Evaluation of Desferrithiocin and Its Synthetic Analogues as Orally Effective Iron Chelators

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Desferrithiocin, a novel microbial siderophore isolated from cultures of *Streptomyces antibioticus* DSM 1865, and a number of its derivatives and analogues are evaluated for their ability to promote iron clearance. The compounds have been designed with the objective of identifying the structural features of desferrithiocin which render this ligand an orally effective iron chelator. The desferrithiocin aromatic hydroxyl and the thiazoline ring carboxyl group are shown to be central to desferrithiocin's activity. The ligand's methyl and the aromatic nitrogen play little role in the compound's efficacy. The animal model chosen for this study, the bile duct cannulated rat, provides information regarding both the chelator-induced total iron output and the kinetics of both biliary and urinary iron excretion.

Introduction

Although primary and secondary hemochromatosis,[1] iron-overload diseases, have received considerable attention in recent years from a variety of scientific disciplines,[2-5] treatment of the diseases remains essentially unchanged. Patients with primary hemochromatosis are still managed by frequent phlebotomy, while patients suffering from hemochromatosis secondary to blood transfusions must be maintained on chelation therapy.

The subcutaneous infusion of desferrioxamine B, a hexacoordinate hydroxamate iron chelator produced by large-scale fermentation of a strain of *Streptomyces pilosus*, is still widely regarded as the method of choice for treatment of transfusional iron overload.[6] While the drug's efficacy and long-term tolerability have been well-documented, there remain two major shortcomings: patient compliance associated with the drug's required mode of administration and the relatively high cost of treatment. As a result, considerable effort is being invested in the search for alternative iron chelators, in particular, those which are orally effective.

In this paper we describe the synthesis and biological evaluation of a series of compounds related to the novel siderophore desferrithiocin (1) (Figure 1). This ligand was first isolated from the culture broth of *S. antibioticus* DSM 1865,[7] and its structure and absolute configuration were determined by x-ray crystallographic studies. The molecule is noteworthy in that unlike most other microbial siderophores, it is neither a hydroxamate[8] nor a catecholamide[9] iron chelator. Structurally, desferrithiocin can be separated into two distinct segments: a 3-hydroxypyridine and a thiazoline ring system that can be thought of as arising from a cyclodehydration condensation of L-α-methylcysteine with 3-hydroxypyridine-2-carboxylic acid. The ferric siderophore complex, ferrithiocin, exists in a 2/1 ligand-to-metal ratio with an iron formation constant of $K_f = 10^{29.6}$ M^{-1}.[9] Although desferrithiocin is highly selective for ferric ion, the ligand also binds other metals including aluminum, zinc, and magnesium, however not as tightly.[10] While the precise structure of the complexes has yet to be determined, analogy with the catecholamide chelators would implicate the phenolic oxygen, the thiazoline nitrogen, and a carboxyl oxygen in the chelation of ferric ion.

Preliminary experiments have clearly demonstrated that desferrithiocin increases the rate of iron excretion in rats when administered either orally or subcutaneously (unpublished results). These results have prompted further investigations in order to evaluate the potential of desferrithiocin and its synthetic derivatives as orally effective agents in the treatment of transfusional iron-overload disease. The compounds described in this study have been designed and synthesized in order to identify the structural features of the parent molecule which are requisite for effective promotion of iron excretion. Biological evaluation of the test compounds has been accomplished by utilizing a non-iron-overloaded rat model in which drug-promoted iron excretion is monitored in both the bile and the urine.

Design and Synthesis

The compounds evaluated in this study (Figure 1) can be separated into six structural types: (A) the parent molecule, desferrithiocin (1), and its monosodium salt (2), (B) desmethyldesferrithiocin derivatives **3-5**, (C) the 2-pyridyl condensed thiazoline derivatives **6** and **7**, (D) the salicoyl condensed thiazoline derivative **8**, (E) the L-threonine-containing desferrithiocin analogue **9**, and (F) the terminal bis-desferrithiocin spermidine derivative **10**.

Previous studies[7] suggested that desferrithiocin forms a 2/1 complex with iron and that the aromatic hydroxyl, the thiazoline nitrogen, and a carboxyl oxygen are the ligating atoms. However, the importance, if any, of the pyridine nitrogen, the thiazoline sulfur, and the methyl group in the molecule's activity was completely unclear prior to this study. In evaluating the role of each of these structural components in the desferrithiocin's oral chelating efficacy, we have prepared a group of molecules, each with a particular functionality missing, and have compared the iron-clearing properties of these compounds with those of desferrithiocin.

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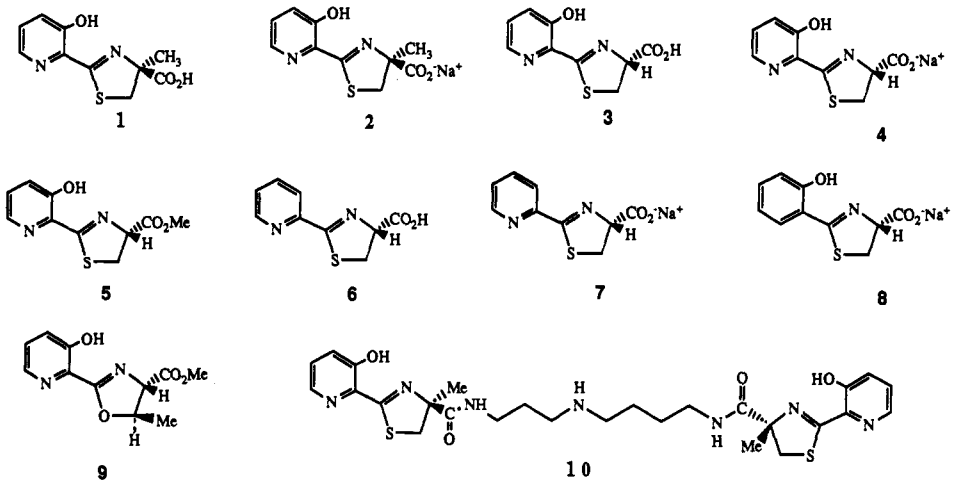
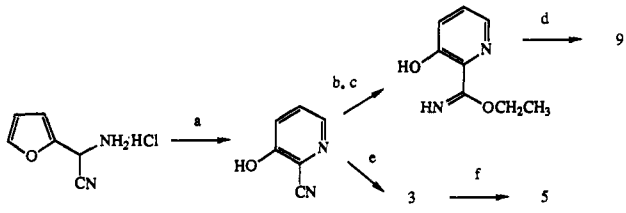


Figure 1. Structures of the siderophores desferrithiocin (1), desferrithiocin sodium salt (2), desmethyldesferrithiocin (3), desmethyldesferrithiocin sodium salt (4), desmethyldesferrithiocin methyl ester (5), dehydroxydesmethyldesferrithiocin (6), dehydroxydesmethyldesferrithiocin sodium salt (7), desazadesmethyldesferrithiocin sodium salt (8), desferrithiocin oxazoline methyl ester analogue (9), and N^1,N^8-bis(desferrithiocinoyl) spermidine (10).

Scheme I.[a] General Synthetic Scheme for Desferrithiocin and its Analogues and Derivatives



[a] Reagents: (a) 6 N HCl/Cl_2; (b) HCl/EtOH; (c) K_2CO_3; (d) L-Thr, Me ester/MeOH; (e) L-Cys/MeOH/0.1 M phosphate buffer; (f) 2,2-dimethoxypropane/*p*-TsOH/CH_2Cl_2.

The fact that desmethyldesferrithiocin is far more water soluble than desferrithiocin itself suggests that it is less lipophilic. The possibility that the desferrithiocin α-methyl group is augmenting the molecule's lipophilicity, thus promoting its oral absorption, is an interesting one. Its importance is further amplified by the difficulty in accessing the parent amino acid L-α-methylcysteine for the synthesis of desferrithiocin and its derivatives. If the methyl group were critical to the action of the chelator, the kinds of structural analogues possible would be limited. The synthesis of the desmethyl analogue 2-(3′-hydroxypyrid-2′-yl)-Δ^2-thiazolin-4(R)-carboxylic acid (**3**), begins with α-amino-2-furanacetonitrile hydrochloride (Scheme I). Oxidation of this intermediate was carried out with chlorine in hydrochloric acid which generated the key intermediate 3-hydroxy-2-cyanopyridine.[11] Direct condensation of the nitrile with L-cysteine in a phosphate buffer solution containing methanol afforded the desired thiazoline-containing derivative **3**. Treatment of acid **3** with aqueous sodium hydroxide furnished the corresponding sodium salt **4**. Preparation of methyl ester **5** was effected by treatment of carboxylic acid **3** with 2,2-dimethoxypropane in methylene chloride containing *p*-toluenesulfonic acid.

While the aromatic hydroxyl is expected to be a critical component in the ligand's metal-binding properties, one could also envisage an alternate mode of chelation involving the aromatic nitrogen, the thiazoline ring nitrogen, and the carboxyl. To determine the importance of the hydroxyl group, dehydroxydesmethyldesferrithiocin (**6**) was synthesized, and its iron-clearing properties were compared with those of desmethyl compound **3**. Synthesis of the 2-pyridyl condensed thiazoline derivative **6** was accomplished by a reaction similar to that described for **3** utilizing 2-cyanopyridine. Analogously, treatment of 2-hydroxybenzonitrile with L-cysteine furnished 2-(2-hydroxyphenyl)-Δ^2-thiazoline-4(R)-carboxylic acid, sodium salt (**8**).

The necessity of the thiazoline ring sulfur in desferrithiocin-promoted iron clearance was evaluated by comparing the ability of the threonine analogue to clear iron with that of the desmethyldesferrithiocin methyl ester. Synthesis of a threonine-containing analogue (**9**) required slightly different methodology (Scheme I). Because of the decreased nucleophilicity of the threonyl hydroxyl moiety, direct condensation with 3-hydroxy-2-cyanopyridine was not possible. Activation of the nitrile precursor was effected by treatment with saturated ethanolic hydrogen chloride. The resulting unstable imino ether hydrochloride was immediately transformed into the free base by reaction with potassium carbonate. Next, the ethyl imidate was allowed to react with L-threonine in refluxing methanol to generate oxazoline ring containing derivative **9**.

Finally, the role of the carboxyl in in vivo chelation was evaluated by comparing the ability of the methyl ester of desmethyldesferrithiocin (**5**) and N^1,N^8-bis(desferrithiocinoyl)spermidine (**10**) to clear iron with that of the parent compounds. The synthesis of the spermidine derivative was predicated on the synthesis of the naturally occurring tetracoordinate catecholamide chelator N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine.[12] For the preparation of derivative **10**, desferrithiocin was converted into an active ester employing dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) in THF, and this ester was reacted with spermidine to furnish, with regioselective control, the corresponding N^1,N^8-bis-functionalized spermidine analogue **10**.

Biological Evaluation

Biological evaluation of the lead and target compounds as orally effective iron-chelating agents was performed with a non-iron-overloaded bile duct cannulated rat model. The drug-promoted iron excretion was monitored in both the bile and the urine, and the results were compared to a

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Table I. A Comparison of the Total Iron Output (μg/kg) Induced by 1–10, DFO (po), and DFO (sc), over 24 h

time, h	control	DFO (po)	DFO (sc)	compound 1	compound 2	compound 3	compound 4
			Bile				
3	9.1 (±1.2)	14.7 (±5.2)	43.6 (±18.2)	64.5 (±4.9)	21.8 (±7.4)	28.7 (±9.3)	9.0 (±3.2)
6	17.4 (±2.3)	43.9 (±13.7)	105.9 (±21.2)	143.0 (±20.0)	70.0 (±16.6)	98.2 (±19.1)	28.1 (±4.7)
9	24.7 (±2.3)	76.8 (±19.6)	152.8 (±22.3)	205.3 (±69.6)	111.7 (±28.6)	154.0 (±43.4)	40.7 (±5.1)
12	31.6 (±2.9)	97.1 (±21.9)	173.9 (+22.9)	234.8 (±76.2)	125.8 (±28.8)	182.3 (±48.5)	48.5 (±5.2)
15	37.1 (±3.0)	110.8 (±22.9)	196.2 (±25.9)	245.6 (±76.3)	135.9 (±29.0)	194.0 (±48.7)	56.3 (±5.2)
18	42.4 (±3.0)	119.8 (±22.9)	210.7 (±27.8)	253.7 (±76.3)	146.8 (±29.1)	201.3 (±48.7)	65.3 (±5.2)
21	47.9 (±3.4)	128.7 (±23.0)	219.8 (±27.8)	263.1 (±76.4)	153.2 (±29.1)	209.6 (±48.7)	74.8 (±6.3)
24	53.5 (±3.6)	135.0 (±23.1)	228.4 (±27.9)	268.5 (±76.4)	159.3 (±29.1)	217.2 (±48.7)	81.9 (±6.3)
			Urine				
24	11.7 (±1.3)	16.5 (±1.0)	49.2 (±19.8)	27.0 (±8.6)	38.43 (±7.87)	14.1 (±9.1)	21.0 (±0.2)
total induced iron	65.2 (±3.8)	151.5 (±23.1)	277.6 (±34.2)	295.5 (±76.9)	197.7 (±30.2)	231.3 (±49.5)	102.9 (±6.3)
(treated–control)		86.3	212.4	230.3	132.5	166.1	37.7

time, h	compound 5	compound 6	compound 7	compound 8	compound 9	compound 10
			Bile			
3	42.9 (±19.2)	11.8 (±3.2)	7.1 (±1.8)	28.6 (±10.5)	11.3 (±1.6)	13.2 (±3.9)
6	53.3 (±19.3)	22.5 (±4.2)	16.6 (±4.7)	91.6 (±25.0)	21.3 (±1.8)	30.0 (±4.6)
9	59.9 (±19.4)	31.2 (±4.9)	26.2 (±4.8)	143.8 (±26.2)	20.2 (±2.5)	45.2 (±5.1)
12	68.7 (±19.4)	39.5 (±5.1)	34.9 (±5.2)	184.9 (±27.3)	37.3 (±2.9)	56.6 (±5.4)
15	74.7 (±19.5)	47.1 (±5.4)	42.2 (±5.4)	210.6 (±28.1)	43.7 (±3.0)	65.8 (±5.7)
18	81.0 (±19.6)	56.2 (±5.8)	49.6 (±5.4)	226.8 (±28.5)	49.4 (±3.2)	74.0 (±6.2)
21	85.3 (±19.6)	64.1 (±5.9)	56.8 (±5.5)	238.1 (±28.6)	55.1 (±3.3)	82.2 (±6.6)
24	93.3 (±19.6)	71.9 (±6.1)	62.1 (±5.5)	246.9 (±28.6)	60.8 (±3.4)	92.8 (±7.2)
			Urine			
24	18.7 (±6.4)	14.2 (±6.6)	11.9 (±0.3)	18.67 (±6.55)	7.6 (±1.7)	18.0 (±4.2)
total induced iron	112.0 (±20.7)	86.1 (±9.0)	74.0 (±5.5)	265.6 (±29.4)	68.4 (±3.8)	110.9 (±8.3)
(treated–control)	46.8	20.9	8.8	200.4	3.2	45.7

standard desferrioxamine dose given either orally (po) or subcutaneously (sc). As it is not clear what the stoichiometry of the ferrithiocin–iron complex would be under physiological conditions, or for that matter the stoichiometry of any of the complexes, we simply compared the effectiveness of the various molecules on a molar basis, 150 μmol/kg. If one chooses to make assumptions about the stoichiometry of ligand/metal complex, e.g. 1/1 for the desferrioxamine–iron complex or 2/1 for the desferrithiocin–iron complex, the results can also be analyzed in these terms. The iron excretion data for the drugs evaluated in this study are reported in Table I with comparative data for sc and po desferrioxamine. The data are presented in total "induced" iron excreted over 24 h, per kilogram of rat weight. The values are derived from the difference between the total iron excreted in test animals vs control animals on a per weight basis.

Because of the differences in water solubility among the various drugs, its was necessary to employ a vehicle in which all of the compounds could be administered. We utilized a mixture of Cremophor RH-40 and water (40/60 v/v). Furthermore, as dietary iron can reduce or in some cases enhance the amount of biliary iron excreted, all animals were fasted for 48 h before the administration of the drug, and the fast was maintained throughout the course of the experiment. Although fasting reduces the bile flow, data interpretation is facilitated. In addition, dietary iron can complex with the ligand, reducing its availability for absorption, or alternatively, the iron-chelator complex can itself be absorbed and then excreted in the bile, further complicating the results.

Analytical Methods. In all of our previous investigations we measured the iron concentration in urine and bile by utilizing a bathophenanthroline-based colorimetric method (CM).[13] We wanted to compare the data from this procedure with data generated from atomic absorption spectrophtometry (AA) to assure both the accuracy and precision of the data. Although the iron concentrations in the bile were nearly identical, agreement between the two methods in the urine iron levels was not as consistent. Because of the rather meager contribution of urine iron to the total iron output, the importance of these small discrepancies was minimal. Both AA and CM values were averaged to generate the final output values.

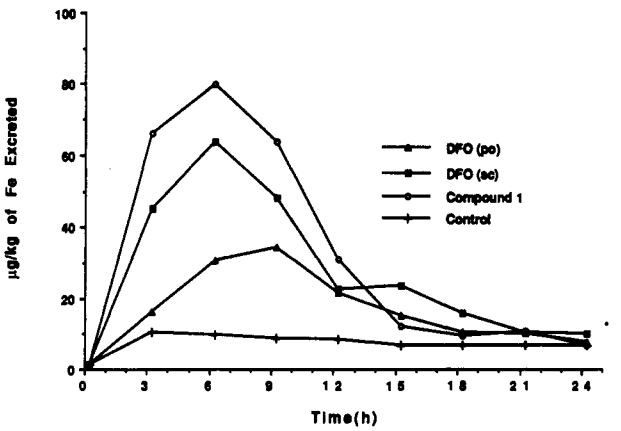

Figure 2. A comparison of the biliary iron clearance induced by desferrithiocin administered orally with the biliary iron clearance induced by desferrioxamine when given orally or subcutaneously.

Structure–Activity Correlations. In comparing desferrithiocin (1) with desferrioxamine when both ligands are given orally, desferrithiocin is approximately 2.7 times as effective in promoting cumulative iron excretion (Table I). When compared with subcutaneously administered desferrioxamine (DFO), orally administered desferrithiocin is about as effective. Biliary iron clearance induced by a single subcutaneous injection of DFO is back to baseline clearance in 15 h, with its maximum output at 6 h. Desferrithiocin-induced iron clearance also reaches baseline in about 15 h, with a similar spike at 6 h. Although DFO given orally in Cremophor to rats does promote the excretion of iron, it is not nearly as effective as subcutaneously administered DFO, and the clearance time is rather

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protracted: levels return to baseline only after 18 h (Figure 2). With all of the compounds studied, urinary iron clearance was over within 24 h of drug administration.

A comparison of compounds **6** and **7** with **3** and **4** clearly indicates the importance of the aromatic hydroxyl in iron clearance. The nonhydroxylated compounds put out iron at essentially baseline levels. This is in keeping with the literature model for the desferrithiocin/iron complex in which the aromatic hydroxyls comprise two of the six donor sites in the 2/1 desferrithiocin/iron complex.

Unlike the hydroxyl, removing the pyridine nitrogen does not diminish the activity of the compound, but in fact seems to actually enhance its iron-clearing ability. This is clear when comparing the salicyloyl deivative, a sodium salt, compound **8**, with the desmethyldesferrithiocin salt **4**. Compound **8** is approximately 5.3 times as effective as compound **4** and slightly better than desmethyldesferrithiocin acid **3**. This is also consistent with the literature suggestion that the pyridine nitrogen is not actually involved in chelation.

Interestingly, although the thiazoline sulfur is not implicated as a donor in the chelate either, its removal from the molecule does seem to result in some diminished activity. Compound **9**, an oxazoline methyl ester, is not active, but the corresponding desmethyldesferrithiocin methyl ester (**5**) shows weak activity. However, the comparison may not be a fair one because of the additional methyl on the oxazoline ring. Further investigation is currently underway.

The conversion of desmethyldesferrithiocin (**3**) to the corresponding methyl ester **5** results in a molecule whose activity is reduced by 72% and is about as active as the sodium salt, while conversion of desferrithiocin (**1**) to bis-amide **10** reduces desferrithiocin's activity to 20%.

Finally, in evaluating the role of the desferrithiocin thiazoline ring methyl, two rather interesting observations can be made: (1) although removal of the methyl group does not substantially diminish desferrithiocin's activity, the impact is far more obvious when comparing desferrithiocin sodium salt (**2**) with the corresponding desmethyl salt **4**, and (2) with both desferrithiocin (**1**) and desmethyldesferrithiocin (**3**), the acid is far more active than the corresponding salt. Desmethyl sodium salt **4** is 30% as efficacious as the corresponding desferrithiocin sodium salt. Desmethyl carboxylic acid **3** is about 72% as effective as the the parent desferrithiocin acid at promoting iron excretion. Desferrithiocin salt **2** is 57% as effective in promoting iron excretion as the parent acid **1**, while desmethyl salt **4** is only about 23% as effective as the corresponding acid **3**.

The differences in activity between the acid and the corresponding salt are rather surprising as both go through the stomach and experience the same pH. Therefore, the species present should be the same regardless of which form one administers. This, then, suggests that the vehicle, Cremophor, must be at the origin of the differences. It is not possible to compare the behavior of desferrithiocin and its sodium salt when both are given orally in aqueous solutions because of the poor solubility of the acid. However, both desmethyl sodium salt **4** and its acid **3** are sufficiently soluble in water to compare their oral activity when administered as aqueous solutions. It is not surprising that the ability of the sodium salt and the acid to clear iron is essentially identical under these conditions (Figure 3). Precisely how the Cremophor promotes this selective absorption is currently under investigation.

In all of the chelators evaluated, the major fraction of iron excreted was found in the bile with a high of 94% in the case of desmethyldesferrithiocin (**3**), and a low of 81% in the case of desferrithiocin sodium salt (**2**). On the basis of the total biliary iron cleared, the compounds can be separated into three different groups: (1) the four best chelators, which are all comparable, compounds **1**, **3**, **8**, and DFO (sc), (2) ligands of intermediate iron clearing capacity, **2** and DFO (po), and (3) those ligands which do not promote iron clearance much above baseline, **5**, **6**, and **10** (Figure 4). The kinetics of iron clearance promoted by the four most effective compounds, **1**, **3**, **8**, and DFO (sc), are all similar with a maximum output occurring at 6 h (Figure 5).

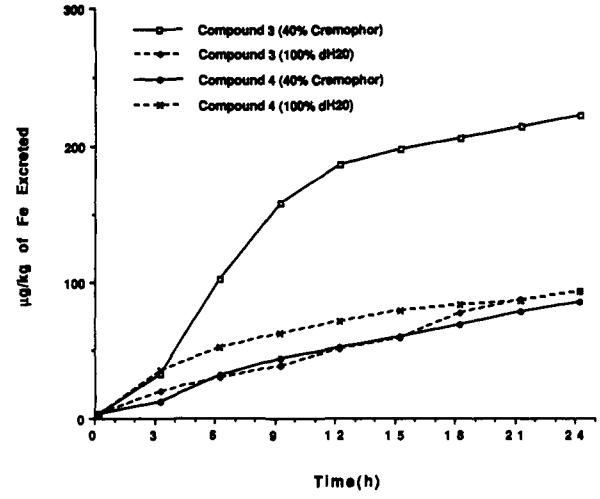

Figure 3. A comparison of the cumulative biliary iron clearance induced by desmethyldesferrithiocin (**3**) with the cumulative biliary iron clearance induced by desmethyldesferrithiocin sodium salt (**4**) when both drugs are administered orally in water.

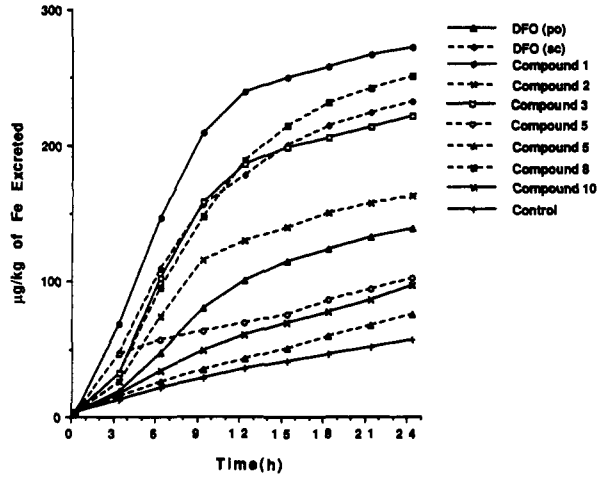

Figure 4. A comparison of the cumulative biliary iron clearance induced by desferrithiocin and its analogues with the biliary iron clearance induced by desferrioxamine when given orally or subcutaneously.

The data are consistent with the idea that the desferrithiocin aromatic hydroxyl and the free carboxyl are requisite for the compound's iron-clearing properties. It is also true that although the desferrithiocin methyl group does augment the compound's activity, its presence is not nearly as important as the other two functionalities. As the methyl group arises from the rather unusual amino acid L-α-methylcysteine and adds an additional step in the synthesis of the analogues, this finding simplifies further studies. Finally, not only is the role of the carboxyl in chelation underscored when comparing diamide **10** with the parent acid, or on comparing desmethyldesferrithiocin (**3**) with its methyl ester (**5**), but the idea of employing the

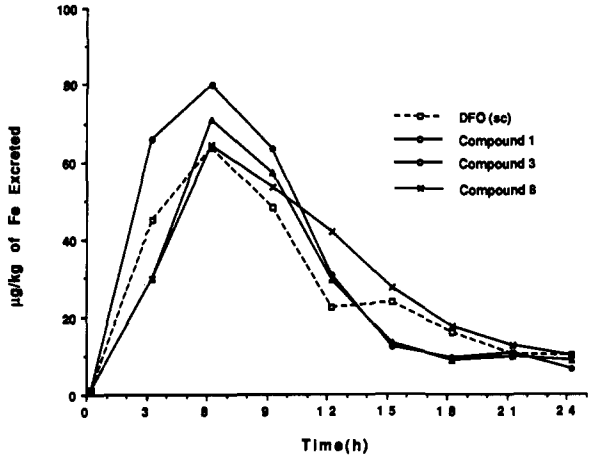

Figure 5. A comparison of the ferrokinetic clearance properties of compounds 1, 3, and 8 with that of subcutaneously administered desferrioxamine.

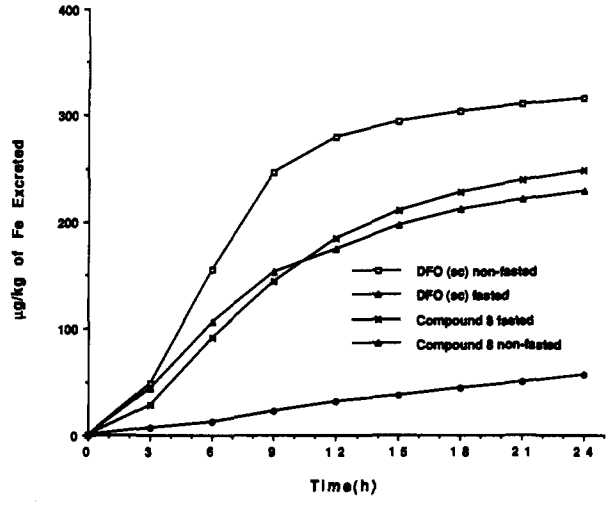

Figure 6. A comparison of the effect of diet on the cumulative biliary iron clearance induced by desazadesmethyldesferrithiocin sodium salt (8) with the biliary iron clearance induced by desferrioxamine when given subcutaneously.

carboxyl as a synthon for coupling two desferrithiocins to optimize activity is clearly shown to be ineffective. However, this does not imply that coupling two desferrithiocin molecules with the concept of improving on the entropy component of binding in the desferrithiocin is not a good one. These kinds of connective entropic advantages have been well-exploited in nature as exemplified in the binding of an iron atom by three molecules of 2,3-dihydroxybenzoic acid versus the binding by a single mole of enterobactin, a macrocyclic tris-2,3-dihydroxybenzoyl amide. The data simply suggest that any connection of two desferrithiocins should not involve the critical chelating atoms.

Animal Model. The animal model chosen for this study, the non-iron-overloaded bile duct cannulated rat, has three major advantages: (1) biliary iron clearance can be monitored continually, (2) measuring the iron in the bile and urine can be accomplished with facility, and (3) the problems associated with iron-overloading animals prior to chelator evaluation are absent.

Earlier models evaluated biliary iron output by monitoring fecal iron, a method which does not lend itself to the study of the kinetics of chelator-induced iron clearance. The procedures employed in this study allow us to measure both the rate at which various iron chelators induce iron clearance in the bile and the total iron cleared.

Iron concentration in the bile and urine is determined colorimetrically, by employing a method predicated on the complexation of iron(II) by bathophenanthroline[13] and by atomic absorption spectroscopy. The bile samples do not require the extensive and labor-intensive preparation that is required for feces samples, e.g. ashing and dissolution in nitric acid. Furthermore, one is no longer faced with the problems of trying to evaluate small differences between large numbers, a difficulty associated with the high iron background in the feces and the relatively small chelator-induced iron values. Although the fecal iron background can be reduced by maintaining the animals on a low-iron diet,[14] this represents additional problems which are absent in the current model. Finally, radioisotopes are not required in this method, a feature which can be of some significance in the absence of proper facilities for counting and handling radioactivity.

In the past, the testing of most iron chelators employed iron-overloaded rats. Animals were iron overloaded utilizing either iron dextran or by injection of senescent red blood cells. Concurrently, the animals were given a radioactive isotope, e.g. ^{59}Fe or ^{55}Fe, in order to facilitate measuring iron output. In this model, the injected iron was allowed to equilibrate before the animals were exposed to test chelators.[15] The animals' fecal and urinary radiolabeled iron output was measured until a constant baseline was attained.

The equilibration time can be of some importance in terms of how the iron is distributed among the various pools.[16] For example, all iron chelators cannot be expected to have the same volume of distribution and therefore cannot be expected to have access to the same iron pools. If the radioactive iron finds its way into a pool to which the ligand does not have access, the chelator-induced iron clearance will appear unsatisfactory. As the model employed in this study does not require iron overloading nor isotopic equilibration of iron, access of chelators to limited pools is not a problem. In a previous study utilizing a cannulated rat model in which both normal and iron-overloaded rats were employed, Pippard et al. suggested that rats with normal stores of iron might well be satisfactory for a quick screen of chelator activity.[17]

One of the critical factors in the successful application of this model system is associated with dietary considerations. In every instance we have been able to show that fasted animals respond to the orally administered chelators much more effectively than those that are fed ad libitum. For example, the induced total iron output in fasted rats by desazadesmethyldesferrithiocin (8) is reduced from 265 to 79 µg/kg when the rats are maintained on an iron-supplemented diet during the course of the experiment (Figure 6).

When fasted and fed rats are given desferrioxamine subcutaneously and the iron output compared, the differences in iron clearance are as expected (Figure 6). The fasted rats put out 15% less bile in the same time period and ca. 22% less iron overall. Adjusting for bile flow, the chelator-promoted iron clearance in both animal sets is very similar. The only effect the diet has in this instance

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is associated with the bile flow. Although bile flow in the fasted animals is somewhat dimished, there is no dietary iron with which to complex. Most commercial rodent foods consist of components high in iron and are often further supplemented with ferrous sulfate. Chelators administered orally can easily bind this iron, and thus the level of free ligand available for absorption and iron clearance is reduced. Alternatively, the iron/chelator complex generated in the gut may itself be absorbed and excreted through the kidney or in the bile, thus confusing data interpretation.

There are some problems associated with the cannulated rat model. The interruption of enterohepatic circulation can, of course, lead to overestimation of fecal iron excretion. Under normal circumstances, when the bile duct is not cannulated, the iron/chelator complex in the bile is passed through the duodenum, where it may be reabsorbed by the intestine. In spite of this potential problem, this model still represents a very useful and rapid initial screen of new chelators. If the ligand of interest does not induce iron clearance in the bile or the urine, then additional investigations are unnecessary. Furthermore, once having established that a ligand clears iron in the bile, the role of enterohepatic circulation can be evaluated simply by administering radiolabeled iron chelate to animals and monitoring radioactivity in the blood. However, the chelate must be introduced in the duodenum where the bile would normally enter the intestine since simple oral administration of the iron chelate could result in decomposition of the complex because of the low pH of the stomach. Alternatively, a non-bile duct cannulated rat model or primate model can be utilized.

Conclusion

Orally administered desferrithiocin promotes urinary and biliary iron excretion even more effectively than desferrioxamine given subcutaneously. The desferrithiocin synthetic analogues evaluated in this study suggest that the desferrithiocin aromatic hydroxyl and its carboxyl group are central to the ligand's activity, while the pyridine nitrogen is of little importance. The α-methyl group of desferrithiocin, although not a requisite for oral activity as exemplified by the behavior of desmethyl compounds **4**, **6**, and **7**, does improve on the parent compound's efficacy. These results suggest that relatively simple molecules predictated on the desferrithiocin framework can now be constructed, molecules without the complicating amino acid L-α-methylcysteine or the pyridine nitrogen. The data not only point to the necessity of the two ligating groups, the aromatic hydroxyl and the carboxyl, but also to the problems associated with altering the latter, e.g. by converting it to an ester or amide. For example, although connecting two desferrithiocin units to gain an entropic advantage is conceptually sound, this cannot be accomplished by converting the carboxyl to an amide functionality.

The non-iron-overloaded bile duct cannulated rat model employed in this study proved to be a very effective method for comparatively evaluating the ability of chelators to clear iron. There is no time required to iron overload the rats, the use of radioisotopes, e.g. ^{55}Fe or ^{59}Fe, is unnecessary, and the ferrokinetics of biliary iron excretion can be measured directly. Such ferrokinetic measurements make it possible to easily optimize chelator dosing.

Experimental Section

Melting points were determined on a Büchi 520 melting point apparatus and are uncorrected. ^1H NMR spectra were obtained on a Bruker AM 360 (360 MHz) instrument. Infrared spectra were recorded with a Perkin-Elmer 983 G spectrometer. Mass Spectra were determined on a VG ZAB-HF spectrometer (VG Analytical, Manchester, UK). Elemental analyses were performed by the Analytical Research Division, CIBA-GEIGY. Results were within 0.4% of theoretical values, unless otherwise stated. All organic extracts were dried over sodium sulfate prior to evaporation. Cremophor 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). Intramedic polyethylene tubing PE 50 was obtained from Fisher Scientific (Pittsburgh, PA). All other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) or Fluka Chemical (Buchs, Switzerland).

2-(3′-Hydroxypyrid-2′-yl)-4-methyl-Δ^2-thiazoline-4(*S*)-carboxylic Acid (Desferrithiocin, 1). The natural product was isolated and purified from a *Streptomyces* strain as described in the literature.[7] The product was recrystallized from water then dried under high vacuum for 72 h: mp 154 °C dec; ^1H NMR (CD_3OD) δ 1.67 (s, 3 H), 3.29 (d, 1 H), 3.83 (d, 1 H), 7.39 (d, 2 H), 8.13 (t, 1 H); IR (CH_2Cl_2) 3470, 2980, 1750, 1720, 1590, 1450, 1300, 985 cm^{-1}; FABMS m/z $(M + H)^+$ 239. Anal. ($C_{10}H_{10}N_2O_3S$) C, H, N, O, S.

2-(3′-Hydroxypyrid-2′-yl)-Δ^2-thiazoline-4(*R*)-carboxylic Acid (3). A solution of L-cysteine (60.58 g, 500 mmol) in a mixture of degassed methanol (1400 mL) and 0.1 M phosphate buffer, pH 5.95 (900 mL), was treated with 3-hydroxy-2-cyanopyridine (30.03 g, 250 mmol) in portions. The resulting brown reaction mixture was stirred for 48 h at 30 °C. After stirring for 5 h, the pH was adjusted to 6 with concentrated sodium hydroxide. Following removal of volatile components under reduced pressure, the solution was cooled in ice and concentrated phosphoric acid was added dropwise to a final pH of 2.8. The resulting white precipitate was vacuum filtered and the solid washed with ice-cold water. Drying under high vacuum afforded 47.83 g (85%) of product (**3**): mp 108–109 °C; ^1H NMR (CD_3OD) δ 3.59–3.69 (m, 2 H), 5.45–5.54 (m, 1 H), 7.40 (d, 2 H), 8.12 (t, 1 H); IR (KBr) 3430, 3180, 1725, 1585, 1470, 1380, 1305 cm^{-1}, FABMS m/z $(M + H)^+$ 225. Anal. ($C_9H_8N_2O_3S \cdot 0.1H_2O$) C, H, N, O, S, H_2O.

2-(3′-Hydroxypyrid-2′-yl)-Δ^2-thiazoline-4(*R*)-carboxylic Acid, Sodium Salt (4). Sodium hydroxide (1 equiv) was added to a suspension of **3** (8.00 g, 35.68 mmol) in water (80 mL). The resulting solution was stirred for 1 h and lyophilized. The yellow-green solid was dissolved in hot methanol and decolorized (Norit). The solution was filtered, concentrated, and recrystallized from acetone to furnish 7.25 g of **4** (83%): mp 225 °C dec; ^1H NMR (CD_3OD) δ 3.58 (d, 2 H), 5.25 (t, 1 H), 7.37 (m, 2 H), 8.10 (m, 1 H); IR (KBr) 3600, 3320, 3240, 1660, 1610, 1575, 1455, 1415 cm^{-1}; FABMS m/z $(M + H)^+$ 247. Anal. ($C_9H_7N_2NaO_3S \cdot H_2O$) C, H, N, Na, S, H_2O.

Methyl 2-(3′-Hydroxypyrid-2′-yl)-Δ^2-thiazoline-4(*R*)-carboxylate (5). A solution **4** (11.21 g, 50 mmol) in methylene chloride (130 mL) was treated with 2,2-dimethoxypropane (52.1 g, 500 mmol) and *p*-toluenesulfonic acid (11.4 g, 60 mmol). The reaction mixture was stirred at room temperature for 6 h, then a 0.5 M phosphate buffer solution, pH 7.4, was added and the product extracted with methylene chloride. The combined organic extracts were washed with water, dried, and evaporated under reduced pressure. The crude residue was recrystallized from ether/heptane to afford 10.43 g (88%) of **5**: mp 75–76.5 °C; ^1H NMR (CD_3OD) δ 3.56–3.68 (m, 2 H), 3.38 (s, 3 H), 5.54 (dd, 1 H), 7.40 (d, 2 H), 8.13 (t, 1 H); IR (CH_2Cl_2) 3060, 2950, 1740, 1590, 1400, 1300, 1180, 980 cm^{-1}; FABMS m/z $(M + H)^+$ 239. Anal. ($C_{10}H_{10}N_2O_3S \cdot 0.02H_2O$) C, H, N, O, S, H_2O.

2-(Pyrid-2′-yl)-Δ^2-thiazoline-4(*R*)-carboxylic Acid (6). 2-Cyanopyridine (10.41 g, 100 mmol) dissolved in methanol (100 mL) was added dropwise to a stirred solution of L-cysteine (24.24 g, 200 mmol) in a mixture of degassed methanol (600 mL) and 0.1 M phosphate buffer, pH 5.95 (450 mL). The resulting light brown solution was heated to 40 °C with continued stirring for 48 h. The reaction mixture was then filtered, and volatile components were removed under reduced pressure. Concentrated phosphoric acid was added dropwise to the ice cooled solution (pH 2) and the mixture extracted with methylene chloride. The combined organic layers were washed with water, dried, and concentrated to afford crude product. Recrystallization from methylene chloride furnished 13.26 g (64%) of **6**: mp 114–115 °C; ^1H NMR (CD_3OD) δ 3.57–3.71 (m, 2 H), 5.35–5.43 (t, 1 H),

7.51 (m, 1 H), 7.91 (m, 1 H), 8.15 (m, 1 H) 8.62 (m, 1 H); IR (CH_2Cl_2) 3460, 3050, 1770, 1725, 1610, 1570, 1470, 1440, 1350 cm^{-1}; MS m/z (M^+) 208. Anal. ($C_9H_8N_2O_2S$) C, H, N, O, S.

2-(Pyrid-2'-yl)-Δ^2-thiazoline-4(R)-carboxylic Acid, Sodium Salt (7). Compound **7** was prepared from carboxylic acid **6** as described for **4**: mp 135 °C dec; ^1H NMR (CD_3OD) δ 3.54–3.69 (m, 2 H), 5.18 (t, 1 H), 7.49 (m, 1 H), 7.88 (m, 1 H), 8.18 (m, 1 H), 8.58 (m, 1 H); IR (KBr) 3420, 3060, 1600, 1465, 1440, 1395, 790 cm^{-1}; FABMS m/z $(M + H)^+$ 231. Anal. ($C_9H_7N_2NaO_2S \cdot 0.8H_2O$) C, H, N, Na, S, H_2O.

2-(2'-Hydroxyphenyl)-Δ^2-thiazoline-4(R)-carboxylic Acid, Sodium Salt (8). Compound **8** was prepared as described for **6**, by utilizing 2-hydroxybenzonitrile, in 54% yield. Conversion into the salt form was accomplished with standard procedures: mp 219–220 °C; ^1H NMR (CD_3OD) δ 3.62 (d, 2 H), 5.16 (t, 1 H), 6.87 (m, 1 H), 6.93 (m, 1 H) 7.33 (m, 1 H), 7.43 (m, 1 H); IR (KBr) 3580, 3160, 1670, 1620, 1590, 1500, 1460, 1400 cm^{-1}; FABMS m/z $(M + H)^+$ 246. Anal. ($C_{10}H_8NNaO_3S \cdot 0.4H_2O$) C, H, N, Na, S, H_2O.

Methyl 2-(3'-Hydroxypyrid-2'-yl)-5(R)-methyl-Δ^2-oxazoline-4(S)-carboxylate (9). Dry absolute ethanol (200 mL) in a four-neck flask, equipped with stir bar, gas sparger, and thermometer, was cooled to 0 °C and saturated with dry HCl for 2 h. The mixture was then cooled with a dry ice/acetone bath followed by the addition of 3-hydroxy-2-cyanopyridine[11] (5.10 g, 42.46 mmol). After purging of the suspension for an additional 1 h with dry HCl, the reaction mixture was allowed to warm slowly until a clear orange solution was obtained. The solution was allowed to stand at −20 °C for 72 h. The solution was poured over ice-cold ether and the resulting solid collected then washed with additional cold ether. The solid was added carefully to an ice-cold saturated potassium carbonate solution, then extracted with ether. The combined organic layers were dried, filtered, and concentrated in vacuo to afford the crude imino ether (4.71 g, 67%). L-threonine methyl ester (4.4 g, 25.9 mmol), in methanol (75 mL), was added to a flask containing the imino ether (3.92 g, 23.6 mmol). The mixture was heated to reflux for 14 h, then the solvent removed under reduced pressure. The residue was purified on silica gel eluting with methylene chloride to afford **9** (3.41 g, 61%) as an oil: ^1H NMR ($CDCl_3$) δ 1.64 (d, 3 H), 3.82 (s, 3 H), 4.59 (d, 1 H), 5.04–5.12 (m, 1 H) 7.37 (m, 2 H), 8.26 (m, 1 H), 11.81 (br, 1 H); IR (CH_2Cl_2) 2980, 2960, 1740, 1630, 1455, 1200 cm^{-1}; MS m/z (M^+) 236. Anal. ($C_{11}H_{12}N_2O_4 \cdot 0.05\ H_2O$) C, H, N, O.

N^1,N^8-Bis[[2-(3'-hydroxypyrid-2'-yl)-4-methyl-Δ^2-thiazolin-4(S)-yl]carbonyl]spermidine (10). Desferrithiocin (1) (4.76 g, 20 mmol) and 1-hydroxybenzotriazole (3.30 g, 22 mmol) are added to a solution of spermidine (1.45 g, 10 mmol) in 100 mL of absolute tetrahydrofuran. The resulting suspension was cooled and dicyclohexylcarbodiimide dissolved in 50 mL of tetrahydrofuran added dropwise. The reaction mixture was stirred at room temperature for 15 h, then filtered and concentrated. Purification by column chromatography (230 g of SiO_2, 40/10/1 CH_2Cl_2/MeOH/NH_4OH) led to a band that was concentrated with ethanol, dissolved in CH_2Cl_2, dried, filtered, and concentrated to give 5.45 g of **10** (93% yield). The product was crystallized from ethanol as the fumarate salt: mp 174–176 °C; ^1H NMR (DMSO-d_6) δ 1.38–1.50 (m, 4 H), 1.54 (s, 6 H), 1.60–1.76 (m, 2 H), 2.60–2.77 (m, 4 H) 3.02–3.30 (m, 6 H), 3.70 (d, 2 H), 6.41 (s, 1 H), 7.48 (m, 4 H), 8.13 (m, 1 H), 8.16–8.32 (m, 3 H), 10.06 (br, 2 H). Anal. ($C_{29}H_{37}N_7O_6S_2 \cdot 0.38H_2O$) C, H, N, H_2O.

Colorimetric Iron Determination. The iron indicator solution, solution A, was prepared by diluting a stock aqueous solution of 0.134 mM mercaptoacetic acid and 1.9 mM bathophenanthrolinedisulfonic acid disodium salt with 5 parts water and 5 parts saturated aqueous sodium acetate. Solution B is prepared as above but without the bathophenanthroline.

An equal volume of a 10% trichloroacetic acid solution in 3 M HCl (solution C) was added to a urine sample (2.5 mL), and the sample heated in a 65 °C bath for 4 h. After filtration through nylon filters, two 1-mL aliquots were removed. Solution A (5 mL) was added to the sample, and solution B (5 mL) was added to the blank.

Bile samples were prepared as the urine samples; however the volumes were reduced. Standard iron curves were generated with each assay. All samples were transferred to polystyrene cuvettes and the absorbance measured at 535 nm.

Atomic Absorption Iron Determinations. 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 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.

Bile Duct Cannulation. Male Sprague–Dawley rats averaging 400 g were housed in Nalgene plastic metabolic cages and were 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.

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

Registry No. 1, 76045-30-2; **2**, 105635-69-6; **3**, 81744-98-1; **4**, 133817-87-5; **5**, 133817-88-6; **6**, 133817-89-7; **7**, 133817-90-0; **8**, 133817-91-1; **9**, 133817-92-2; **9** imino precursor, 133817-94-4; **10**, 133817-93-3; 3-hydroxy-2-cyanopyridine, 932-35-4; L-cysteine, 52-90-4; 2-cyanopyridine, 100-70-9; 2-hydroxybenzonitrile, 611-20-1; L-threonine methyl ester, 3373-59-9; spermidine, 124-20-9; iron, 7439-89-6.