Esters and Lactones of Phenolic Amino Carboxylic Acids: Prodrugs for Iron Chelation

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The new iron chelator *N,N'*-bis(2-hydroxyphenyl)ethylenediamine-*N,N'*-diacetic acid (**1**), its dilactone **2**, *N,N'*-bis(2-hydroxybenzyl)-2-hydroxypropylene-1,3-diamine-*N,N'*-diacetic acid (**3**), and its methyl ester lactone **4** and a series of esters of *N,N'*-bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (**5**) were prepared and their iron chelating efficacy and toxicity determined by using the hypertransfused mouse model of iron overload. The biological activities were compared with results obtained with use of the hypertransfused rat. Esterification enhanced the oral iron chelating activity but also increased toxicity. The diisopropyl ester of **5** exhibited the highest therapeutic index. In vitro measurements showed that the rate of ester hydrolysis at pH 7.5 increased by a factor of 10^4 in the presence of 5×10^{-4} M ferric ion, which may account for the utility of esters and lactones as prodrugs. Seventeen other chelating agents were screened but showed no intraperitoneal or oral activity.

The recent discovery[1] of a mouse model of β-thalassemia offers greater hope that genetic manipulation may ultimately correct this fatal genetic disease, which arises from errant biosynthesis of the β-chain of hemoglobin. Presently, however, β-thalassemia can only be treated by a regular, life-long transfusion regimen, which itself ultimately produces a fatal hemosiderotic condition associated with cardiotoxicity, hepatic cirrhosis, and pancreatic dysfunction.[2] Partial removal of the excess iron from the body may be accomplished by chelation therapy using deferoxamine (DFB). This drug, a trihydroxamic acid derived from microbial sources, is the only iron-specific compound now approved for human use.[3] Its utility is limited by low oral activity, rapid metabolism, and only moderate efficacy, and it is necessary to resort to inconvenient and expensive modes of administration such as sustained subcutaneous or intravenous infusion[4-6] to achieve a net loss of iron.

These deficiencies have prompted a search for more effective chalators, in particular ones that are orally active. A variety of compounds, chosen on the basis of the presence of functional groups with a high and selective affinity for iron(III), have been screened.[7] Two hexadentate chelating agents, *N,N'*-bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (**5**) and ethylenediamine-*N,N'*-bis(2-hydroxyphenylacetic acid) (**6**) (Figure 1) were particularly effective when tested in the mouse and rat models of iron overload; preliminary data indicated these compounds were orally active provided ionization of the carboxy groups was temporarily blocked by esterification.[8,9] This paper describes the activity of two related hexadentate iron chelating agents, *N,N'*-bis(2-hydroxyphenyl)ethylenediamine-*N,N'*-diacetic acid (**1**) and *N,N'*-bis(2-hydroxybenzyl)-2-hydroxypropylene-1,3-diamine-*N,N'*-diacetic acid (**3**) in which the carboxylic acid groups may be masked as lactones, as well as a more complete series of esters of **5**. Measurements of the rates of hydrolysis of these lactones and esters in vitro show that the presence of iron(III) markedly enhances the rate of regeneration of the free carboxyl groups, a fact that may explain their efficacy as prodrugs.

Results

Chemical Synthesis. Compound **1** was prepared by sequential alkylation of *o*-anisidine with 1,2-dibromoethane and then methyl α-bromoacetate. The lactones of **1** and **3** were prepared by treatment of the carboxylic acids with thionyl chloride. Esters of **5** were prepared by treatment of the carboxylic acids with thionyl chloride in the presence of the alcohol. Only the *tert*-butyl ester could not be prepared by this method. The latter was obtained less directly by preparation of di-*tert*-butyl ethylenediamine-*N,N'*-diacetate and then alkylation with 2-acetoxybenzyl bromide. Other compounds were prepared by literature procedures.

Biological Results. The efficacies of the various chelating agents were determined by using the hypertransfused mouse model. the published procedure[8,10] was expanded to include the determination of fecal iron but was otherwise unchanged. The changes in spleen and liver iron levels and the urinary and fecal iron excretion of test animals, relative to a transfused control group, are listed in Table I (derivatives of **1** and **3**) and Table II (derivatives of **5**). DFB administered ip at a dose of 250 mg/kg was used as a standard in each assay. The results of more than 24 assays of this compound were consistent with previously published data on 12 assays obtained by using the hypertransfused mouse model; that is, there was a substantial decrease in the hepatic but not the splenic iron levels, and the major excretory pathway was via the urine with little fecal iron loss.

Compound **5** administered ip at 200 mg/kg depleted both splenic and hepatic iron and produced increases in urinary and fecal iron (Table II). All test animals showed

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Table I. Changes in Splenic, Hepatic, Fecal, and Urinary Iron in Transfused BDF_1 Male Mice Treated with 1 and 3 and Their Lactones

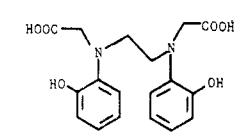
test compound	dose, mg/kg	route	no. of survivors	% iron changes vs. control			
				spleen	liver	feces	urine
DFB	250	ip	240/240	±2	−24	+7	+270
1 disodium salt	200[a]	ip	5/10	+22	−22	−15	+143
1 disodium salt	100	ip	10/10	+7	−22	+3	+106
1 dilithium salt	129	ip	9/10	−7	−49	−38	+28
2	200	ip	8/10	−17	−68	−65	+157
2	200	po	10/10	−30	−56	−2	+93
3	300	ip	10/10	+6	−50	−32	+127
4	300	po	10/10	−18	−14	−7	−3

[a] Reduced to 100 mg/kg on day 2.

Table II. Changes in Splenic, Hepatic, Fecal, and Urinary Iron in Transfused BDF_1 Male Mice Treated with 5 and Its Esters for 7 Days

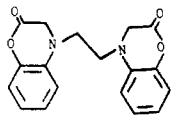
test compound	dose, mg/kg	route	no. of survivors	% iron changes vs. control			
				spleen	liver	feces	urine
DFB	250	ip	240/240	±2	−24	+7	+270
5	200	ip	10/10	−5	−11	+38	+50
5	200[a]	po	9/10	−9	−3	−7	+14
5 dimethyl ester	25	po	10/10	−7	−20	−10	+30
	50	po	10/10	−12	−27	+8	+49
	100	po	10/10	−12	−42	−2	+34
	200[a]	po	10/10	−29	−53	−71	+127
5 diacetyl, dimethyl ester	200	ip	10/10	−22	−56	+5	+280
	375	ip	10/10	−5	−62	−4	+303
	400	po	6/10	−46	−60	−55	+164
	200	po	10/10	−13	−40	−41	+138
5 diethyl ester	100	ip	10/10	+4	−49	+26	+240
	200[a]	po	8/10	−34	−63	−77	+215
5 diisopropyl ester	75	po	6/6	−10	−34	+1	−10
	150	po	6/6	+2	−32	+23	+33
	300	po	6/6	−7	−45	−1	+76
	400	po	10/10	−13	−45	−5	+233
	600	po	6/6	−27	−56	+5	+141
5 di-*tert*-butyl ester	200	ip	10/10	−5	+10	−1	−1
	200	po	10/10	+5	+6	−13	−27
5 diacetyl, di-*tert*-butyl ester	200	po	10/10	−6	+16	−1	−4
5 dipentyl ester	100	ip	10/10	−2	−33	+24	+117
	100	po	10/10	+12	−12	−3	+43
	150	po	8/10	−11	−60	−69	+132
5 dibenzyl ester	250	po	10/10	−21	−10	−10	+62

[a] Reduced to 100 mg/kg on day 4.



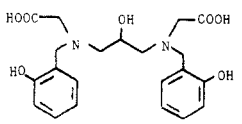
N,N'-Bis(2-hydroxyphenyl)ethylenediamine-N,N'-diacetic Acid

1



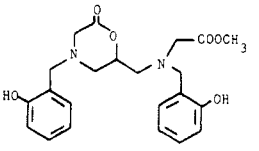
Dilactone of 1

2



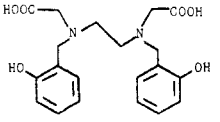
N,N'-Bis(2-hydroxybenzyl)-2-hydroxypropylene-1,3-diamine-N,N'-diacetic Acid

3



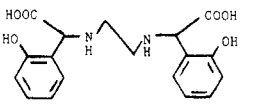
Methyl Ester, Lactone of 3

4



N,N'-Bis(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic Acid

5



Ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)

6

Figure 1. Structures of the hexadentate iron(III) chelating agents.

signs of moderate CNS depression and body weight loss during the first 3 days of the treatment period but recovered subsequently. The toxicity increased when **5** was administered orally, and severe CNS inhibition and anorexia contributed to a decline in body weight. Reducing the dose to 100 mg/kg on day 4 did not prevent one death, and there was a loss of iron chelating activity although the reduction in excreta limited the criteria of activity to splenic and hepatic iron changes.

Conversion of **5** to its methyl ester restored the iron chelating activity but did not eliminate the toxicity. Animals receiving an oral dose of 25 mg/kg were slightly weakened, and increasing the dose caused body weight loss and moderate CNS depression. Liver iron depletion was dose related, while urinary iron was constant except at the highest dose. The reduction in fecal iron values was caused by inhibition of fecal excretion. Masking the phenolic group as the easily hydrolyzable acetate derivative did not reduce the CNS activity of the methyl ester, and while oral administration of this compound produced a very large hepatic iron reduction (40–60%) and increased urinary iron output, defecation was blocked, motor activity was reduced, and 4 of 10 animals died at the dose of 400 mg/kg.

Similar results were obtained with the ethyl ester of **5**, 2 of 10 animals dying after oral administration of 200 mg/kg. In contrast, the isopropyl ester exhibited much reduced toxicity while retaining iron chelation properties. A limited dose–response relationship was derived by oral administration of doses of 75, 150, 300, 400, and 600 mg/kg.

Table III. First-Order Rate Constants (min^{-1}) and Half-Lives (min) of Hydrolyses of 2 and the Diesters of 5 in the Absence and Presence of Metals: Determined Titrimetrically and Spectrophotometrically

substrate	metal ion	titrimetric		spectrophotometric	
		10^2k_1	$t_{1/2}$	10^2k_1	$t_{1/2}$
2	Fe(III)	0.970 ± 0.047	71.6 ± 3.5	0.587 ± 0.005	118 ± 1
5 dimethyl ester	none[a]	0.325 ± 0.016	214 ± 11		
5 dimethyl ester	Fe(III)	3.52 ± 0.02	19.7 ± 0.1	2.19 ± 0.14	31.8 ± 2.0
		12.9 ± 1.1[b]	5.4 ± 0.5[b]		
5 dimethyl ester	Zn(II)	3.66 ± 0.30[b]	19.1 ± 1.6[b]		
5 dimethyl ester	Cu(II)	0.513 ± 0.015[b]	135 ± 4[b]		
5 dimethyl ester	Co(II)	0.436 ± 0.007[b]	163 ± 1[b]		
5 diethyl ester	Fe(III)	1.99 ± 0.03	34.8 ± 0.5	1.14 ± 0.02	60.6 ± 0.9
5 diisopropyl ester	Fe(III)			0.542 ± 0.036	129 ± 9

[a] pH 10.5; other measurements at pH 7.5. [b] Measured in water; other measurements in 50% aqueous methanol.

Only the highest dose produced toxic symptoms, specifically a 10% reduction in body weight, a 29% reduction in liver weight, and a 28% reduction in spleen weight. Except for the highest dose, urinary iron excretion was dose related, as was the reduction in hepatic iron levels. There was no gross pathology on necropsy for any dose.

The *tert*-butyl ester of **5** produced weakness and decreased defecation, as did the compound derived from acetylation of the phenolic hydroxyl groups of this ester. However, unlike the lower homologues in this series of esters, these two compounds did not promote measurable loss of iron from the liver and spleen, nor excretion of the metal. The benzyl and pentyl esters were tested to examine the effect of further increases in hydrophobicity but not steric hindrance. The pentyl ester produced the same toxic symptoms and 2 out of 20 animals succumbed at an oral dose of 150 mg/kg. Significant reductions in liver iron and increases in excreted urinary iron were observed.

The new chelating agent **1** was prepared because of its structural similarity to **5** and **6** and because its dilactone **2** represents a non-ionized derivative suitable for oral administration. The lithium salt of **1** administered ip (129 mg/kg) was toxic (Table I). CNS inhibition was manifested by weakness, ungroomed appearance, dyspnea, 17% mean body weight loss, and the death of 1 of 10 animals. Significant depletion of hepatic iron was observed. The sodium salt of **1** elicited gross toxic signs at doses between 200 and 250 mg/kg (ip). However, at 100 mg/kg (ip) there were no deaths and no toxic signs. The reduction of hepatic iron and the increase in urinary iron were similar to the values obtained with DFB. The lactone **2** administered ip (200 mg/kg) was also toxic, causing anorexia (which was reversible), weakness, and ungroomed appearance, and hypersensitivity to handling. Two of 10 animals died. Liver iron loss was substantial (68%), and urinary iron output also increased (157%). Oral administration (200 mg/kg) of the same lactone substantially reduced the toxic signs, while the iron chelating properties were retained. Some hepatoxicity was implied by a 20% decrease in liver weight, and there was a 25% decrease in spleen weight. Iron loss from these organs was 56% and 30%, respectively, but there was not a corresponding increase in fecal iron.

Compound **3** effected a large decrease in hepatic iron when administered ip but was toxic. It produced a progressive increase in water consumption and a 25% decrease in liver weight, and the 32% decrease in fecal output paralleled the reduced fecal iron. The methyl ester lactone of **3** was ineffective when administered orally but retained the toxic properties of the parent drug.

A number of other chelating agents, listed in the Experimental Section and shown in Figure 2 (supplementary material), had no significant iron chelating activity compared with **1** and **5**.

In Vitro Hydrolysis. The rates of hydrolysis of the different esters were determined in order to obtain an estimate of their relative susceptibility to nonenzymatic cleavage in the presence and absence of metals in vivo. The rates were measured by using an autotitrator to monitor the rate of consumption of base at a constant pH and, in the case of iron promoted hydrolysis, spectrophotometrically in buffer (Table III). The rate constants determined by each method agreed within a factor of 2.

The rate of hydrolysis of the dimethyl ester of **5** in the absence of added metals was immeasurably slow at pH 7.5 but at pH 10.5 (38 °C, 0.05 M KCl in 50% aqueous MeOH) exhibited first-order kinetics with a rate constant of 3.2×10^{-3} min^{-1}. This corresponds to a half-life of 108 days at pH 7.5. In the presence of 1 equiv of ferric iron, the rate increased by a factor of 10^4, the half-life at pH 7.5 decreasing to 5–20 min depending on the solvent employed. When the iron/diester ratio was reduced to 0.5:1, the rate of hydrolysis was unchanged but the reaction ceased when half of the diester was consumed. The metal ions Zn(II), Cu(II), and Co(II) were not as effective in accelerating the hydrolysis (Table III), while Ca(II) and Mg(II) were ineffective. The iron(III)-promoted hydrolysis of the series of esters followed the order: *i*-Pr < Et < Me. The rate of hydrolysis of the diisopropyl ester was too slow to measure accurately by the autotitration method but was determined spectrophotometrically. The rate of hydrolysis of the lactone **2** fell between the rates of the ethyl and isopropyl esters of **5**. No distinction between the rates of hydrolysis of the first and second ester groups could be made.

Discussion

The oral efficacy of the phenolic amino acids as chelating agents depends on the extent to which they are absorbed from the GI tract and survive first pass metabolism. Assuming their passage through the GI epithelium is a passive diffusion process, conversion to the ester or lactone provides a more lipophilic, non-ionic form of the drug, which may pass through the epithelial membrane. After absorption the efficacy further depends on the rate of hydrolysis of the lactone or ester, to regenerate the hexadentate ligand. For most esters, aqueous hydrolysis at pH 7.5 is slow, and the use of esters as prodrugs depends on the action of esterases.[11] The measurements of the rate of hydrolysis of the esters and lactones of the subject chelators suggest that a second in vivo mechanism is available, hydrolysis promoted by the metal that is to be chelated. While metal promotion of ester hydrolysis was first noted in 1952,[12] and there are now numerous examples of this process,[13] iron(III) has been conspicuous by its

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Table IV. Biological Activities[a] of 3, 5, and 6 and Their Esters, Measured Using the Rat Model of Iron Overload (Pitt, 1981)

compound	route	LD_{50}, mg/kg	% iron changes vs. control	
			feces	urine
3	ip	≥800	+255	+118
	po		+20	+30
5	ip	≥800	+195	+196
	po		+53	+40
5 dimethyl ester	ip	≥800	+390	+694
	po		+369	+588
5 dipentyl eser	ip		+114	+215
	po		+268	+390
6	ip	175	+111	+484
	po		+41	+19
	po		-18	+31
6 dimethyl ester	ip	≥800	+104	+188
	po		+89	+115
6 diethyl ester	po	≥800	+25	+109

[a] Biological activities were kindly determined by R. W. Grady using the rat model of iron overload developed at Rockefeller University.[28]

absence from the list of metals known to be effective. In fact, it is reported that iron(III) does not promote the hydrolysis of the ethyl ester of another chelator, nitrilotriacetic acid.[14] The effectiveness of iron(III) in the present case, particularly relative to the endogenous metals, Zn, Cu, and Co, is probably related to the presence of phenolic ligands; the latter have a high affinity for iron(III).[15]

While the in vitro kinetics results are not unambiguous proof that metal-promoted hydrolysis is operative in vivo, there is no question that the active form of these chelators is the free carboxylic acid. The carboxylic ester group has a very low affinity for metals, and as a result, the binding affinities of the unhydrolyzed esters of 5 will be comparable to *N,N'*-bis(2-hydroxybenzyl)ethylenediamine. This compound and analogues that are tetradentate and lack carboxylic acid groups bind iron(III) much less effectively than 5,[15] which has an iron(III) binding constant of $10^{39.7}$. It is known that *N,N'*-bis(2-hydroxybenzyl)ethylenediamine does not remove iron from hypertransfused mice.[8] The lactone 2, with both phenolic and carboxy groups masked, is a bidentate chelator comparable to ethylenediamine and not expected to have a high iron(III) affinity prior to hydrolysis. It is also significant that the di-*tert*-butyl ester of 5, the only ester that is not hydrolyzed at a measurable rate in the presence of iron(III), is also the only inactive ester *in vivo*.

The mean iron levels in the liver and spleen of the hypertransfused mice were 5×10^{-3} and 1×10^{-2} molal, respectively, while hepatic and serum iron levels in persons suffering from iron toxicity are typically at least 10^{-1} molal and 10^{-4} M, respectively.[16] These iron concentrations are comparable to or greater than those used in the in vitro kinetic studies, although it must be recognized that the contribution of iron(III) promoted ester hydrolysis in vivo will also be dependent on the availability of iron. Iron is present in both intra- and extracellular forms and bound by a variety of endogenous ligands.[17] Only the excess iron will not be bound as ferritin and transferrin. The concentration of iron available to promote ester hydrolysis will be determined by the relative binding affinities of the chelator drug and these endogenous ligands.

The oral acitivity of the esters of 5, first observed in the rat screen (Table IV),[10,18] was confirmed in this study. Whereas none of these esters was reported to be toxic in the rat screen, significant CNS activity was found with use of the hypertransfused mouse screen. This toxicity was more pronounced when the esters were orally administered and so would appear to be related to first pass metabolism. Temporary protection of the phenolic hydroxyl groups as the acetate did not diminish either toxicity or iron-chelating ability. Separation of toxicity and chelation activity was achieved by extension of the series of esters. The isopropyl ester had the optimum therapeutic index, with no deaths and no toxic effects until the dose was increased to 600 mg/kg; effective substantial hepatic iron loss was observed at a dose of 75 mg/kg.

Although the iron binding constants of 1 and 3 have not been determined, their structural resemblance to 5 suggests a high and selective affinity for iron(III). The large reduction in hepatic iron produced by oral administration of these compounds supports this expectation, although their toxicity reduced fecal output and prevented an assessment of their ability to excrete iron.

The change in hepatic iron was considered to be the most useful measure of iron-chelating ability in the mouse model. Liver is the major iron-storage organ, and the hepatic iron level is sensitive to both the transfusion regimen and to the control drug, DFB. Hepatic iron levels are less affected by the toxic side effects of the drug than is the amount of excreted iron. While the urinary and fecal iron output are critical measures of iron chelating efficacy, fecal output in particular is diminished substantially by CNS-active drugs. Provided hepatic iron depletion in the mouse screen was compared with fecal iron in the rat model, there was good agreement for those compounds tested in both animal screens.

Experimental Section

Synthetic Methods. Melting points were determined on a Kofler block and are uncorrected. IR absorption spectra were obtained with a Perkin-Elmer Model 222 spectrophotometer. Unless otherwise mentioned, NMR spectra were obtained with a Varian Model 360 (60 MHz) spectrometer using $CDCl_3$ as a solvent and Me_4Si as an internal standard. Elemental analyses of crystalline products were performed by Atlantic Microlab. Elemental analyses of noncrystalline products were determined by high-resolution mass spectroscopy (AEI-MS902) after the purity of the sample was verified by TLC and ^1H NMR. All reactions were carried out under an atmosphere of dry nitrogen.

5 Dimethyl Ester. Thionyl chloride (16 mL, 0.22 mol) was slowly added to a stirred solution of 5 (12 g, 0.031 mol) in dry MeOH (50 mL) at 40 °C or below. The mixture was stirred at 60 °C for 3 h, concentrated in vacuo, redissolved in $CHCl_3$, and washed with $NaHCO_3$. Elution of the crude product from silica gel with $CHCl_3$ gave 6.8 g (63%) of the diester as a white crystalline solid: mp 71–74 °C; IR ($CHCl_3$) 1740 cm^{-1}; ^1H NMR δ 2.70 (s, 4 H), 3.27 (s, 4 H), 3.70 (s, 10 H), 6.55–7.27 (m, 8 H). Anal. ($C_{22}H_{28}N_2O_6$) C, H, N.

The following esters of 5 were prepared by the same procedure.

(13) For recent examples, see: (a) Fife, T. H.; Przystas, T. J. *J. Am. Chem. Soc.* **1985**, *107*, 1041. (b) Hay, R. W.; Pujari, M. P. *J. Chem. Soc., Dalton Trans.* **1984**, 1083. (c) Buckingham, D. A. In *Biological Aspects of Inorganic Chemistry*; Addison, A. W., Cullen, W. R., Dolphin, D., James, B. R., Eds.; Wiley-Interscience: New York, 1976; pp 141–196.
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Diethyl ester: mp 73–74 °C; ^{1}H NMR δ 1.2 (t, 6 H, J = 7 Hz), 4.17 (q, 4 H, J = 7 Hz). Anal. ($C_{24}H_{32}N_2O_6$) C, H, N. Diisopropyl ester: mp 37–39 °C; ^{1}H NMR δ 1.20 (d, 12 H, J = 6 Hz), 4.67–5.30 (m, 2 H). Anal. ($C_{26}H_{36}N_2O_6$) C, H, N. Dibenzyl ester: mp 101–103 °C; ^{1}H NMR δ 2.67 (s, 4 H), 7.20 (s, 10 H). Anal. ($C_{34}H_{36}N_2O_6$) C, H, N. Dipentyl ester: ^{1}H NMR δ 0.65–1.77 (m, 18 H), 2.70 (s, 4 H), 3.21 (s, 4 H), 3.70 (s, 4 H), 4.05 (t, J = 7 Hz, 4 H), 6.43–7.23 (m, 8 H). Anal. Calcd for $C_{30}H_{44}N_2O_6$ m/z 528.3200, found m/z 528.3206.

5 Dimethyl Ester Diacetate. Compound **5** dimethyl ester (2.8 g, 6.7 mmol) was dissolved in acetic anhydride (6 mL, 0.06 mol) and pyridine (30 mL, 0.37 mol). After 4 h, excess reagents were removed in vacuo, and the residue in CH_2Cl_2 was washed with brine. The product (2 g, 61%) crystallized after addition of hexane: mp 77–80 °C; IR ($CHCl_3$) 1760 (OAc), 1740 (COOMe) cm^{-1}; ^{1}H NMR δ 2.21 (s, 6 H), 2.77 (s, 4 H), 3.58 (s, 6 H), 3.67 (s, 4 H), 6.73–7.47 (m, 8 H). Anal. ($C_{26}H_{32}N_2O_8$) C, H, N.

5 Di-*tert*-butyl Ester. Benzyl chloroformate (26.2 mL, 0.200 mol) in ether (140 mL) was added dropwise to a vigorously stirred mixture of ethylenediamine-*N,N'*-diacetic acid (5.0 g, 0.090 mol) and sodium bicarbonate (30.1 g, 0.40 mol) in water (140 mL) at a rate that maintained the aqueous layer at pH 8. The aqueous layer was separated, adjusted to pH 2 with HCl, extracted with CH_2Cl_2, and concentrated to give *N,N'*-bis(benzyloxycarbonyl)-ethylenediamine-*N,N'*-diacetic acid (33.6 g, 89%). This unpurified product in CH_2Cl_2 (300 mL) and concentrated H_2SO_4 (4 mL) was cooled to 0–5 °C and isobutene was bubbled through the mixture until gas absorption diminished. After the mixture was allowed to stand for 3 days at room temperature, isobutene was bubbled into the mixture for 1 h at 0–5 °C, and the mixture was kept at room temperature for 24 h. After concentration, the residue in CH_2Cl_2 was washed with water, dried (Na_2SO_4), and eluted from SiO_2 (350 g) with ether/hexane (1:1). The product (7.0 g) in EtOH (300 mL) and 10% Pd on C (0.8 g) was stirred under hydrogen for 72 h and then filtered to give ethylenediamine-*N,N'*-diacetic acid di-*tert*-butyl ester (3.6 g). This compound (1.6 g, 5.7 mmol), *o*-acetoxybenzyl bromide (3.3 g, 14 mmol), and anhydrous K_2CO_3 (1.6 g, 11.5 mmol) in acetone (100 mL) were refluxed under N_2 for 10 h, filtered, and concentrated. Elution from silica gel (50 g) with 15% ethyl acetate in hexane afforded the di-*tert*-butyl ester of **5** diacetate as a crystalline solid (1.4 g, 42%): mp 70–73 °C; IR 1760 (PhOAc), 1740 (COOBu-*t*) cm^{-1}; ^{1}H NMR δ 1.43 (s, 18 H), 2.25 (s, 6 H), 2.78 (s, 4 H), 3.18 (s, 4 H), 3.73 (s, 4 H), 6.77–7.50 (m, 8 H). Anal. ($C_{32}H_{44}N_2O_8$) C, H, N.

The diacetate (1.3 g, 2.2 mmol) in MeOH (50 mL) and Et_3N (0.06 mL) was refluxed overnight, concentrated in vacuo, and then eluted from SiO_2 (50 g) with 7.5% ethyl acetate in hexane to give **5** di-*tert*-butyl ester as a colorless gum: IR 1740 (COOBu-*t*) cm^{-1}; ^{1}H NMR δ 1.40 (s, 18 H), 2.63 (s, 4 H), 3.10 (s, 4 H), 3.67 (s, 4 H), 6.43–7.23 (m, 8 H). Anal. Calcd for $C_{28}H_{40}N_2O_6$ m/z 500.288, found m/z 500.288.

***N,N'*-Bis(2-hydroxyphenyl)ethylenediamine-*N,N'*-diacetic Acid (1).** *N,N'*-Bis(2-methoxyphenyl)ethylenediamine was prepared from *o*-anisidine and 1,2-dibromoethane by a literature procedure.[19] This compound (19.6 g, 0.0700 mol), $CaCO_3$ (14.0 g, 0.140 mol), and methyl bromoacetate (42.8 g, 0.280 mol) in DMF (150 mL) were heated at 60 °C for 2 h. After removal of the solvent in vacuo, the residue in $CHCl_3$ was washed with water, dried, and concentrated; yield 29.9 g. This product (21 g, 0.050 mol) and lithium thioethoxide (34.0 g, 0.500 mol) in DMF (700 mL) were heated at 100 °C for 8 h. The lithium salt of the product separated as a crystalline solid on cooling and was filtered, washed with acetone, and dried: IR (KBr) 1600 cm^{-1} (COOLi). Anal. ($C_{18}H_{18}N_2O_6Li_2{\cdot}2H_2O$) C, H, N, Li.

Disodium Salt of 1. The dilithium salt (2.5 g, 6.7 mmol) was sonicated with 2 N HCl (30 mL). After 15 min, the precipitated dihydrochloride was filtered, washed with water and acetone, and dried (1.21 g, 42%): mp 133 °C dec; IR (KBr) 1690 cm^{-1} (COOH). Anal. ($C_{18}H_{20}N_2O_6{\cdot}2HCl{\cdot}H_2O$) C, H, N, Cl. This compound (1.2 g, 2.8 mmol) was neutralized with 4 equiv of aqueous NaOH and then lyophilized to obtain the disodium salt as a white solid.

1 Lactone. Thionyl chloride (6.7 g, 57 mmol) was added dropwise to the dilithium salt of **1** (5.40 g, 14.5 mmol) in MeOH (40 mL) at 0 °C. The mixture was then heated at 50 °C for 3.5 h before concentration in vacuo. The residue in $CHCl_3$ was washed with aqueous $NaHCO_3$, concentrated, and heated in toluene (200 mL) at 100 °C for 2 h. The concentrated residue was crystallized from EtOAc: yield 0.8 g (17%); IR 1760 (COOAr) cm^{-1}; ^{1}H NMR δ 3.47 (s, 4 H), 3.88 (s, 4 H), 6.57–7.20 (m, 8 H). Anal. ($C_{18}H_{16}N_2O_4$) C, H, N.

3 Methyl Ester Lactone. Thionyl chloride (7 mL, 0.1 mol) was added dropwise to **3** (2.0 g, 4.8 mmol) in MeOH (25 mL), and the mixture was heated at 40 °C for 6 h. After concentration in vacuo, the residue in $CHCl_3$ was washed with aqueous $NaHCO_3$, again concentrated, and eluted from SiO_2 (150 g) with $CHCl_3$/acetone/MeOH (56:2:1) to give the methyl ester lactone (1.3 g, 60%) as an oil: IR 1740 cm^{-1} (COOR); ^{1}H NMR (250 MHz) δ 2.42–3.13 (m, 4 H), 3.42–3.83 (m, 11 H), 4.59 (m, 1 H), 6.77–7.26 (m, 8 H). Anal. Cald for $C_{22}H_{26}N_2O_6$ m/z 414.1791, found m/z 414.1791.

2-[(*o*-Hydroxybenzyl)amino]phenol (9). Sodium cyanoborohydride (0.90 g, 14 mmol) in MeOH (10 mL) was added dropwise to 2-[[(*o*-hydroxyphenyl)imino]methyl]phenol[20] (3.0 g, 14 mmol) in methanol (300 mL) and then stirred overnight at room temperature. After concentration in vacuo, the residue was treated with brine (50 mL) and diethyl ether (100 mL). The pH of the aqueous layer was adjusted to 7 with dilute HCl and extracted several times with diethyl ether. The residue (3.3 g) from the combined extracts was eluted from SiO_2 (50 g) with 20% ethyl acetate in hexanes to give a solid (1.8 g), which was crystallized from CH_2Cl_2/hexanes, yielding crystals of the product (1.1 g, 37%), mp 98–100 °C. Anal. ($C_{13}H_{13}NO_2$) C, H, N.

2-[[(*o*-Carboxyphenyl)amino]methyl]pyridine (13). Sodium cyanoborohydride (0.4 g, 7 mmol) in MeOH (15 mL) was added dropwise to 2-[[(o-carboxyphenyl)imino]methyl]pyridine (1.6 g, 7.0 mmol) in MeOH (150 mL) and then stirred overnight at room temperature. After concentration in vacuo, the residue was partitioned between CH_2Cl_2 and water whose pH was maintained at 6. Extraction with methylene chloride and crystallization from CH_2Cl_2 gave the product (1.2 g, 75%), mp 150–152 °C. Anal. ($C_{13}H_{12}N_2O_2$) C, H, N.

***N,N'*-Bis(2,3-dihydroxybenzoyl)-1,4-diaminobutane (14).** 2,3-Dimethoxybenzoic acid (10 g, 55 mmol) and thionyl chloride (40.0 mL, 550 mmol) were refluxed for 1 h and concentrated in vacuo. The resulting acid chloride in diethyl ether (80 mL) was added dropwise to a cooled, stirred solution of 1,4-diaminobutane (11.0 mL, 110 mmol) in Me_2SO (50 mL). After 1 h at room temperature, the reaction mixture was treated with H_2O (100 mL) and 1 N NaOH (60 mL). The aqueous phase was extracted with $CHCl_3$ and the combined organic layer was extracted with 2 N HCl (200 mL). Concentration of the organic phase gave *N,N'*-bis(2,3-dimethoxybenzoyl)-1,4-diaminobutane (7.7 g). This unpurified intermediate (3.0 g, 7.2 mmol) in CH_2Cl_2 (250 mL) at −78 °C was treated dropwise with BBr_3 (2.1 mL, 22 mmol). The resulting suspension was stirred for 1 week at room temperature, diluted with 6 N HCl (5 mL) and MeOH (50 mL), stirred for 5 min and concentrated in vacuo. The residue was treated with MeOH and concentrated several times to remove volatile borate esters. The residue was boiled with H_2O (50 mL) and the mixture cooled, and the H_2O-insoluble product was collected and dried to give the product (2.4 g, 92%), mp 208–210 °C. Anal. ($C_{18}H_{20}N_2O_6$) C, H, N.

***N,N'*-Bis[4-(2,3-dihydroxybenzamido)butyl]-2,3-dihydroxyterephthalic Acid Diamide (15).** 2,3-Dimethoxyterephthalic acid (1.2 g, 5.2 mmol) was refluxed with thionyl chloride (15 mL, 0.21 mol) for 1 h and concentrated in vacuo. The resulting acid chloride was heated to 80 °C with *N*-(2,3-dimethoxybenzoyl)-1,4-diaminobutane (3.0 g, 12 mmol), prepared as above, triethylamine (3.3 mL, 24 mmol), and 4-(dimethylamino)pyridine (73 mg, 0.60 mmol) in DMF (20 mL) for 2 days. The mixture was concentrated in vacuo and the residue in $CHCl_3$ was washed with dilute NaOH followed by dilute HCl. The crude product remaining in the $CHCl_3$ phase was eluted from silica gel (EM60, 150 g) with use of $CHCl_3$/acetone/MeOH (70:5:2.5) to give the tetramide (1.4 g, 38%), whose structure was verified by

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^{1}H NMR. Boron tribromide (1.7 mL, 18 mmol) was added dropwise to this intermediate (1.4 g, 2.0 mmol) in CH_2Cl_2 (100 mL) at −78 °C. The resulting suspension was stirred at room temperature for 3 days, treated with 6 N HCl (5 mL) and methanol (50 mL), stirred for 5 min, and concentrated in vacuo. The residue was treated with MeOH and concentrated several times to remove borate esters. The total crude was washed with diethyl ether and boiled with H_2O (20 mL). Upon cooling, the H_2O-insoluble material was filtered and dried to give the product (1.1 g, 92%), mp 98–100 °C. Anal. ($C_{30}H_{34}N_4O_{10}\cdot 2.5H_2O$) C, H, N.

(*o*-Aminobenzal)acethydrazide (16). A mixture of *o*-aminophenol (2.0 g, 16 mmol) and acethydrazide (1.2 g, 16 mmol) in 100% EtOH (20 mL) was refluxed overnight, cooled, and concentrated, when pale yellow crystals of product separated (1.8 g, 65%), mp 160–164 °C. Anal. ($C_9H_{11}N_3O$) C, H, N.

(*o*-Aminobenzal)benzhydrazide (17). *o*-Aminophenol (2.0 g, 16 mmol) and benzoylhydrazine (2.3 g, 16 mmol) in absolute EtOH (20 mL) were refluxed overnight, cooled, and concentrated, and the resulting yellow crystals were filtered. Recrystallization from absolute EtOH gave the product (3.4 g, 88%), mp 180–182 °C. Anal. ($C_{14}H_{13}N_3O$) C, H, N.

2-[[(*o*-Hydroxyphenyl)imino]methyl]-8-acetoxyquinoline (18). A mixture of 8-acetoxyquinoline-2-carboxaldehyde (1.1 g, 5.0 mmol), *o*-aminophenol (0.80 g, 7.5 mmol), and molecular sieves (Linde 5 Å, 2.0 g) in toluene (50 mL) was stirred overnight and filtered, and the residue from the filtrate was crystallized from diethyl ether to give the product (1.1 g, 76%), mp 125–128 °C. Anal. ($C_{18}H_{14}N_2O_3$) C, H, N.

2-[[(*o*-Hydroxyphenyl)imino]methyl]pyridine (19). 2-Pyridinecarboxaldehyde (3.8 mL, 40 mmol) in H_2O (10 mL) was added dropwise to *o*-aminophenol (4.4 g, 40 mmol) in H_2O (300 mL) with stirring at room temperature. After 0.5 h, the mixture was extracted with $CHCl_3$ (3 × 100 mL) and the residue from $CHCl_3$ was crystallized from CH_2Cl_2/hexanes to give the product (5.1 g, 65%), mp 107–108 °C. Anal. ($C_{12}H_{10}N_2O$) C, H, N.

2-[[(*o*-Hydroxyphenyl)amino]methyl]pyridine (20). Sodium cyanoborohydride (0.6 g, 10 mmol) in MeOH (20 mL) was added dropwise to 2-[[(*o*-hydroxyphenyl)imino]methyl]pyridine (2.0 g, 10 mmol) in MeOH with stirring overnight at room temperature. After concentration in vacuo, the residue was partitioned between saturated NaCl solution (50 mL) and CH_2Cl_2 (100 mL). The pH of the aqueous layer was adjusted to 7 with dilute HCl, when further extraction with CH_2Cl_2 gave the crude product (2.2 g), which was eluted from silica gel (EM60, 50 g) with use of a gradient of 25% EtOAc/hexanes to 50% EtOAc/hexanes. Fractions containing the desired product were combined and concentrated, and the residue was cyrstallized from CH_2Cl_2/hexanes to yield the pure product (0.80 g, 43%), mp 143–145 °C. Anal. ($C_{12}H_{12}N_2O$) C, H, N.

2-[(*o*-Hydroxybenzyl)imino]pyridine (21). 2-Aminopyridine (4.0 g, 40 mmol) and salicylaldehyde (4.5 mL, 40 mmol) were heated on a steam bath for 15 min, cooled, and crystallized from CH_2Cl_2/hexanes to give the product (5.8 g, 73%), mp 65–66 °C. Anal. ($C_{12}H_{10}N_2O$) C, H, N.

2-[(*o*-Hydroxybenzyl)amino]pyridine (22). Sodium cyanoborohydride (0.60 g, 10 mmol) in MeOH (20 mL) was added dropwise to 2-[(*o*-hydroxybenzyl)imino]pyridine (2.0 g, 10 mmol) in MeOH (200 mL), and the mixture was stirred at room temperature. After 18 h, the mixture was concentrated in vacuo and the residue was partitioned between CH_2Cl_2 (100 mL) and saturated NaCl solution (50 mL, pH adjusted to 7 with dilute HCl). The CH_2Cl_2 phase yielded a product, which was eluted from silica gel (EM60, 50 g) with use of 20% EtOAc in hexanes. Fractions containing the product were combined and crystallized from CH_2Cl_2/hexanes to give the pure product (0.80 g, 40%), mp 106–108 °C. Anal. ($C_{12}H_{12}N_2O$) C, H, N.

Kinetic Measurements. A Metrohm autotitrator (pH stat mode), equipped with a thermostated titration vessel, microburet, and pH meter with a calomel electrode calibrated with standard buffers at pH 7.0 and 10.0, was used. Hydrolyses were measured at 38.0 ± 0.1 °C in 0.05 M KCl in 50% aqueous methanol or water under argon with the use of 5×10^{-4} M concentrations of the metal and the chelator and 0.05 M NaOH to maintain a constant pH.

Spectrophotometric studies were conducted in 50% aqueous methanol buffered at pH 7.5 with Hepes and thermostated at 38 °C. The concentrations of the ester and ferric nitrate were both 1.7×10^{-4} M, and rates were determined from the increase in absorption at 480 nm. The kinetics were analyzed by a nonlinear least-squares curve fitting program (NLIN) capable of deconvoluting consecutive first-order processes.[21]

The formation of the iron(III) complex of **5** under the conditions of the kinetic measurements was verified by paper chromatographic analysis ($BuOH/H_2O/HOAc$; 4:6:1; upper phase), which was capable of visualizing the metal complexes of both **5** and its diesters.

Biological Testing. The mouse model developed by Gralla[10] and subsequently modified by Rosenkrantz[8] was employed. Male BDF_1 hybrid mice (Taconic Farms, Germantown, NY, or Simonsen Laboratories, Gilroy, CA) ca. 6–7 weeks old and weighing 16–20 g were randomized into groups of 10, and each group was housed in a 26 × 19 × 22 cm stainless steel wire mesh metabolic suspension cage. Commercial pellet feed (Wayne Lab-Blox, Chicago, IL) and water were freely available. All animals were isolated in one room regulated for a 12-h light/dark circadian cycle, with an ambient temperature of 23 ± 2 °C and with eight cycles of fresh air each hour. Two purebred adult male beagle dogs (Marshall Beagles, Inc., North Rose, NY), retained nearby in a separate room, were the constant source of transfusion blood. It was demonstrated that canine erythrocytes evoked no immunological distress in the mice. On the day of transfusion an approximate 60-mL pool of canine heparin-treated whole blood was collected, and the centrifuged red blood cells were washed twice with isotonic saline and exposed to 50 °C for 30 min in a water bath. No hemolysis occurred under these conditions, and no occult blood was detected in urine specimens after transfusion. Each mouse received a single ip transfusion of 0.6 mL of suspended erythrocytes every other day until three transfusions had been performed. A total of 1.8 mL was transfused, which was approximately equivalent to 2 mouse blood volumes. Little or no ferricosuria was observed after the third transfusion.

Treatment and Analytical Procedures. Each bioassay consisted of a nontransfused control group, a transfused control group, a reference standard group (DFB), and generally seven groups for test compounds. Test compounds were dissolved in distilled water or fine aqueous suspensions were prepared with the aid of a drop of Polysorbate 80 (final concentration of 2%). The injection volume was less than 0.5 mL. Two days after the last transfusion, each mouse was given a single daily ip abdominal injection of drug or vehicle for 7 consecutive days. Observations of toxicity signs were conducted daily. At each 24-h interval, group urine specimens were collected and volumes were recorded. Specimens were cleared of sediment by centrifugation and supernatants were directly aspirated into the burner of an atomic absorption spectrometer for the determination of iron. Each daily fecal pool was weighed and combined with the previous day's specimen, until a weekly pool for each group was collected. Fecal specimens were stored at <−20 °C until analysis. The pools were blended at room temperature for 15 min with use of a mortar and pestle. Triplicate samples (100 mg), one from each of three different locations, were analyzed by heating with 10 mL of 1 N hydrochloric acid at 90 °C for 30 min. The hydrolysate was cooled to room temperature and centrifuged (2000*g*) for 15 min, and the iron content was determined by atomic spectroscopy. Iron analyses were conducted on a Model 303 atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) calibrated with a standard commercial ferric chloride solution (Harleco Co., Minneapolis, MN).

At the completion of drug treatment, the mice were anesthetized with diethyl ether and the abdominal aorta severed to exsanguinate organs; the liver and spleen were massaged in situ to facilitate removal of blood. The latter organs were blotted and weighed, and individual organs were processed to 1–5% homogenates in 0.073 M saline with a Teflon-glass motor-driven homogenizer. The cellular debris was removed by centrifugation, and the supernatant was analyzed for iron by atomic absorption spectroscopy. Iron recovery was monitored by addition of exogenous iron to some aliquots of homogenates, and iron standards and calibration curves were prepared as needed.

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It was found that 0.073 M saline afforded optimal extraction of hepatic and splenic iron, superior to isotonic saline or other molarities between 0.014 to 0.145. The recoveries of exogenous iron in the 2–5-μg range were 82–104% for tissues and 84–114% for excreta (12 determinations for each tissue and excreta). The precision of atomic absorption determination indicated a 2% coefficient of variation for urine and 10% for liver homogenates. In 36 separate bioassays with DFB at a dose of 250 mg/kg, the mean decrease ± SE in liver iron was 23.8 ± 4.7% and the increase in urinary iron was 270 ± 14%.

The following compounds failed to exhibit iron chelating activity when tested in the above screen: 2,2′-dihydroxy-5,5′-dimethylbiphenyl (**7**),[22,23] 2,6-bis(2-hydroxy-5-methylphenyl)-4-methylphenol (**8**),[22,23] 2-[(*o*-hydroxybenzyl)amino]phenol (**9**),[23] 2-[[(*o*-hydroxyphenyl)imino]methyl]phenol (**10**),[20] 2-(*o*-hydroxyanilino)-Δ^2-penten-4-one (**11**),[24] 2-[[(*o*-carboxyphenyl)imino]methyl]pyridine (**12**),[25] 2-[[(*o*-carboxyphenyl)amino]methyl]pyridine (**13**), *N,N′*-bis(2,3-dihydroxybenzoyl)-1,4-diaminobutane (**14**), *N,N′*-bis[4-(2,3-dihydroxybenzamido)butyl]-2,3-dihydroxyterephthalic acid diamide (**15**), (*o*-aminobenzal)acethydrazide (**16**), (*o*-aminobenzal)benzhydrazide (**17**), 2-[[(*o*-hydroxyphenyl)imino]methyl]-8-acetoxyquinoline (**18**), 2-[[(*o*-hydroxyphenyl)imino]methyl]pyridine (**19**), 2-[[(*o*-hydroxyphenyl)amino]methyl]pyridine (**20**), 2-[(*o*-hydroxybenzyl)imino]pyridine (**21**), 2-[(*o*-hydroxybenzyl)amino]pyridine (**22**), 2-(8-quinolylamino)-Δ^2-penten-4-one (**23**),[26] 8-[(α-pyridylmethylene)amino]quinoline (**24**).[27]

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Registry No. **1**, 10328-28-6; **1**·2Li, 102212-18-0; **1**·2HCl, 102212-19-1; **1**·2Na, 102212-20-4; **2**, 21972-77-0; **3**, 63651-93-4; **4**, 102212-27-1; **5**, 35998-29-9; **5** (dimethyl ester), 85120-52-1; **5** (diethyl ester), 102212-10-2; **5** (diisopropyl ester), 102212-11-3; **5** (dibenzyl ester), 102212-12-4; **5** (dipentyl ester), 98318-32-2; **5** (dimethyl ester diacetate), 102212-13-5; **5** (di-*tert*-butyl ester), 102212-14-6; **5** (di-*tert*-butyl ester diacetate), 102212-17-9; **6**, 1170-02-1; **6** (dimethyl ester), 90044-13-6; **6** (diethyl ester), 98318-25-3; **9**, 36282-74-3; **13**, 5691-02-1; **13** (imino precursor), 78604-78-1; **14**, 71636-73-2; **14** (bis dimethoxy precursor), 73630-96-3; **15**, 102212-21-5; **16**, 102212-22-6; **17**, 102212-23-7; **18**, 102212-25-9; **19**, 3860-58-0; **20**, 102212-26-0; **21**, 1823-47-8; **22**, 70301-52-9; $PhCH_2O_2CCl$, 501-53-1; $HO_2CCH_2NH(CH_2)_2NHCH_2CO_2H$, 5657-17-0; $HO_2CCH_2N(Z)(CH_2)_2N(Z)CH_2CO_2H$, 102212-15-7; *t*-$BuO_2CCH_2NH(CH_2)_2NHCH_2CO_2Bu$-*t*, 102212-16-8; *o*-$AcOC_6H_4CH_2Br$, 704-65-4; *o*-$MeOC_6H_4NH(CH_2)_2NHC_6H_4OMe$-*o*, 37460-52-9; MeO_2CCH_2Br, 96-32-2; *o*-$HOC_6H_4CH{=}NC_6H_4OH$-*o*, 1761-56-4; $AcNHNH_2$, 1068-57-1; $NH_2(CH_2)_4NH_2$, 110-60-1; *o*-$NH_2C_6H_4OH$, 95-55-6; $PhCONHNH_2$, 613-94-5; Fe, 7439-89-6; 2,3-dimethoxybenzoic acid, 1521-38-6; 2,3-dimethoxyterephthalic acid, 7168-95-8; *N*-(2,3,dimethoxybenzoyl)-1,4-diaminobutane, 102212-24-8; 8-acetoxyquinoline-2-carboxaldehyde, 36456-52-7; 2-pyridinecarboxaldehyde, 1121-60-4; 2-aminopyridine, 504-29-0; salicylaldehyde, 90-02-8.

Supplementary Material Available: Figure 2 shows the structure of the chelators **7**–**24** (1 page). Ordering information is given on any current masthead page.

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Spin Probes as Mechanistic Inhibitors and Active Site Probes of Thymidylate Synthetase

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C-4- and C-5-substituted analogues of dUMP were examined as inhibitors of thymidylate synthetase and as topographical probes of its active site by electron spin resonance (ESR). The C-5-substituted spin-labeled analogues pDUAP (**2**) and a pDUTT (**3**) as well as the unlabeled AAdUMP (**1**) were competitive inhibitors with K_i's of 9.2, 89, and 7.9 μM, respectively. The C-4-spin-labeled pls^{4}dU (**4**) displayed no inhibition activity. Scatchard plots as determined by ESR gave similar association constants for **2** (K_{assoc} = 1.9 × 10^5 M^{-1}) and for **3** (K_{assoc} = 2.4 × 10^5 M^{-1}). Both of these values are similar to the K_{assoc} of FdUMP indicating that the bulky substituent in position 5 does not interfere with the formation of the binary complex. The enzyme–C-5-spin-labeled nucleotide complexes indicate the presence of similarly immobilized spin labels by ESR, whereas no binding and immobilization were noticed with the C-4-spin-labeled nucleotide. A model for the active-site geometry of the enzyme was derived which suggests that the C-5 substituents point toward the opening of the binding cavity whose depth is at least 12 Å. Also, the approximate 10-fold increased inhibitory activity of **2** as compared to that of **3** may be attributed to the significant electron withdrawing properties of the C-5 substituent in **2**. Finally, the set of probes used for the binding and inhibition of thymidylate synthetase gives direct experimental evidence that an electron-withdrawing C-5 substituent primarily affects the formation of the ternary complex and will not substantially influence the stability of the binary complex.

Thymidylate synthetase, the key enzyme in the sole de novo pathway for thymidylate synthesis, is recognized as a clinically effective target enzyme for the control of neoplastic cell proliferation.[1,2] The active form of the drug, 5-fluorouracil, in the chemotherapeutic control of certain forms of cancer at the enzymatic level has been shown to be 5-fluoro-2′-deoxyuridylic acid (FdUMP), which acts as a powerful inhibitor of the enzyme. The enzyme catalyzes the conversion of dUMP and dTMP in the presence of the cofactor 5,10-methylene tetrahydrofolate (Scheme I).

The generally accepted mechanism of action of the enzyme involves the initial formation of a reversible binary

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