated (65 °C, 4 H), and filtered before analysis by flame AA. Bile samples were handled in the same manner as the urine specimens, except that the sample volume was reduced and filtration was unnecessary.

Preparation of Drugs. Drug solutions were prepared in 60% water, 40% Cremophor RH-40 (v/v).

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