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Synthesis of Polyfunctional Hydroxamic Acids for Potential Use in Iron Chelation Therapy

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Two new multidentate *N*-methylhydroxamic acids were prepared and characterized. β-Cyclodextrin was esterified by treatment with succinic anhydride. The resulting carboxyl groups (14 per cyclodextrin) were converted to the *N*-hydroxysuccinimide esters and then on to the hydroxamic acids by treatment with *N*-methylhydroxylamine. Tetracyanoethylation of cyclohexanone followed by hydrolysis of the nitrile and conversion of the carboxylic acid to hydroxamic acid produced a tetrahydroxamic acid derivative of cyclohexanone. Infrared, ^{1}H NMR, and ^{13}C NMR were consistent with the proposed structures. The hydroxamic acids were water soluble and formed the typical red-brown iron complexes. Stability constants (log *K*) of 29–30 for the iron complexes indicated a strong chelate effect. Animal tests indicated that the two compounds were only weakly effective in removing iron *in vivo* from iron-overloaded mice. The potency was only 0.1 that of the standard drug desferrioxamine-B.

INTRODUCTION

Because of their remarkable ability to sequester iron(3+), hydroxamic acids are receiving considerable attention, particularly in medicine where iron chelation therapy has become an important treatment for iron overload. Iron overload can arise from a number of causes, the most important of which are iron poisoning and the disease Cooley's anemia (*1*). In either of these situations, it is necessary to remove iron from the patient efficiently and rapidly without severe side effects. Current treatment involves the administration of the naturally occurring trishydroxamic acid desferrioxamine-B (DFB)[1] (*2, 3*) which is available commercially as the methane sulfonate salt under the name Desferal (Ciba Pharmaceutical Co.). DFB has been successfully employed in the clinic over the past 20 years, but there are some disadvantages in its use (*4*). For this reason, a program is underway to synthesize and evaluate new iron chelators for potential clinical application (*5–11*).

Prior work suggests that in the case of hydroxamic acids, *in vivo* iron chelation is generally more effective when the compounds bear several hydroxamic acids

[1] Abbreviations used: DFB, desferrioxamine-B; TMS, tetramethylsilane; NHS, N-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; DMF, dimethylfuran; DMSO, dimethyl sulfoxide; HA, hydroxamic acid.

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arranged to provide a good chelate effect (*6*). In order to expand upon our knowledge concerning the relation between structure and *in vivo* iron chelation, we have synthesized and characterized two new polydentate hydroxamic acids, one derived from β-cyclodextrin and the other from 2,2,6,6-(tetracarboxyethyl)cyclohexanone, and have examined the effectiveness of these compounds for removing iron via an iron chelation bioassay.

EXPERIMENTAL

Infrared spectra were recorded on the Beckman IR-8, and visible–uv spectra on the Bausch and Lomb Spectronic 2000. ^1H and ^{13}C NMR spectra were recorded on the JEOL GX-270 NMR spectrometer (270 MHz in ^1H) using TMS as internal standard. β-Cyclodextrin, methylhydroxylamine hydrochloride, *N*-hydroxysuccinimide (NHS), and dicylcohexylcarbodiimide (DCC) were obtained from Aldrich Chemical Company. Elemental analyses were carried out by Galbraith Laboratories, Knoxville, Tennessee.

β-Cyclodextrinsuccinate (**I**). This compound was prepared by a method similar to that reported for the synthesis of succinic esters of starch and dextran (*12*). β-Cyclodextrin was heated at 80°C under vacuum to constant weight. To a mixture of β-cyclodextrin (11.9 g, 0.073 mol) and succinic anhydride (44.1 g, 0.44 mol) in 100 ml dry formamide, 36.7 ml of pyridine was added and the solution was heated at 70°C for 48 h. The reaction mixture was poured into 500 ml of ether and then methanol was added until a pasty mass had separated. The paste was dissolved in methanol and reprecipitated by adding ether. After several reprecipitations, the solid was dissolved in water and freeze-dried to yield 21.2 g white flaky compound **I** (80% yield). Infrared ν (cm^{-1}, Nujol): 3300–3500 (O–H), 1730 (COOH). ^1H NMR δ (ppm, DMSO-d_6): 2.6 (br), 4.0–5–5 (series of six broad bands).

Anal. Calcd for $(C_{14}H_{18}O_{11})_7$: C, 46.40; H, 4.97%. Found: C, 46.53; H, 5.28%.

The number of COOH groups per gram of compound was determined by titration with aqueous KOH. *Anal.* Calcd on the basis of 14 COOH groups per molecule: 5.55 meq COOH/g. Found: 5.59 meq/g.

NHS ester of β-cyclodextrinsuccinate (**II**). *N*-Hydroxysuccinimide (12 g, 0.104 mol) and the cyclodextrin ester **I** (8 g, 0.022 mol) were dissolved in DMF (70 ml) and the solution was cooled in an ice bath. DCC (21.4 g, 0.104 mol) was added and the reaction mixture was stirred in an ice bath for 2 h. The precipitated dicyclohexylurea was removed by filtration and the filtrate was stirred at room temperature for 2 days, whereupon more dicyclohexylurea precipitated to give a total of 9.0 g (0.04 mol). The solution was evaporated under vacuum to a small volume and excess ether was added to cause precipitation. The pasty material was thoroughly washed with ether and then triturated with 60 ml of methanol, whereupon the paste was converted to a fine solid. The solid was filtered, washed with methanol, reprecipitated from DMF–methanol, and vacuum dried to yield 11 g of product (90% yield). Infrared ν (cm^{-1}, Nujol): 3300–3500 (O–H), 1820 and 1785 (succinimide C=O), 1735 (ester C=O).

Anal. Calcd for $C_{22}H_{24}N_2O_{15}$: C, 47.48; H, 4.31; N, 5.03%. Found: C, 47.06; H, 4.36; N, 5.85%.

β-Cyclodextrinsuccinate(N-methyl)hydroxamic acid (**III**). To a solution of methylhydroxylamine hydrochloride (2.33 g, 0.028 mol) in DMF (15 ml) was added triethylamine (3.9 ml, 0.028 mol). After cooling and stirring for 30 min, the triethylamine hydrochloride was removed and the filtrate was added to a solution of NHS ester (**II**) (7 g, 0.013 mol) in 10 ml DMF. The reaction mixture was stirred for 2 h at room temperature and poured into excess ether. The oily product was dissolved in methanol and reprecipitated with ether. After six or seven reprecipitations from methanol–ether, the product gradually solidified to yield 3.0 g of hydroxamic acid **III** (55% yield), which was insoluble in water and methanol. Infrared ν (cm^{-1}, Nujol): 3250 (OH), 1740 (ester C=O), 1635 (hydroxamic acid C=O). ^1H NMR δ (ppm, DMSO-d_6): 2.6 (br $-CH_2CH_2-$), 3.07 ($-CH_3$), 4.0–5.5 (series of broad weak unresolved bands), 9.86 (–NOH). Ratio of $-CH_2CH_2-$:$-CH_3$:–NOH = 4:3:1.

Anal. Calcd for $C_{16}H_{24}N_2O_{11}$: C, 45.71; H, 5.71; N, 6.67%. Found: C, 45.88; H, 6.08; N, 6.92%.

2,2,6,6-Tetra(β-cyanoethyl)cyclohexanone (**IV**). This compound was prepared from acrylonitrile and cyclohexanone according to the literature method (*13*) and purified by recrystallization from acetone, mp 165°C (lit. mp 165°C). Infrared ν (cm^{-1}, Nujol): 2260 (C≡N), 1680 (C=O), ^1H NMR δ (ppm, DMSO-d_6) 1.8 (14H, m, $-CH_2-$), 2.35 (8H, t, $-CH_2CN$). ^{13}C NMR δ (ppm, DMSO-d_6), 11.5, 15.5, 30.6, 31.4, 49.5, 120.5 (C≡N), 214.8 (C=O).

2,2,6,6-Tetra(carboxyethyl)cyclohexanone (**V**). Alkaline hydrolysis of nitrile **IV** yielded the acid **V**, which was recrystallized from water, mp 180°C (lit. mp (*13*) 179–180°C). Infrared ν (cm^{-1}, Nujol): 1700 br (ester and acid C=O). ^1H NMR δ (ppm, DMSO-d_6): 1.7 (14H, m, $-CH_2-$), 2.05 (8H, t, $-CH_2C=O$), 12.1 (4H, br, –COOH).

2,2,6,6-Tetra(succinimidylpropionate)cyclohexanone (**VI**). To a cooled solution of NHS (27.6 g, 0.24 mol) and acid **V** (11.6 g, 0.03 mol) in dry DMF (600 ml) was added DCC (49.5 g, 0.24 mol). After 8 h stirring at room temperature, the dicyclohexylurea was removed by filtration and the filtrate was evaporated to a small volume. Addition of excess methanol–ether mixture (1/1) caused the separation of a pasty product which solidified after repeated washings with methanol. The crude yield was 14 g (60%). The product was recrystallized from acetone–methanol mixture (mp 185°C). Infrared ν (cm^{-1}, KBr) 1810 and 1780 (succinimide C=O), 1720–1750 cm^{-1} (ketone and ester C=O).

Anal. Calcd for $C_{34}H_{38}N_4O_{17}$: C, 52.71; H, 4.91; N, 7.24%. Found: C, 55.51; H, 6.39; N, 8.05%.

2,2,6,6-Tetra(propio-N-methylhydroxamic acid)cyclohexanone (**VII**). Triethylamine (7 ml, 0.05 mol) was added to a cooled solution of *N*-methylhydroxylamine hydrochloride (4.2 g, 0.05 mol) in DMF (30 ml). The mixture was stirred for 30 min and triethylamine hydrochloride was removed by filtration. The filtrate was added to 50 ml DMF solution containing the NHS ester **VI** (7.8 g, 0.01 mol). The solution was stirred for 2 h and evaporated under reduced pressure. The residue was washed several times with ethyl acetate and cooled, whereupon a solid product

was obtained. The solid was dissolved in water and freeze-dried to yield 3 g (59%) of **VII**, mp 162–165°C. Infrared ν (cm^{-1}, Nujol): 3400 (OH), 1720 (ketone C=O), 1620 (hydroxamic acid C=O). ^1H NMR δ (ppm, DMSO-d_6): 1.7 (14H, br, $-CH_2-$), 2.2 (8H, t, br, $CH_2C=O$), 3.1 (12H, s, $N-CH_3$), 9.8 (4H, br, N–OH). ^{13}C NMR δ (ppm, DMSO-d_6): 16.2, 26.4, 31.1, 32.6, 35.6, 49.3, 172.6 (C=O), 216.9 (C=O).

Anal. Calcd for $C_{22}H_{38}N_4O_9$: C, 52.59; H, 7.57; N, 11.16%. Found: C, 50.85; H, 7.91; N, 10.84; Calcd for $C_{22}H_{38}N_4O_9 \cdot H_2O$: C, 50.77; H, 7.69; N, 10.77%.

Determination of stoichiometry of the Fe : HA Complex. The stoichiometry of the iron(3+) complex formed by the multidentate hydroxamic acids **III** and **VII** was determined by the mole ratio method. A stock solution of **VII** was prepared by dissolving 0.066 g in aqueous Na_2CO_3, immediately acidifying the solution with concentrated HNO_3, and diluting to 5 ml. A stock solution of **III** was prepared by dissolving 0.063 g in 5 ml water. To aliquots of hydroxamic acid solution (0.25 ml of **III** or 0.15 ml of **VII**) were added varying amounts of a 0.0339 M stock solution of iron(3+) perchlorate, the solutions were diluted to 10 ml with water, and absorption spectra were recorded between 600 and 300 nm. The absorbance at 450 nm was plotted against the Fe/HA ratio.

Periodate oxidation. Aqueous solutions of the β-cyclodextrinsuccinate (**I**) and the parent β-cyclodextrin were separately mixed with an 8% excess of sodium periodate solution and diluted to 10 ml with water. The absorbance due to periodate at 300 nm was recorded at regular time intervals over a 5-day period and the consumption of periodate was calculated using the value 137.3 for the molar absorbtivity of IO_4^- at 300 nm (*14*).

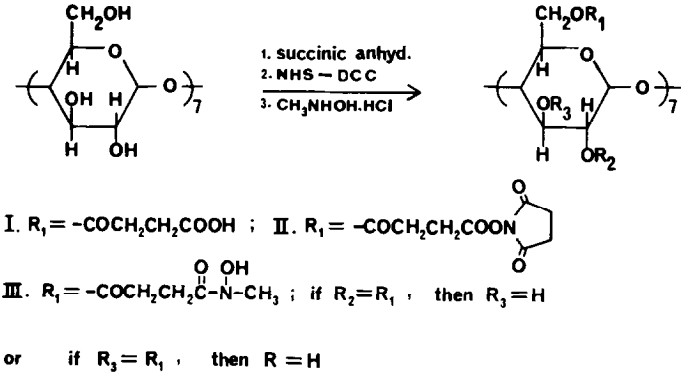
Bioassay for iron removal from iron-overloaded mice. The bioassay for iron chelation activity was performed by EG&G Mason Research Institute and the procedure has been reported in detail elsewhere (*6*). A brief discription follows:

The bioassay was conducted on 6- to 7-week-old male BDF_1 hybrid mice. The iron chelator screen essentially encompasses drug formulation, attainment of acute toxicity data, hypertransfusion of mice with canine red blood cells, a 7-day treatment interval, daily collection of fecal and urinary pools, and iron analysis by atomic absorption and statistical evaluation of iron chelation efficacy. With each assay, the clinically effective Desferal is tested to provide a reference of relative potency of test compounds. At the end of the treatment period livers and spleens were dissected, blotted, weighed, homogenized, and then analyzed for iron. A gross pathological examination of major organs *in situ* was performed during necropsy.

RESULTS AND DISCUSSION

Synthesis and Characterization of β-Cyclodextrinsuccinate(N-methyl)hydroxamic Acid (III)

The procedure for synthesizing the hydroxamic acid derivative of β-cyclodextrin is shown in Fig. 1. β-Cyclodextrin was first converted to the succinate ester by reaction with succinic anhydride. Titration revealed the presence of 14 carboxyl groups per molecule for an average of 2 per glucose unit. The elemental



I. $R_1 = -COCH_2CH_2COOH$; II. $R_1 = -COCH_2CH_2CO$-N(succinimide)

III. $R_1 = -COCH_2CH_2C(=O)-N(OH)-CH_3$; if $R_2 = R_1$, then $R_3 = H$

or if $R_3 = R_1$, then $R = H$

FIG. 1. Synthetic scheme for converting β-cyclodextrin to the hydroxamic acid derivative.

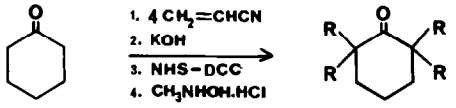
analysis agreed with this result. In order to determine how these 14 succinate esters were distributed among the 21 available hydroxyls of the β-cyclodextrin, a periodate oxidation was performed for the purpose of detecting the presence of vicinal hydroxyl groups.

In the case of the parent β-cyclodextrin, 7 eq of periodate was consumed as predicted on the basis of the seven glycol units. On the other hand, the succinic ester **I** failed to consume any periodate, which indicated the absence of vicinal hydroxyls. Thus, the esterification of β-cyclodextrin with succinic anhydride must have proceeded to cause substitution on at least one of the secondary hydroxyl groups of each glucose of the cyclodextrin. Since the primary hydroxyls are somewhat more reactive than the secondary hydroxyls, it seems reasonable to conclude that each glucose unit is substituted by two succinic esters, one on a primary hydroxyl and the other on one of the two secondary hydroxyl groups. The NMR spectrum was consistent with such a structure, but the lines for the ring protons, being broad and indistinct, were of little help in establishing the exact positions of the succinate esters.

The carboxyl groups were cleanly converted to the NHS esters by treatment with NHS and DCC. Reaction of this ester with methylhydroxylamine gave the hydroxamic acid derivative **III**. The NMR spectrum clearly revealed the $-CH_2-CH_2-$, the $N-CH_3$, and the N–OH of the succinate–hydroxamic acid side chains.

Synthesis and Characterization of 2,2,6,6-Tetra(propio-N-methylhydroxamic acid) Cyclohexanone

The scheme for the synthesis of the tetrahydroxamic acid derivative of cyclohexanone is shown in Fig. 2. Cyanoethylation of cyclohexanone proceeded readily to give the tetranitrile **IV** as reported (*13*). The fact that substitution had occurred at all four α positions was confirmed by the ^{13}C NMR spectrum which exhibited seven lines for the seven nonequivalent carbons of a tetra substituted product. The nitriles were hydrolyzed to the carboxylic acid and the acids were converted to the NHS esters by treatment with NHS and DCC.



IV. R = $-CH_2CH_2CN$; V. R = $-CH_2CH_2COOH$

VI. R = $-CH_2CH_2COON$; VII. R = $-CH_2CH_2\overset{\overset{O}{\|}}{C}-\overset{\overset{OH}{|}}{N}-CH_3$

FIG. 2. Synthesis of tetrahydroxamic acid **VII**.

Although the elemental analysis of the ester was not particularly good, the infrared spectrum clearly showed the two bands typical of NHS groups. Dicyclohexylurea, one of the products of the reaction, is often difficult to remove, even on repeated crystallizations, and thus, contamination from this source is suspected. Finally, reaction of the NHS ester with methylhydroxylamine gave the desired tetrahydroxamic acid **VII**.

The infrared, ^1H NMR, and ^{13}C NMR were all consistent with the proposed structure. In the infrared spectrum, bands for the hydroxyl and the two carbonyls were present. In the ^1H NMR spectrum, all protons were accounted for. In the ^{13}C NMR spectrum, lines for all eight carbons were present, two of which were in positions associated with carbonyl carbons. The elemental analysis was correct for a monohydrate. This additional molecule of water was also revealed by an increase in the water peak in the NMR spectrum of the sample, compared with the DMSO-d_6 solvent itself, as determined by integration.

Complexation of Hydroxamic Acids **III** *and* **VII** *with Iron*(3+)

Addition of iron(3+) perchlorate to aqueous solutions of the two hydroxamic acids produced the red-brown color characteristic of the iron(3+) complex. Mole ratio plots (Fig. 3) were prepared by plotting the absorbance at 450 nm against the Fe/HA ratio. In the case of **III**, the concentration of hydroxamic acid was calculated on the basis of 14 groups per molecule. In the case of **VII**, the concentration was based on 4 groups per molecule. Intersections at 0.30 and 0.33 for **III** and **VII**, respectively, are consistent with a normal 3 : 1 HA : Fe complex and are further confirmation that compound **III** possesses 14 hydroxamic acids and **VII**, 4 hydroxamic acids.

A preliminary evaluation of the stability constant of the iron(3+) complexes was carried out by measuring the competition between the hydroxamic acid and another ligand such as EDTA for iron(3+) (*15*). Only a few measurements at pH 2 were carried out, but the results indicated a log K of the order of 29–30, a clear indication that the hydroxamic acids were in sufficient proximity to exert a chelate effect. These values are of the same order of magnitude as reported for other iron : hydroxamic acids (*10, 15, 16*). Also, addition of a large excess of iron did not result in a conversion of the 3 : 1 HA : Fe complex to the 1 : 1 complex. Instead,

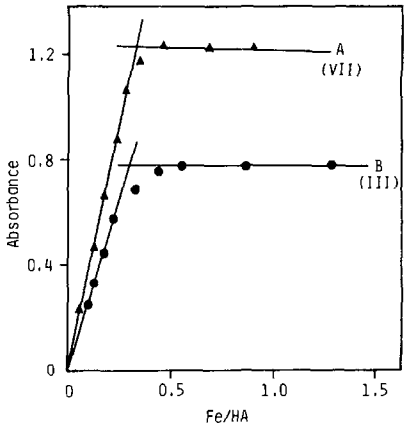


FIG. 3. Mole ratio plot, absorbance vs Fe^{3+}/HA ratio.

the λ_{max} shifted from the 450 nm (3 : 1 complex) to 465 nm characteristic of the 2 : 1 complex. The failure of the complex to be converted to a 1 : 1 complex is further indication of the presence of a chelate effect resulting from neighboring hydroxamic acids.

Bioassay Results

Results of the bioassay are given in Table 1 in terms of the average percentage increase (+) or decrease (−) in the iron level of spleen, liver, feces, and urine for the mice receiving the drug to the same data for control mice hypertransfused, but receiving no drug. The relative potency (P) of a test drug is determined by comparing the percentage iron changes for the drug with similar data obtained for DFB as follows.

$$P = \frac{(S_x + L_x + F_x + U_x)/\text{dose}_x}{S_{\text{Std}} + L_{\text{Std}} + F_{\text{Std}} + U_{\text{Std}}/\text{dose}_{\text{Std}}}$$

S,L,F, and U are the absolute values of the average percentage iron changes in spleen, liver, feces, and urine, respectively. Subscript x refers to the test drug and subscript Std to the standard drug, DFB. A more detailed description of the bioassay is given elsewhere (*6*).

TABLE 1

RESULTS OF BIOASSAY FOR IRON CHELATION ACTIVITY

Test compound	Dose (mg/kg)	Route	Number of survivors	% Iron changes vs control				Potency (P)
				Spleen	Liver	Feces	Urine	
DFB	250	ip	130/130	± 2	−28	±3	+234	1.0
III	500	ip	7/7	+53	+10	−2	+ 24	0.1
VII	500	ip	10/10	+ 8	+13	−4	+ 56	0.1

Both compounds **III** and **VII** exhibited lower activity than the standard desferrioxamine-B. The compounds showed little toxicity at the dose levels used (500 mg/kg body wt) which supports the earlier observations that in general hydroxamic acids are nontoxic.

Potential Use of the β-Cyclodextrin Hydroxamic Acid Derivative as a Catalyst for Ester Hydrolysis

Gruhn and Bender (*14*) have observed that a monohydroxamic acid derivative of α-cyclodextrin exhibited catalytic activity in the hydrolysis of certain esters. It is possible that our 14-hydroxamic acid substituted β-cyclodextrin derivative could exhibit similar behavior.

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