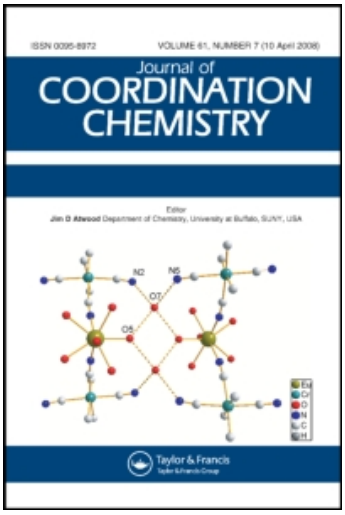

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SYNTHESIS AND IRON COMPLEXATION STUDIES OF BIS-HYDROXAMIC ACIDS

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SYNTHESIS AND IRON COMPLEXATION STUDIES OF BIS-HYDROXAMIC ACIDS

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A series of *bis*-hydroxamic acids was prepared by the condensation of the aminoacids glycine, β-alanine, and γ-aminobutyric acid, with succinyl and adipoyl chlorides followed by conversion of the terminal carboxyl groups to hydroxamic acids (HA). Mole ratio plots of iron chelation indicated that 3:1 HA:Fe complexes exist at pH 4 and 6, but that 2:1 HA:Fe complexes are the prevalent species at pH 2. Differences in spacing had little effect on the iron complexing ability as measured through competition equilibria with EDTA. The six *bis*-hydroxamic acids were subjected to a bioassay screen designed to select compounds possessing *in vivo* iron removal activity for potential use in treating iron overload in Cooley's Anemia. All of the six compounds had some activity. The *bis*-hydroxamic acid derived from succinic acid and γ-aminobutyric acid was particularly active, almost as active as the standard drug desferrioxamine-B.

Keywords: Iron, hydroxamates, Cooley's anemia, synthesis, testing

INTRODUCTION

The effect of structure on the complexation of hydroxamic acids with iron has long been of interest, especially since the discovery of several families of naturally occurring tris-hydroxamic acids[1-4] (called siderophores), such as desferrioxamine and ferrichrome, shown in Figure 1. Early work of Schwarzenbach and co-workers[5-7] showed that compounds of this type exhibit strong chelate effects which can raise iron complex stability constants several orders of magnitude above those of simple monohydroxamic acid complexes.

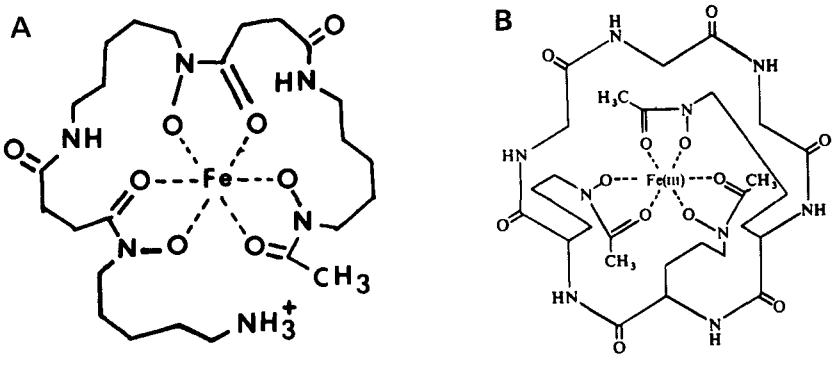

FIGURE 1 Naturally occurring siderophores. A, Ferrioxamine-B; without iron it is called desferrioxamine-B. B, Ferrichrome.

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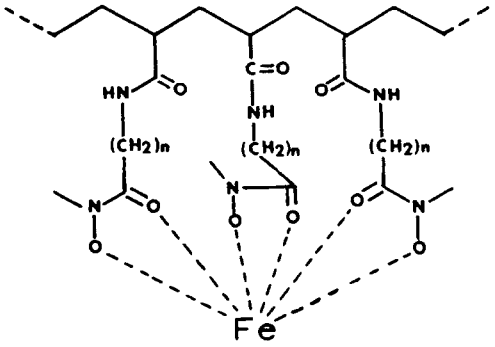

FIGURE 2 Iron complex of hydroxamic acid polymer.

Within a family, the iron complex stability constant can be influenced by subtle changes in the structure and arrangement of the hydroxamic acids. The influence of spacing distance was demonstrated by a study of a series of polymers bearing side chains terminated by hydroxamic acids.[8,9] (Figure 2). With side chains of sufficient length, three hydroxamic acids from neighboring side chains can easily fit the octahedral coordination sphere of the iron, and the stability constant is appropriately high. With less distance between hydroxamic acids (shorter side chains), the complex stability is lower, a result reflecting a decreased ability of the hydroxamic acids to coordinate iron. With very short side chains, the iron complexes of the polymers precipitate immediately on addition of iron. The logical conclusion is that when the side chains are too short to favour intramolecular complexation, intermolecular complexation occurs and the extensive cross-linking results in insolubility.

Hydroxamic acids form either 3:1 (orange-brown), 2:1 (red-brown), or 1:1 (purple) HA:Fe complexes depending upon the relative proportions of iron to hydroxamic acid. In the case of monohydroxamic acids, addition of iron first causes the formation of the 3:1 complex. On continued addition of iron, the 3:1 complex is converted to the 2:1 and finally to the 1:1 form, (equations *1–3*).

$$3HA + Fe^{+3} \rightarrow FeA_3 + 3H^+ \quad (1)$$

$$2FeA_3 + Fe^{+3} \rightarrow 3FeA_2^{+1} \quad (2)$$

$$FeA_2^{+1} + Fe^{+3} \rightarrow 2FeA^{+2} \quad (3)$$

This process can be observed spectrophotometrically by a shift of λ_{max} from 440 nm (3:1) to 470 nm (2:1) to 520 nm (1:1).[2] In the case of desferrioxamine and the hydroxamic acid polymers, 3:1 complexes are readily converted to 2:1 species by the addition of iron. However, even after the addition of large amounts of iron, the 2:1 complex is stable and does not convert to the 1:1 species.[8] It would appear that chelation is more effective in stabilizing the 2:1 than the 3:1 complex. The pH is also an important factor, with stability decreasing with decreasing pH. In the case of the polymers, mole ratio plots indicated that the 3:1 complex is stable down to a pH of at least 2.[8]

In the present paper, we report the synthesis and characterization of a series of *bis*-hydroxamic acids with varying spacings between hydroxamic acids. The objective is to see if changes in the HA spacing would result in any significant changes in the structure of the iron complex and also to see the effect of pH on the dissociation of a free hydroxamic acid not chemically linked to the other two forming the complex.

EXPERIMENTAL

Melting points were determined on a Uni-Melt (Arthur H. Thomas) and are uncorrected. Spectra were recorded on a Beckman IR-8 for IR, a Varian EM 360 for NMR, and a Bauch and Lomb Spectronic 2000 for UV-visible. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Sources of reagents were Sigma: *N*-hydroxysuccinimide and γ-aminobutyric acid; Aldrich: methylhydroxylamine, β-alanine, dicyclohexylcarbodimide, succinyl chloride, and adipoyl chloride; Alpha: ferric perchlorate.

Dicarboxylic acids

A solution containing 0.4 mol NaOH and 0.2 mol of amino acid (glycine, β-alanine, or γ-aminobutyric acid) in 50 cm^3 of water was prepared and cooled to 0–5°. To this solution was added dropwise 0.1 mol of the diacid chloride (succinyl or adipoyl) over a period of 25 min at 0–5°. Stirring was continued at 5–10° for 1 hr and at room temperature for 2 hrs. The reaction mixture was acidified with conc. HCl to pH of 2, whereupon the diacid precipitated. The diacid was collected and purified by recrystallization.

N-Hydroxysuccinimide Esters of Diacids

To a solution of 0.05 mol diacid and 0.1 mol NHS in 150 cm^3 DMF was added 0.1 mol of DCC. After 4 hrs stirring, the dicyclohexylurea (DCU) was removed by filtration. After 4 additional hrs, more DCU had precipitated and this was also removed. The reaction mixture was concentrated on a rotary evaporator and the NHS ester was precipitated by the addition of ether and alcohol, and then recrystallized.

Bis-hydroxamic acids

To a solution of 0.06 mol of methylhydroxylamine hydrochloride in 30 cm^3 DMF was added 0.06 mol triethylamine (TEA) and the mixture was stirred for 15 min and then cooled. The TEA· HCl was removed by filtration and the filtrate was added to a solution of 0.02 mol of the NHS ester in 120–150 cm^3 DMF. After stirring at room temperature for 4 hrs the solution was concentrated on the rotary evaporator. On addition of diethyl ether, a pasty solid was produced which was collected and recrystallized twice to give a white crystalline solid.

Mole Ratio Study

Stock solutions of *bis*-hydroxamic acids were prepared by dissolving a known amount in water. To 0.03 cm^3 aliquots of aqueous solution of hydroxamic acid were added varying amounts (0.02 cm^3 to 0.3 cm^3) of iron(III) perchlorate. Nitric acid was added to the solution to a pH of 2 and the solution was diluted to 10 cm^3. The absorption spectra of the solutions were recorded between 600 and 300 nm. Several runs at pH 2, 4 and 6 were also carried out in perchloric acid medium at ionic strength of 0.8 M maintained by adding sodium perchlorate. The results were unchanged from those obtained in the absence of sodium perchlorate.

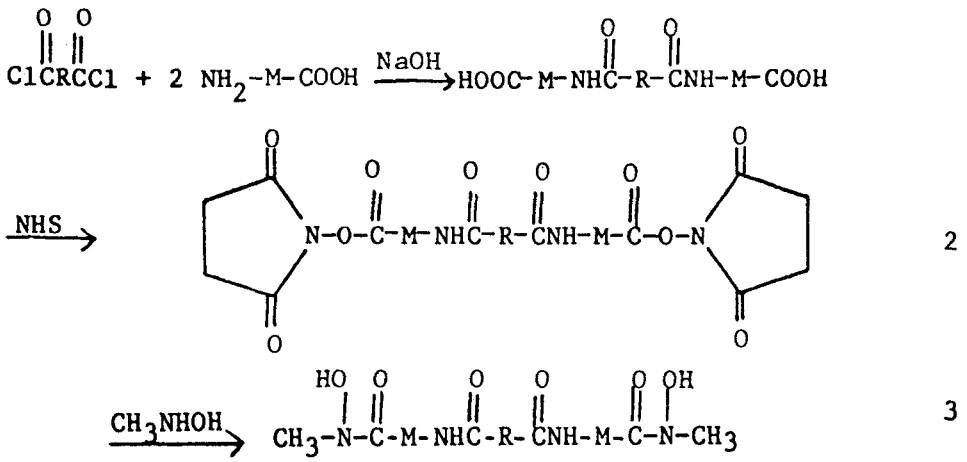

FIGURE 3 Synthetic scheme for the synthesis of *bis*-hydroxamic acids. NHS, *N*-hydroxysuccinimide

Competition Study

In a 10 cm^3 flask, aqueous solutions of accurately known concentration of *bis*-hydroxamic acid and EDTA were placed. Standardized Fe^{+++} solution was added, the pH was adjusted to 2 or 4, and the solution was diluted to 10 cm^3. The solutions were allowed to equilibrate for 18 hrs at room temperature and the absorbance at 450 and 460 nm was recorded.

RESULTS AND DISCUSSION

A series of *bis*-hydroxamic acids was prepared by the synthetic sequence shown in Figure 3. Condensation of succinyl or adipoyl chloride with glycine, β-alanine and

TABLE I
Data for the *bis*-Hydroxamic Acids.

Compd. Code	Type	R	M	Cryst. Solv.	Yield (%)	mp (deg)
1a	acids	$(CH_2)_2$	CH_2	C[a]	30	216
b			$(CH_2)_2$	C	38	215
c			$(CH_2)_3$	C	46	157
d		$(CH_2)_4$	CH_2	B	77	198
e			$(CH_2)_2$	B	69	196
f			$(CH_2)_3$	B	56	143
2a	NHS esters	$(CH_2)_2$	CH_2	A	78	202
b			$(CH_2)_2$	A	82	172
c			$(CH_2)_3$	A	75	147
d		$(CH_2)_4$	CH_2	A	79	165
e			$(CH_2)_2$	A	83	173
f			$(CH_2)_3$	A	75	147
3a	Hydr. Acids	$(CH_2)_2$	CH_2	A	75	196
b			$(CH_2)_2$	A	71	158
c			$(CH_2)_3$	A	77	143
d		$(CH_2)_4$	CH_2	A	52	186
e			$(CH_2)_2$	A	35	185
f			$(CH_2)_3$	A	48	140

[a]A, methanol; B, water; C, methanol-water.

TABLE II
Mole Ratio Data for the Iron Complexes of the *bis*-Hydroxamic Acids.

Compd.	HA Atomic Spacing[a]	pH	mole ratio Fe/HA	λ_{max} range, nm
3a	8	2	0.43	466–467
3b	10	2	0.49	464–468
3c	12	2	0.50	463–467
3d	10	2	0.46	460–466
3e	12	2	0.41	452–470
3f	14	2	0.48	447–468
3b	10	4	0.35	442–469
3b	10	6	0.27	421–436

[a]No. of atoms in chain separating hydroxamic acid groups.

γ-aminobutyric acid followed by conversion of the carboxyl groups to hydroxamic acids produced a series of *bis*-hydroxamic acids having 8, 10, 12 and 14 atomic spacing between HA units. Specific compounds are listed in Table I, together with the solvent of crystallization, yield, and melting point. Elemental analyses and the IR and NMR spectra were consistent with the proposed structures. In the infrared, acid carbonyls appeared at 1680–1690 cm^{-1}, esters at 1735 cm^{-1}, succinimide carbonyls at 1780 and 1820 cm^{-1}, and the amide I and amide II bands at 1635 and 1540 cm^{-1}, respectively. In the NMR spectra, lines characteristic of the various protons were clearly evident and in all cases confirmed the proposed structures.

A mole ratio study was made of the iron complexation of each of the six *bis*-hydroxamic acids at pH 2. An example is shown in Figure 4. The intersections of the plots, which give the mole ratio of iron to hydroxamic acid in the complex, are presented in Table II. The results ranged from a low of 0.41 to a high of 0.50 with the probable error being estimated as about ±0.03 Fe:HA. These results indicate that at pH 2 the complex is approaching a 2:1 HA:Fe complex. In the visible spectrum, λ_{max} values in the neighborhood of 460 nm are also consistent with the existence of the 2:1 complex at this pH.

At pH 4 a mole ratio intersection at 0.35 (Figure 4) is near the theoretical value of 0.33 for a 3:1 HA:Fe complex. At pH 6 the intersection at 0.27 is a little low for a 3:1 complex, but at this high pH, precipitation of iron complex or hydroxide often occurs to produce a slight turbidity that can distort the results. A further indication of a 3:1 complex at high pH is indicated by λ_{max} values in the range 420–449 nm during the initial stages when hydroxamic acid is in excess. It thus appears that at pH >3 or 4 this series of *bis*-hydroxamic acids form intermolecular 3:1 HA:Fe complexes that are almost completely converted to 2:1 complexes at pH 2. The effect is also in accord with the observations of Monzyk and Crumbliss[10] for the case of desferrioxamine, which also appears to convert to the 2:1 HA:Fe complex on passing from pH 3 to 2.

This behaviour is quite different from that observed for hydroxamic acid polymers (Figure 2).[8] In these cases the chelate effect arising from the linking together of large numbers of hydroxamic acids through polymerization is sufficiently great to stabilize the 3:1 HA:Fe complex down to a pH of 2.

In the presence of excess iron, the 3:1 HA:Fe complex of monohydroxamic acids normally convert to 2:1 and then to 1:1 complexes, as evidenced by a bathochromic shift from 420 to 467 to 520 nm, respectively. For both the *bis*-hydroxamic acids and the hydroxamic acid polymers, the 2:1 complex is evidently stable in the presence of excess iron as λ_{max} fails to move much above 470 nm. When the spacing between hydroxamic

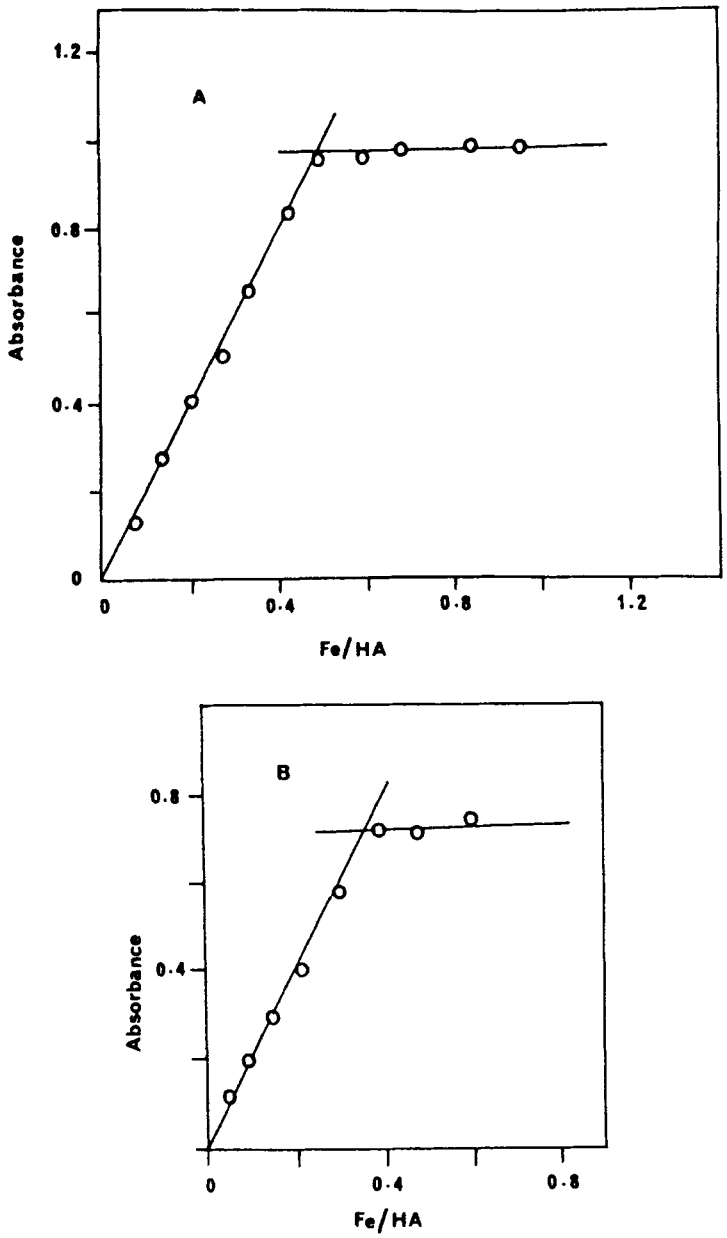

FIGURE 4 Mole ratio plots for *bis*-hydroxamic acid 3b derived from succinic acid-β-alanine condensation product. A. pH 2. B. pH 4.

acids is sufficient to permit the hydroxamic acids to fit the octahedral geometry of the iron, the chelate effect for the formation of the 2:1 complex appears to be particularly strong.

In order to determine if the changing atomic spacing between hydroxamic acid groups would produce differences in iron chelation ability, competition studies were

carried out between hydroxamic acid, EDTA, and iron.[7,8] The procedure is based on the equilibria expressions of Eqs. *4* and *5*,

$$Fe(Sid)' + Z' \rightarrow FeZ' + Sid' \tag{4}$$

$$K_I = \frac{[FeZ]'\,[Sid]'}{[Fe(Sid)]'\,[Z]'} \tag{5}$$

where Sid stands for siderochrome and Z is the competing ligand, EDTA. The concentrations of the species are indicated by the sign (') and are defined as the sums of the species at all degrees of protonation.

The absorbance, D, of the Fe-HA complex was determined both in the absence and presence of the competing chelating agent Z. From the equilibrium concentration of Fe(Sid)', as measured by the absorbance, and from the total concentrations of Fe, Sid and Z, the quantities required for evaluating K_I may be determined, eqs. *6–9*, where ε is the molar absorptivity and d the path length (1 cm). Since the iron-EDTA complex has little absorption in the visible region under the experimental conditions, the second term of Eq. *6* is negligible.

$$D = \varepsilon_{FeSid}[FeSid]'\,d + \varepsilon_{FeZ}[FeZ]'\,d \tag{6}$$

$$[Fe]_t = [FeSid]' + [FeZ]' \tag{7}$$

$$[Sid]_t = [Sid]' + [FeSid]' \tag{8}$$

$$[Z]_t = [Z]' + [FeZ]' \tag{9}$$

Comparison of K_I values, Table III, shows that there is little or no discernable difference in chelating ability between samples with the shortest spacing and those with the longest. It appears that if the spacing is sufficiently great to allow the hydroxamic acid groups to fit the octahedral coordination sphere of the iron, the chelating ability is particularly strong. We would expect, however, that if the spacing were exceedingly long the chelate effect would decrease, as was actually observed in the case of certain hydroxamic acid copolymers.[11]

The synthesis of this series of *bis*-hydroxamic acids were conducted as part of a program to design new and effective iron chelators to remove the iron that accumulates as a result of the extensive transfusion regimen used in treating the genetic disease β-thalassemia, or Cooley's Anemia.[12–14] The drug now being used for this purpose is desferrioxamine (Figure 1) but because of some inherent problems,[11] new iron chelators are being sought.

TABLE III
Competition Data for the the *bis*-Hydroxamic Acids and EDTA for Iron.

Compd. Code	R	M	pH	ε^a, nm	$K_I^b(n)^c$	SD[d]
3a	$(CH_2)_2$	CH_2	2.0	1.90	3.15(8)	2.67
3c	$(CH_2)_2$	$(CH_2)_3$	2.0	1.99	3.18(4)	0.88
3c	$(CH_2)_2$	$(CH_2)_3$	4.0	1.99	5.70(3)	2.99
3f	$(CH_2)_4$	$(CH_2)_3$	2.0	1.94	2.41(3)	0.61

[a]Millimolar absorptivity. [b]Average value. [c]No. of measurements used to determine average. [d]Standard deviation.

TABLE IV
Relative Potency of the *bis*-Hydroxamic Acids in Treating Iron Overload

Compound	HA Atomic Spacing	Relative Potency
Desferrioxamine-B	9	1.0
3a	8	0.1
3b	10	0.4
3c	12	0.9
3d	10	0.1
3e	12	0.3
3f	14	0.5

The effectiveness of a new iron chelating drug is measured by its ability to remove iron from iron-overloaded mice. The detailed methodology is described elsewhere,[15] but, in essence, the bioassay involves raising the iron level in mice through transfusions of canine red blood cells, administering the test drug over a one week period, collecting urine and faeces, collecting spleens and livers at the end of the test period, reduction of samples to forms suitable for analysis, and analysis for iron by atomic absorption. The iron levels in spleen, liver, urine and faeces are combined to give a relative potency based on the standard drug desferrioxamine (P=1).[15] The results are shown in Table IV. The nature of the amino acid spacer appears to be more important than the actual atomic spacing, with the potency increasing with increasing number of CH_2 groups in the amino acid portion. Compound 3c, derived from succinic acid and γ-aminobutyric acid is almost as effective as desferrioxamine itself and could be considered as a viable candidate for further biological study.

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