Biomimetic Synthesis of a *Paracoccus denitrificans* Siderophore Analogue

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A biomimetic approach to the synthesis of N^4-acylated N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine siderophores is described. The key to the sequence is the use of Cu(II) as a transitory protecting group for the catechols of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine in the condensation of this synthon with the model acylating agent *N*-(2-hydroxybenzoyl)glycine. An alternative route to this siderophore analogue employing classical methods is offered as proof of structure. Preliminary investigations aimed at characterizing the chelate of interest are also described. Proton NMR paramagnetic line broadening studies confirm the catechols of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine to be the chelating functionality. The stoichiometry of the complex is determined by spectrophotometric methods while the cumulative formation constants of the various protonated species are established by potentiometric techniques.

In recent years, a great deal of effort has been focused on the isolation, identification, and synthesis of iron chelators.[1-5] The reason for this effort is closely related to the lack of a satisfactory therapeutic iron chelator for the treatment of various iron-overload syndromes.[6,7] Synthetic sequestering agents have largely been modeled after catechol-[5,8,9] or hydroxamate-containing[10] natural products.

Our interest in polyamines[11] led us to investigate the synthesis of the catecholamides, spermidine siderophores first isolated from *Paracoccus denitrificans* and later from *Agrobacterium tumefaciens*.[1] These compounds are characterized by 2,3-dihydroxybenzoyl moieties fixed to the terminal N^1,N^8-positions of spermidine. They differ in the nature of the substituent at the N^4-position of spermidine; e.g., parabactin[1,12] incorporates an oxazoline derivative of *N*-(2-hydroxybenzoyl)threonine while agrobactin has an oxazoline derivative of *N*-(2,3-dihydroxybenzoyl)threonine at the N^4-position.

In a previous study,[13] we demonstrated N^4-benzylspermidine to be an ideal reagent for selective N^1,N^8-terminal acylations of spermidine. Although in this paper we will focus on the N^4-benzylspermidine system, the sequences described as proof of structure can be carried out on N^5-benzylhomospermidine and *N*,*N*-bis(3-aminopropyl)benzylamine as well.[14] With N^4-benzylspermidine and the acylating agents 2,3-methylenedioxybenzoyl chloride and 2,3-diacetoxybenzoyl chloride, respectively, we were able to prepare N^1,N^8-bis[2,3-(methylenedioxy)benzoyl]spermidine (I) and N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine (II),[9] the biochemical precursor to the *Paracoccus denitrificans* siderophore, in good yield. Both compounds I and II were generated in approximately equal yields based on their respective acids. In an earlier paper[13] we mentioned in a footnote that we had managed to N^4-acylate these compounds. We will now report on the details of these experiments.

Although both compounds I and II each provide a route to N^4-acylated N^1,N^8-bis(acylated)spermidine derivatives, the choice of intermediate weighs heavily on the nature of the N^4 substituent desired. In the first instance, if a synthon is fixed to the N^4-position of N^1,N^8-bis[2,3-(methylenedioxy)benzoyl]spermidine, the methylenedioxy protecting group must still be removed. Removal of this group requires fairly strong Lewis acids, e.g., BBr_3, BCl_3, or HI, which sets some limits on the nature of the synthon appended to the N^4-position. As most of the acyl groups we ultimately want to append to the N^4-position will be acid labile, such a sequence is simply not feasible. On the other hand, while N^4-acylation of compound I is relatively simple, the free hydroxyls of compound II complicate matters by introducing the probability of polyacylation. This paper describes a biomimetic approach which effectively surmounts this problem, thus allowing incorporation of the acid-labile substituents at the N^4-position as required in the synthesis of the siderophores of interest.

In his original investigation, Tait demonstrated that *Paracoccus denitrificans* uses compound II in its biosynthesis of *N*-[3-(2,3-dihydroxybenzamido)propyl]-*N*-[4-(2,3-dihydroxybenzamido)butyl]-2-(hydroxyphenyl)-5-methyloxazoline-4-carboxamide (III); i.e., when ^{14}C-labeled N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine was fed to the microorganism, ^{14}C-labeled compound III was obtained.[1] This implies that in vivo, the catechol hydroxyls are in some way protected from acylation. This "effective" protection could be accomplished by hydroxyl acylation, chelation, or simple enzymatic selectivity.

In attempting to mimic such a reaction, we decided to consider the possibility of protecting the catechol hydroxyls by chelating them with Cu(II). Copper is well-known to form strong tetracoordinate complexes with soft anions, the strength of the chelate being directly dependent upon pH. At high pH, with the chelate in the form of the polyanion, the copper complex should be extremely stable. Furthermore, CPK space-filling models reveal that in such a complex the secondary nitrogen would be easily accessible to an acylating agent, certainly more accessible than in the parent compound itself. A characteristic of these complexes we planned to exploit is that as the pH is lowered, their stability is diminished, thus providing a vehicle

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(2) R. C. Hider, J. Silver, J. B. Neilands, I. E. G. Morrison, and L. V. C. Rees, *FEBS Lett.*, **102**, 325 (1979).
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(11) R. J. Bergeron, P. S. Burton, K. A. McGovern, and S. J. Kline, *Synthesis*, in press.
(12) J. B. Neilands, S. A. Ong, and T. Peterson, *J. Biol. Chem.*, **254**, 1860 (1979).
(13) R. J. Bergeron, K. A. McGovern, M. A. Channing, and P. S. Burton, *J. Org. Chem.*, **45**, 1589 (1980).
(14) R. J. Bergeron, S. J. Kline, N. J. Stolowich, K. A. McGovern, and P. S. Burton, submitted for publication in *J. Org. Chem.*

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Scheme I

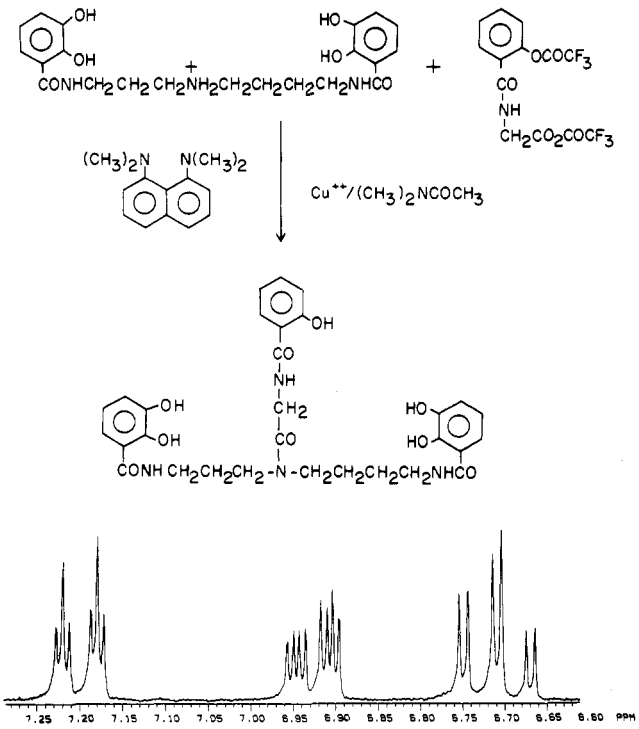


Figure 1. Aromatic region of the 220-MHz spectrum of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine in methanol-d_4.

for removing the copper once having acylated the N^4 nitrogen.

In order to evaluate the feasibility of this approach, we decided to synthesize N^4-[*N*-(2-hydroxybenzoyl)glycyl]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine. The acylating agent required for this coupling model, *N*-(2-hydroxybenzoyl)glycine, has structural components which are common to all potential N^4-acylating agents of interest to us. In this paper, we demonstrate that compound II is a potent Cu(II) chelator and, furthermore, that this chelation can be used to selectively acylate the N^4 nitrogen. The overall scheme involved (1) proving that the ligand could bind copper and determining the stoichiometry of the resulting complex, (2) showing that the chelation sites involved the phenolic hydroxyls, (3) actual coupling of the N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine with *N*-(2-hydroxybenzoyl)glycine, as shown in Scheme I, and (4) an alternate synthesis of the final product from compound I as proof of structure (Scheme II).

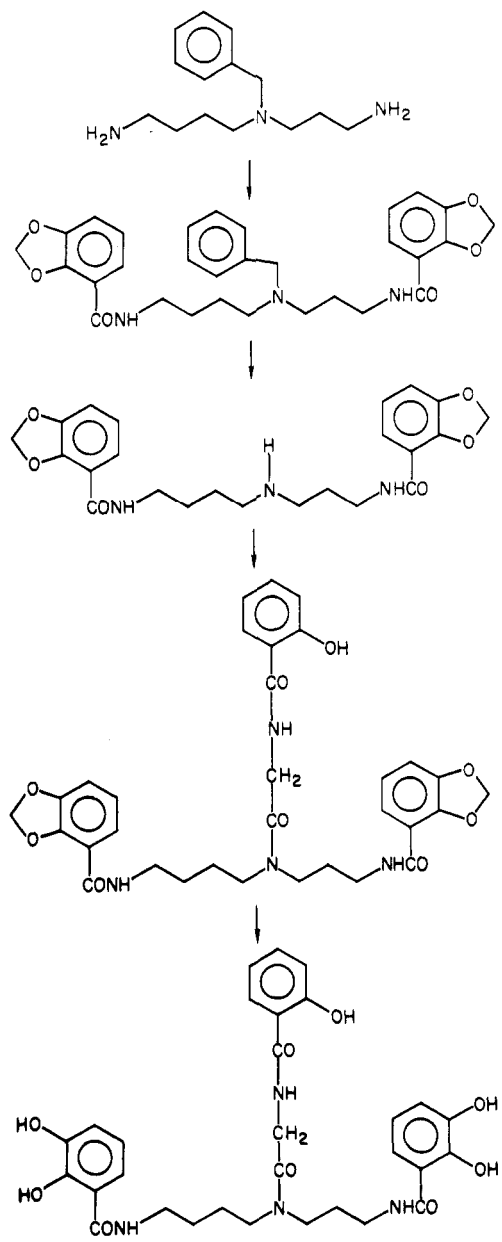
Experimental Section

Sample Preparation for High-Field ^{1}H NMR Experiments of N^1,N^8-Bis(2,3-dihydroxybenzoyl)spermidine. ^{1}H NMR spectra of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine trifluoroacetate salt were recorded in CD_3OD (see Figure 1) on a Nicolet NT 200 spectrometer. All proton assignments were made by using this instrument.

For the copper-induced, line-broadening experiments, a solution 0.05 M in N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine trifluoroacetate salt and 0.2 M in sodium methoxide was prepared in CD_3OD, with methylene chloride (0.04 M) as an internal line-width standard. The sample was titrated with a 5×10^{-3} M stock solution of $Cu(NO_3)_2$ in CD_3OD. Aliquots were added via a microliter syringe directly into the NMR tube. Eight spectra were recorded on a JEOL FX-100 spectrometer, the ligand to metal ratio varying from 500 to 36.

Potentiometric Titrations. All solutions were prepared by using doubly distilled, degassed water and were stored under N_2 which had been passed through a basic solution of pyrogallol. The NaOH solutions were standardized by titration of potassium

Scheme II



hydrogen phthalate to a phenolphthalein end point. Stock copper solutions were prepared by using $Cu(NO_3)_2 \cdot 6H_2O$ and the resulting metal concentrations determined by flame photometry on a Perkin-Elmer Model 290B atomic absorption photometer.

Potentiometric measurements were made in a glass-jacketed titration vessel connected to a constant-temperature circulating water bath. The temperature was maintained at 25.0 ± 0.5 °C. Typically, 20-mL solutions were prepared by appropriate dilutions of a stock solution of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine trifluoroacetate over a concentration range of $(4–8) \times 10^{-4}$ M. Because of the susceptibility of the compound to oxidation when in basic solution, all titrations were conducted under a N_2 atmosphere, the N_2 having again been passed through a basic pyrogallol solution to remove all traces of oxygen. Copper concentrations were adjusted to give a final ligand to metal ratio of 8 in all titrations. The ionic strength of all solutions was adjusted to 0.1 M by addition of KNO_3.

All pH measurements were taken with a Radiometer PHM-84 digital pH meter equipped with a Radiometer combination electrode. Meter calibrations were checked immediately after each titration. A minimum of 14 data points were obtained for each titration, readings being taken after the stirring motor had been turned off and the solutions allowed to equilibrate.

All potentiometric data was evaluated by using the computer program PHFIT.[15] As written, this program handles as many as

4 fundamental and 15 complex species. Initial input includes the composition of all complexes postulated to be of significance, actual values of their cumulative formation constants in logarithmic form when available, and initial estimates of those to be defined as well as the initial concentration of each species. The activity coefficient of H^+ under the experimental conditions employed is also required; this quantity was assigned a value of 0.78 throughout.

The program first calculates, by way of a standard Newton–Raphson iteration, the distribution of species at each data point based on the total concentration of species at that point and the given formation constants for each adduct. Thus, an initial theroetical titration curve is obtained; assuming all significant equilibria have been considered, the difference between this calculated curve and that actually observed is then minimized by further refinement of the estimated formation constants. Refinement is continued until either a given number of cycles have been completed or the fit is improved by less than 0.5%; the final values of these refined overall formation constants along with estimates of their uncertainty are then output.

For convenience, stepwise rather than cumulative formation constants were considered in evaluation of the pK_a titration data; thus, the output uncertainty in this instance refers to the stepwise protonation constants. This interconversion of stepwise and cumulative formation constants was easily handled by means of a simple subroutine.

The data set generated by each titration was evaluated independently of all others. The values obtained for each formation constant were averaged; the errors in these averages were estimated as the square root of the sum of the squared estimated standard deviations associated with each constant divided by the number of constants averaged.

Spectrophotometric Determination of Stoichiometry. Stock solutions of $Cu(NO_3)_2 \cdot 6H_2O$ (9.44×10^{-5} M) and N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine trifluoroacetate (1.05×10^{-4} M) were prepared in 0.1 M KNO_3 as described for the potentiometric titrations. The pH of each was adjusted to 7.6 with NaOH, and a series of solutions was prepared varying in both ligand and metal concentrations while the total number of moles was held constant. A second series containing ligand alone was prepared similarly. Ultraviolet spectra of these solutions were recorded on a Beckman Model 25 UV–vis spectrophotometer.

Synthetic Materials and Methods. All reagents were purchased from Aldrich. Methylene chloride and *N,N*-dimethylacetamide (DMA) were distilled and stored over 3- or 4-Å molecular sieves. Unless specified otherwise, Na_2SO_4 was used as a drying agent. Preparative thin-layer chromatography was done on 20 × 20 cm silica gel plates obtained from Analtech. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. Samples for ^1H NMR were prepared in $DCCl_3$ with chemical shifts given in parts per million relative to an internal Me_4Si standard unless stated otherwise. These spectra were recorded on a Varian T-60 and/or a JEOL FX-100 spectrometer. Samples for IR spectra were prepared in KBr unless stated otherwise, the spectra being recorded on a Beckman IR 4210 spectrophotometer. Results are given in reciprocal centimeters. Elemental analyses were performed by Galbraith Laboratories.

N^4-Benzyl-N^1,N^8-bis[2,3-(methylenedioxy)benzoyl]spermidine Hydrochloride. A solution of N^4-benzylspermidine (0.396 g, 1.68 mmol) and 1,8-bis(dimethylamino)naphthalene (Alder's base; 0.750 g, 3.50 mmol) in 150 mL of CH_2Cl_2 was cooled to 0 °C under N_2. Dropwise addition of 2,3-(methylenedioxy)benzoyl chloride[16] (0.630 g, 3.41 mmol) in 100 mL of CH_2Cl_2 was completed over a 1-h period, and the reaction mixture was allowed to warm slowly to room temperature. After 16 h, the reaction mixture was cooled to 0 °C, washed with ice-cold 3% (w/v) aqueous HCl (3 × 10 mL) and ice-cold water (3 × 10 mL), dried, filtered, and evaporated. The product was dissolved in a minimum amount of CH_2Cl_2 and precipitated with several volumes of Et_2O, yielding 0.902 g (94% crude yield) of the desired product, a white, hygroscopic solid.

(15) D. Leussing, private communications.
(16) Prepared by the method of W. H. Perkin and V. M. Trikojus, *J. Chem. Soc.*, 2925 (1926).

An analytical sample was dissolved in methanol and NaOMe added to a pH of 11. After being stirred 30 min, the mixture was reduced in vacuo. The residue was taken up in CH_2Cl_2, washed with water, dried, filtered, and evaporated. Purification of the resulting amine on silica gel, eluting with 8% MeOH/CH_2Cl_2 (v/v), afforded a brown oil: NMR δ 1.30–2.00 (m, 6 H), 2.20–2.73 (m, 4 H), 3.10–3.68 (m, 4 H), 3.82 (s, 2 H), 5.78 (s, 2 H), 5.85 (s, 2 H), 6.57–7.63 (m, 13 H); IR ($CHCl_3$) 3420, 2960, 1645, 1443, 1050.

Anal. Calcd for $C_{30}H_{33}N_3O_6$: C, 67.78; H, 6.28; N, 7.90. Found: C, 67.70; H, 6.25; N, 7.78.

N^1,N^8-Bis[2,3-(methylenedioxy)benzoyl]spermidine (I). A solution of N^4-benzyl-N^1,N^8-bis[2,3-(methylenedioxy)benzoyl]spermidine hydrochloride (0.867 g, 1.53 mmol) in 45 mL of glacial acetic acid was prepared and $PdCl_2$ (0.103 g, 0.581 mmol) added. The reaction was allowed to proceed at room temperature until hydrogen was no longer taken up. The mixture was then filtered and evaporated, and the residue was dissolved in 35 mL of absolute MeOH. This solution was adjusted to pH 11 with NaOMe and evaporated. The resulting solid was taken up in 25 mL of CH_2Cl_2, washed with cold water (2 × 25 mL), dried, filtered, and evaporated to give 0.660 g (98% crude yield) of product, a brown oil.

An analytical sample was purified on silica gel, eluting with 3% $NH_4OH/CHCl_3$: NMR δ 1.40–2.02 (m, 7 H), 2.42–2.90 (m, 4 H), 3.20–3.78 (m, 4 H), 5.92 (s, 4 H), 6.60–7.68 (m, 8 H); IR ($CHCl_3$) 3420, 2940, 1650, 1520, 1270.

Anal. Calcd for $C_{23}H_{27}N_3O_6 \cdot H_2O$; C, 60.25; H, 6.16; N, 9.17. Found: C, 59.94; H, 5.96; N, 9.15.

N^4-[*N*-(2-Hydroxybenzoyl)glycyl]-N^1,N^8-bis[2,3-(methylenedioxy)benzoyl]spermidine. Trifluoroacetic anhydride (4.46 g, 21.24 mmol) was added to a suspension of *N*-(2-hydroxybenzoyl)glycine (0.402 g, 2.06 mmol) in 10 mL of CH_2Cl_2, and the resulting mixture was refluxed at 45 °C for 2 h. The solution was evaporated in vacuo and the *N*-[2-(trifluoroacetoxy)benzoyl]-glycyltrifluoroacetic anhydride redissolved in 15 mL of CH_2Cl_2. After the mixture was cooled to −78 °C, 1,8-bis(dimethylamino)naphthalene (0.634 g, 2.96 mmol) in 10 mL of CH_2Cl_2 was added, followed by the dropwise addition of N^1,N^8-bis[2,3-(methylenedioxy)benzoyl]spermidine (0.653 g, 1.48 mmol) in 30 mL of CH_2Cl_2. The reaction mixture was allowed to warm to room temperature under N_2. After 45 h, the solution was washed with cold 3% (w/v) aqueous HCl, dried, filtered and evaporated. The residue was then dissolved in methanol and the pH adjusted to 9 by the addition of NaOMe. After the solution was stirred under N_2 for 30 min, methanolic HCl was added at 0 °C to a pH of approximately 3, and the resulting solution was filtered and evaporated. The residue was chromatographed on silica gel, eluting with 5% MeOH/EtOAc (v/v). This purification procedure resulted in 816 mg (89% yield) of the product as a white solid: NMR 1.30–2.30 (m, 6 H), 3.42 (m, 8 H), 4.17 (s, 2 H), 6.00 (s, 4 H), 6.53–7.77 (m, 13 H), 11.99 (s, 1 H); IR 3420, 1640, 1245, 1060, 750.

Anal. Calcd for $C_{32}H_{34}N_4O_9$: C, 62.13; H, 5.54; N, 8.96. Found: C, 61.95; H, 5.59; N, 8.90.

N^4-[*N*-(2-Hydroxybenzoyl)glycyl]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine. To a solution of N^4-[*N*-(2-hydroxybenzoyl)glycyl]-N^1,N^8-bis[2,3-(methylenedioxy)-benzoyl]spermidine (0.230 g, 0.370 mmol) in 20 mL of CH_2Cl_2 was added BBr_3 (0.50 mL, 5.29 mmol) dropwise under N_2 at 0 °C. The mixture was allowed to warm slowly to room temperature. After 22 h, 20 mL of H_2O was added dropwise with vigorous stirring. After the mixture was stirred an additional 2 h, the crude product was collected by filtration, washed thoroughly with water, and dissolved in MeOH, and the solvent was evaporated several times to yield 0.210 g (95% crude yield) of the desired product as a white solid.

An analytical sample was preadsorbed on Sephadex LH-20 and eluted with an ethanol/benzene gradient (5–50% v/v): NMR (CD_2Cl_2), δ 1.32–3.28 (m, 6 H), 3.04–3.72 (m, 8 H), 4.22 (s, 2 H), 6.48–8.12 (m, 15 H), 12.10 (s, 1 H), 12.74 (s, 1 H), 13.09 (s, 1 H); IR 3370, 1635, 1535, 1270, 790.

Anal. Calcd for $C_{30}H_{34}N_4O_9 \cdot 2H_2O$: C, 57.14; H, 6.07; N, 8.88. Found: C, 57.20; H, 5.67; N, 8.77.

N^4-[*N*-(2-Hydroxybenzoyl)glycyl]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine: Biomimetic Synthesis. Trifluoroacetic anhydride (0.300 g, 1.5 mmol) was added with stirring

to a suspension of *N*-(2-hydroxybenzoyl)glycine (0.130 g, 0.66 mmol) in 15 mL of dry CH_2Cl_2. The resulting mixture was allowed to stir at room temperature under N_2 for 0.5 h. The solution was then evaporated to dryness under high vacuum, and the *N*-[2-(trifluoroacetoxy)benzoyl]glycyltrifluoroacetic anhydride residue was redissolved in 25 mL of DMA. The resulting solution was cooled to −10 °C, and 1,8-bis(dimethylamino)naphthalene (0.240 g, 1.12 mmol) in 5 mL of DMA was added. Immediately, a solution of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine hydrochloride (0.252 g, 0.55 mmol), $CuSO_4$ (0.260 g, 1.65 mmol), and 1,8-bis-(dimethylamino)naphthalene (0.470 g, 2.2 mmol) in 10 mL of DMA was added dropwise. The reaction mixture was allowed to warm to room temperature with continued stirring under N_2. After 10 h, the reaction was stopped by the addition of 20 mL of pH 5 phosphate buffer. The resulting yellow solution was extracted with CH_2Cl_2 (10 × 50 mL). The combined extracts were washed with pH 5 phosphate buffer (5 × 25 mL) and H_2O (12 × 25 mL), dried, and passed through a 2 × 1.5 cm column of silica gel, and the solvent was removed in vacuo. The resulting brown gum was dissolved in 25 mL of degassed MeOH, and $NaOCH_3$ was added to a pH of approximately 9. After the mixture was stirred 0.5 h, the pH was adjusted to 2 by addition of methanolic HCl, and the solvent was removed in vacuo. The gummy solid remaining was absorbed onto Sephadex LH-20, applied to a 3 × 10 cm column of Sephadex LH-20, and eluted with 15% ethanol/benzene (v/v). The elution was followed by analytical thin-layer chromatography and the solvent removed, yielding 228 mg (70% yield) of the desired compound; its NMR and TLC properties were identical with those of the compound prepared in the classical way.

Results and Discussion

We recognize the fact that the line broadening, the binding study, and the actual N^4-acylation were done in three different solvent systems. The purpose of the potentiometric and broadening studies was simply to show that the chelate–metal interaction was a very strong one and that it could be expected to occur in DMA. The extrapolation was clearly borne out by the synthesis.

We chose to look at copper chelates of compound II for two reasons: (1) Cu(II) is known to form strong tetracoordinate complexes with a variety of ligands in both aqueous and organic solvents, e.g., aminocyclitol antibiotics,[17] epinephrine,[18] and EDTA,[19] and (2) it is an excellent paramagnetic probe. It was clear that if the Cu(II)/compound II complex was formed through the catechols of compound II as expected, line broadening of the aromatic multiplet would be observed. However, before initiation of the line-broadening study, a ^{1}H NMR assignment study was done on compound II.

Compound II High-Field ^{1}H NMR Spectral Assignments. In order to simplify the ^{1}H NMR spectra of compound II we decided to exchange the phenolic, amido, and amino protons with deuterium by taking all spectra in CD_3OD. This solvent does not present the problems incurred by other workers using solvents such as Me_2SO-d_6 for ^{1}H NMR spectra of these compounds.[20] Because of the viscosity of Me_2SO, the rotational correlation time of a given compound is longer in this solvent than in methanol. These long correlation times shorten the compound's T_1's, and therefore the spectra exhibit broad lines.

The aromatic protons in the range δ 6.65–7.25 consist of three separate sets of signals, demonstrating nonequivalence in the aromatic rings (Figure 1). The two

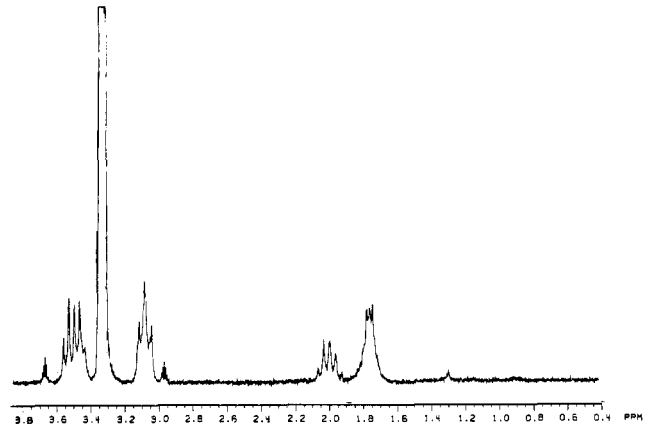


Figure 2. High-field region of the 220-MHz spectrum of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine in methanol-d_4.

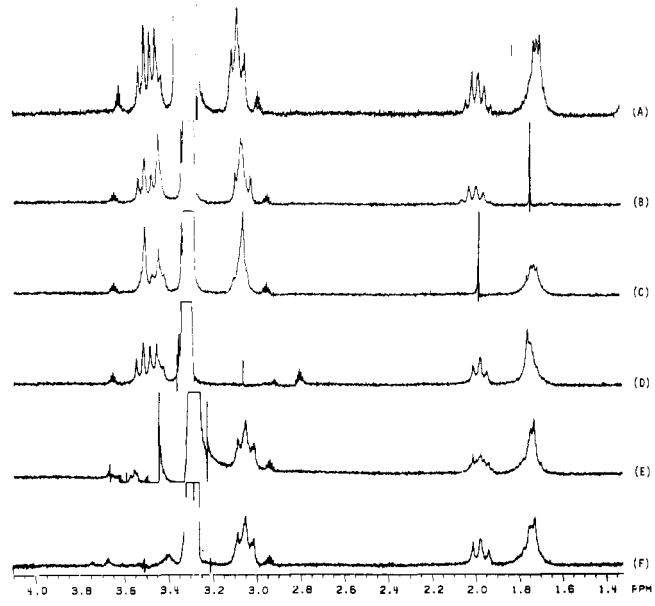


Figure 3. High-field regions of a series of 220-MHz spectra of the same solution used to generate the spectrum shown in Figure 2 (while the difference in scale between Figure 2 and the spectra indicated should be noted, the changes in line shapes upon irradiation at various frequencies are clear); each spectrum (A–E) corresponds to irradiation at a different frequency (see text).

apparent downfield triplets centered at δ 6.70 and 6.72, respectively, integrate to two protons and can be assigned as two nonequivalent meta protons. The meta protons are split by the para protons and again by the ortho protons. These doublets of doublets when superimposed appear as a triplet. Further downfield there are two sets of four lines which correspond to the two protons assignable as the para protons. These protons are split by the meta and again by the ortho protons, resulting in two nonequivalent doublets of doublets. Finally, what appears to be two triplets integrating for two protons can be assigned to the ortho protons. The ortho protons are split by the meta and by the para protons as well. This splitting would be expected to result in two sets of four lines as with the para protons. However, the chemical shift nonequivalence differences between these two sets of protons results in an overlap, producing two apparent triplets.

The remainder of the spectra can be divided into four sets of signals (Figure 2). The high-field set centered at δ 1.75 (four protons) corresponds to the two internal methylenes in the four-methylene bridge of spermidine. What appears to be a downfield quintet (two protons) centered at δ 1.98 corresponds to the internal methylene of the short

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(18) W. G. Levine, Ed., "The Chelation of Heavy Metals", Pergamon Press, Elmsford, NY, 1979.
(19) J. Kragten, "Atlas of Metal–Ligand Equilibria in Aqueous Solution", Ellis Harwood Ltd., Sussex, 1978.
(20) K. K. Bhargara, R. W. Grady, and A. Cerami, *J. Pharm. Sci.*, **69**, 986 (1980).

three-methylene bridge. Irradiation of the high-field signal, δ 1.75 (Figure 3B), has no effect on the δ 1.98 quintet but causes a rather substantial change in the downfield line shapes. The furthest downfield multiplet (δ 3.4–3.55, four protons corresponding to the amide methylenes) reduces from a five- to a four-line signal This decoupling reduces an apparent triplet on the long methylene bridge side to a singlet, leaving the short methylene bridge amino methylene as a triplet centered at δ 3.5. Irradiating the central methylene of the short bridge (Figure 3C) leaves the long-bridge amido methylene as a triplet and reduces the short-bridge amido methylene triplet to a singlet as expected.

Although both irradiations change the line shape of the amino methylenes, analysis of these changes is made difficult by poor resolution of this signal. Irradiation of the amino methylene (Figure 3D) reduces the short-bridge internal methylene quintet to a triplet. However, the long-bridge internal methylenes' signal analysis is also made difficult by lack of resolution. Irradiation of the α-amido protons (Figure 3E) produced identical changes in the line shapes of the short-bridge internal methylenes but slightly different changes in the long-bridge internal methylenes.

Perhaps the most interesting feature of this spectrum is the nonequivalence of the aromatic protons. This nonequivalence is unlikely to have come from the simple asymmetry of the methylene bridges but rather from some preferred conformations. Further NMR studies are currently underway to evaluate contributions from the various solution conformers.

Compound II High-Field ^{1}H NMR Spectra: Copper-Induced Line Broadening. Metal ion probes have been used extensively in the study of coordination complexation.[21-23] Since paramagnetic relaxation is generally quite efficient, it will be the predominant relaxation mechanism for those nuclei within the metal's sphere of influence. Metal probes such as copper, having long electron relaxation times, cause substantial broadening for those nuclei close to the metal.

Preliminary studies of the N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine/Cu(II) complex in aqueous solution revealed tight binding. For verification that the site of chelation involved the catechol hydroxyls, a series of ^{1}H NMR spectra of compound II were run in CD_3OD with varying concentrations of $Cu(NO_3)_2$. Increased broadening of the N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine resonances was observed as the copper concentration was increased.

Interpretation of the broadening data in a quantitative way was not attempted. As a qualitative indicator of the site of coordination the observed line widths were adequate. Since paramagnetic effects are proportional to r^{-6}, the metal's sphere of influence is small, and therefore only protons very close to the site of association will be affected.

In the case of compound II, the aromatic protons rapidly broadened with increasing copper to chelate concentration ratios. At a ligand to metal ratio of 150, the lines collapsed into the base line, rendering line-width measurements difficult. As expected, the internal methylene resonances were observed to broaden at high copper concentrations. Furthermore, when the solution at high metal to ligand ratios was acidified, the original spectrum of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine emerged, indicating the complex had dissociated.

In order to further substantiate that the catechols were involved in copper chelation, we examined a model system. N^1,N^8-Dibenzoylspermidine[13] is a structural analogue of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine lacking the phenolic hydroxyls. When a solution of this model compound in CD_3OD was titrated with $Cu(NO_3)_2$ from a ligand to metal ratio of 200 to 8, no aromatic line broadening was observed. However, the internal methylenes exhibited approximately 1.5-Hz broadening at high metal concentrations. This may be rationalized in terms of some weak interaction between the secondary nitrogen of the spermidine backbone and Cu(II) in solution. Even when the metal concentration exceeded that of the substrate, no observable aromatic line broadening occurred. It seemed clear, then, that chelation of Cu(II) in solution by N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine was occurring at the phenolic oxygens.

On the basis of these results, it was concluded that in the presence of Cu(II) and base, the phenolic oxygens of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine would be protected but the N^4 nitrogen would be free to react. These line-broadening experiments further indicated that the complex could be broken up simply by protonating the phenolic oxanions.

Potentiometric Titrations. Preliminary evaluation of data resulting from titration of the ligand alone indicated that of the ligand's five potentially dissociable protons only two were being titrated within the pH range of the experiments. The experimental curve was best fit by considering the step-wise equilibria shown in eq 1–5, holding

$$L + H \rightleftarrows HL \quad (1)$$

$$HL + H \rightleftarrows H_2L \quad (2)$$

$$H_2L + H \rightleftarrows H_3L \quad (3)$$

$$H_3L + H \rightleftarrows H_4L \quad (4)$$

$$H_4L + H \rightleftarrows H_5L \quad (5)$$

the equilibrium constants of reactions 1–3 at arbitrary, high values of 14.0 each. With the value of pK_w set at 13.787 as calculated from the 17-parameter empirical expression of Sweeton, Mesmer, and Baes for an aqueous solution 0.1 M in KCl at 25 °C,[24] the values of the equilibrium constants for eq 4 and 5 were refined by the program PHFIT as previously described. The average values thus obtained over seven titrations were 6.60 ± 0.08 (pK_{a1}) and 7.80 ± 0.15 (pK_{a2}), corresponding to logarithmic cumulative protonation constants of 56.40 and 49.80, respectively.

For each titration, the calculated curves based on these constants correlated well with those observed. In virtually every case, the greatest deviations between the two occurred in unbuffered regions of the curve, as might be expected, and in the highest pH region where electrode sensitivity is reduced. In the latter case, deviations could not be attributed to the titration of a third proton as such deviations tended to be positive; i.e., the observed pH tended to be higher than that calculated.

The titration of ligand in the presence of copper, of course, introduced a number of species in addition to protonated ligand. Obviously, the hydrolysis of the metal had to be accounted for. Thus, the equilibria shown in eq 6–10 were included. However, evaluation of the experi-

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$$H_2O + M \rightleftarrows M(OH) + H \quad (6)$$

$$2H_2O + M \rightleftarrows M(OH)_2 + 2H \quad (7)$$

$$2H_2O + 2M \rightleftarrows M_2(OH)_2 + 2H \quad (8)$$

$$3H_2O + M \rightleftarrows M(OH)_3 + 3H \quad (9)$$

$$4H_2O + M \rightleftarrows M(OH)_4 + 4H \quad (10)$$

mental data based on these ten equilibria alone produced no reasonable correlation, implying that ligand/metal binding was, in fact, being observed as expected. Subsequently, consideration of the ligand/metal ratio involved in these titrations, coupled with an estimation of the lowered protonation constants of bound ligand, suggested the formation of some or all of the species shown in eq 11–14.

$$L + M \rightleftarrows LM \quad (11)$$

$$L + H + M \rightleftarrows HLM \quad (12)$$

$$L + 2H + M \rightleftarrows H_2LM \quad (13)$$

$$L + 3H + M \rightleftarrows H_3LM \quad (14)$$

The logarithms of the equilibrium constants for reactions 1–3 were set at 14.0 and those for reactions 4 and 5 at their average values as previously ascertained, and the five copper hydrolysis constants were set at their appropriate literature values[19] of −8.2, −17.5, −10.6, −27.8, and −39.1 for equilibria 6–10, respectively; the same calculated value for pK_w was used as described previously. On the basis of these quantities, the cumulative formation constants for the various ligand/metal complexes were refined, and, considering the number of invariant constants imposed and the potential of any one as a source of error, a satisfactory fit was eventually obtained.

Analysis in this manner indicated that inclusion of all four of the ligand/metal species gave a best fit to the observed titration curve. However, only three of the five hydrolyzed metal species proved significant; omission of equilibria 9 and 10 from the calculations affected neither the fit nor the refined values of the formation constants significantly. On the basis of this analysis, the resulting refined cumulative binding constants averaged over three titrations are, in logarithmic form, 30.19 ± 0.66, 39.85 ± 0.51, 45.54 ± 0.36, and 49.1 ± 5.7 for equilibria 11–14, respectively. The relatively high error associated with the last of these values is attributed to the fact that at the initial pH this species is already of little importance. In fact, when this species was omitted completely from the analysis, the correlation between the calculated and the observed titration curves was only mildly affected. Deviations in the initial portions of the curves increased slightly while the values of the formation constants for eq 11–13 were essentially unchanged.

Spectrophotometric Determination of Stoichiometry. In order to verify the validity of the complex species assumed in the analysis of the potentiometric titration data, we experimentally established the stoichiometry of the copper/ligand complex by means of Job's plots.[25]

The ultraviolet spectrum of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine trifluoroacetate salt at pH 7.6 (I = 0.1) exhibits a maximum at 314 nm. Upon complexation with copper, this maximum shifts to 339 nm. A plot of the difference in absorbance at 337 nm between the ligand alone and in the presence of copper against the mole fraction of ligand revealed that the complex was in fact 1:1 as assumed.

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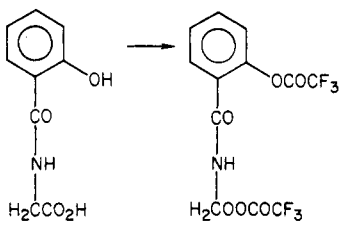
Scheme III



Coupling Reactions. The coupling of *N*-(2-hydroxybenzoyl)glycine with compounds I and II involves two problems: protection of the 2-hydroxyl group and activation of the carboxyl group. If the phenolic hydroxyl is not first protected, activation of the carboxyl group is likely to result in self-condensation of the *N*-(2-hydroxybenzoyl)glycine. For purposes of synthetic economy, we decided to both activate the carboxyl and protect the hydroxyl group in one step by forming the *N*-[2-(trifluoroacetoxy)benzoyl]glycyltrifluoroacetic anhydride (Scheme III). Another advantage to this trifluoroacyl derivative is that once the coupling reaction was completed, the trifluoroacetate group protecting the phenolic hydroxyl could be removed under very mild conditions.

In each case, the reaction was carried out by stirring *N*-(2-hydroxybenzoyl)glycine with an excess of trifluoroacetic anhydride in methylene chloride. Once the activated intermediate was formed, the methylene chloride and remaining trifluoroacetic anhydride were removed under vacuum, the residue was redissolved in either dimethylacetamide or methylene chloride, and the appropriate spermidine derivative was added in the presence of 1,8-bis(dimethylamino)naphthalene. Dimethylacetamide was used for the copper chelate sequence while methylene chloride was used in the alternate synthesis.

Proof of Structure Synthesis. It was decided to make the target compound by an alternate route for two reasons: (1) as a proof of structure and (2) as a means of testing our coupling method. The synthetic scheme for the alternate synthesis is fairly straightforward. The starting reagent N^4-benzylspermidine is acylated with 2,3-(methylenedioxy)benzoyl chloride in methylene chloride in the presence of 1,8-bis(dimethylamino)naphthalene. The resulting N^4-benzyl-N^1,N^8-bis[2,3-(methylenedioxy)-benzoyl]spermidine was debenzylated by hydrogenolysis at atmospheric pressure in acetic acid over $PdCl_2$. Both the acylation and the hydrogenolysis were nearly quantitative. The debenzylated product, N^1,N^8-bis[2,3-(methylenedioxy)benzoyl]spermidine (I), was next coupled with *N*-(2-hydroxybenzoyl)glycine. The activated and protected acid, *N*-[2-(trifluoroacetoxy)benzoyl]glycyltrifluoroacetic anhydride, was reacted with compound I in methylene chloride for 45 h. For removal of the trifluoroacetate protecting group, the solvents were evaporated, the mixture was taken up in methanol, and the solution was adjusted to pH 9 with NaOMe. After 30 min under nitrogen, the solution was acidified to pH 3 to generate N^4-[*N*-(2-hydroxybenzoyl)glycyl]-N^1,N^8-bis-[2,3-(methylenedioxy)benzoyl]spermidine hydrochloride. This compound was taken up in anhydrous methylene chloride and reacted with BBr_3 to remove the methylenedioxy protecting group. The resulting compound after workup had ^1H NMR and chromatographic properties identical with those of the compound prepared by direct acylation of compound II.

Evaluation of Synthetic Schemes. The synthesis of the target compound required consideration of both the problems in selectively substituting spermidine and of protecting and deprotecting the 2,3-hydroxyl groups of the

2,3-dihydroxybenzoic acid moiety. For example, attempted N^1,N^8-diacylation of spermidine with reagents such as cinnamoyl chloride results in <5% yields of the desired products.[26] Similarly, attempts by Grady at acylation of spermidine with unprotected 2,3-dihydroxybenzoic acid (2,3-DHB) also resulted in poor yields of the desired product (~14% based on spermidine).[20] This was partially due to self-condensation of the acid.

Of course, in principle it is possible to improve on N^1,N^8-diacylation of spermidine by employing bulky, sterically hindered acylating agents. Fixing large, easily removable groups to small acylating agents is likely to increase the yield of N^1,N^8-acylation, e.g., 2,3-dimethoxybenzoyl chloride vs. 2,3-bis(benzyloxy)benzoyl chloride. One might further imagine using large, hindered coupling reagents, e.g., dicyclohexylcarbodiimide, in conjunction with the appropriately cumbersome carboxylic acid.

In fact, such approaches have not met with much success. A recent attempt by Neilands to prepare a N^1,N^8-bis[2,3-bis(benzyloxy)benzoyl]spermidine derivative from spermidine and 2,3-bis(benzyloxy)benzoyl chloride resulted in only "about 50% yield".[27] We were able to prepare the analogous intermediate, N^1,N^8-bis[2,3-(methylenedioxy)-benzyl]spermidine, in 92% yield from 2,3-(methylenedioxy)benzoyl chloride and N^4-benzylspermidine without optimizing conditions. Furthermore, our condensation of 2-hydroxyhippuric acid with this intermediate proceeded in 90% yield. It is difficult, however, to compare this condensation with Neilands condensation of the 2,3-dihydroxyhippuric acid analogue as neither yield nor elemental analysis was given. Finally, removal of our methylenedioxy protecting group proceeds in 95% yield as compared to the 34% for removal of the benzyloxy protecting group.

In evaluation of the chelation scheme, two features are noteworthy: the overall good yield and the mild conditions for removal of the copper protecting groups. Although the yield of final product is somewhat lower than that of the proof of structure synthesis, the fact that copper chelation was so successful means we have substantial flexibility regarding the nature of the N^4 substituent. Both acid- and base-labile groups can now be appended.

Conclusions

The problem of selectively acylating the N^4 nitrogen of N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine (II) directly without first protecting the catechol hydroxyls can be overcome by copper chelation.

The key to this sequence was to first show that compound II did indeed chelate Cu(II) and that this chelation involved the catechol hydroxyls. Once having shown this, *N*-(2-hydroxybenzoyl)glycine was condensed with compound II directly. Finally, as proof of the structure, the model system considered, N^4-[*N*-(2-hydroxybenzoyl)-glycyl]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine, was also synthesized by classical methods. The N^4-position of N^1,N^8-bis[2,3-(methylenedioxy)benzoyl]spermidine was acylated with *N*-[2-(trifluoroacetoxy)benzoyl]glycyltrifluoroacetic anhydride, and the protecting groups were removed.

Our interest in this procedure centers around the synthesis of siderophores whose central acyl group is sensitive to the kind of Lewis acid required to remove the catechol protecting groups. For example, in parabactin, the N^4-substituent, an oxazoline derivative of *N*-(2-hydroxybenzoyl)threonine, is very acid labile as evidenced by its opening during isolation. It should be possible to condense this and other acid-labile substituents with compound II in a fashion identical with the *N*-(2-hydroxybenzoyl)glycine condensation. Of course, siderophores with N^4-base-labile substituents can be synthesized by our alternate synthetic scheme very easily. All of the steps in both schemes represent high-yield sequences.

Finally we point out that we have now shown that N^4-[*N*-(2-hydroxybenzoyl)glycyl]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine and its precursor N^1,N^8-bis-(2,3-dihydroxybenzoyl)spermidine are both extremely effective at removing iron from iron-overloaded rats.[28] These findings clearly amplify the significance of these syntheses.

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Registry No. I, 76927-61-2; II·HCl, 73038-10-5; N^4-[*N*-(2-hydroxybenzoyl)glycyl]-N',N^8-bis(2,3-dihydroxybenzoyl)spermidine, 76927-62-3; N^4-[*N*-(2-hydroxybenzoyl)glycyl]-N',N^8-bis[2,3-(methylenedioxy)benzoyl]spermidine, 76927-63-4; N^4-benzyl-N',N^8-bis-[2,3-(methylenedioxy)benzoyl]spermidine hydrochloride, 76927-64-5; N^4-benzyl-N',N^8-bis[2,3-(methylenedioxy)benzoyl]spermidine, 76927-65-6; N^4-benzylspermidine, 73038-05-8; *N*-(2-hydroxybenzoyl)glycine, 487-54-7; 2,3-(methylenedioxy)benzoyl chloride, 66411-55-0; II trifluoroacetate salt, 76927-66-7; Cu, 7440-50-8.

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