fluorescence decay for certain ruthenium complexes in the presence of an electron acceptor in a rigid solution has been recently interpreted[28,35] in terms of an electron-transfer mechanism. We have attempted to fit our experimental decay data for the quinone acceptors to the decay function (6), using $P(t)$ derived from both models, although, unlike fluorescence decay with the CCl_4 acceptor no reasonable values for the parameters v and L were obtained from the analyses for the range of quinones used. The failure to find satisfactory fits of these data to function 6 is likely due to neglecting to include in the calculation the effect of molecular orientation on the electron transfer. Likewise, in the reported analyses[29,34,35] of the electron-transfer reactions in rigid solutions, the effects of molecular orientation on ET rates were also neglected, and the measured rate constants were orientation-averaged quantities. The orientation-dependent ET rate constants, which differ by more than 1 order of magnitude, are suggested in studies of linked PQ molecules;[12] it seems likely that the ET rate will decrease 1 order of magnitude or more for the most unfavorable orientation. Also, if ET is fast enough on a fluorescence time scale, a departure from the random P–Q distribution kinetics is likely to be observed. Therefore, we conclude that the failure to find a suitable kinetic equation for the fluorescence decay for ZnTPP in glassy MTHF solutions containing quinones can be attributed to the orientation effect in this electron-transfer reaction in the diffusionless system. This effect should be more pronounced in the case of the quinone acceptor than in the case of the alkyl chloride acceptor due to the more rigorous structural requirements for electron transfer in the former case.

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Lipophilic Enterobactin Analogues.[1] Stabilities of the Gallium and Ferric Ion Complexes of Terminally N-Substituted Catechoylamides

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The formation constants and metal complex protonation behavior of four lipophilic N-substituted tricatechoylamide analogues of enterobactin with Fe^{3+} and Ga^{3+} have been evaluated. The ligands *N,N''*-diisopropyl-*N,N',N''*-tris(5-sulfonato-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (DiP-3,4-LICAMS), *N,N''*-dibenzyl-*N,N',N''*-tris(5-sulfonato-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (DB-3,4-LICAMS), *N,N''*-dicyclohexyl-*N,N',N''*-tris(5-sulfonato-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (DC-3,4-LICAMS), *N,N',N''*-triisopropyl-*N,N',N''*-tris(5-sulfonato-2,3-dihydroxybenzoyl)-1,3,5-tris(aminomethyl)benzene (TiP-MECAMS) all form tris(catecholato) Fe^{3+} and Ga^{3+} complexes. Comparison of the metal complex stabilities of the N-substituted ligands to those of the nonlipophilic 3,4-LICAMS and MECAMS indicates that the ferric complexes are of similar stability; the gallium complexes are significantly less stable.

Introduction

Interest in the development of new iron chelating agents for their potential medical application in ferric ion decorporation therapy for persons with β-thalassemia[2,3] has spurred a program of ligand design and synthesis.[4,5] These synthetic ligands have also been used as chelating agents for Ga(III) and In(III),[6] since Ga(III) in particular is almost identical with Fe(III) in size.[7] Radiopharmaceuticals incorporating ^{67}Ga and ^{111}In are used for imaging abscesses and tumors.[8] Hexadentate ligands are designed to chelate excess ^{67}Ga(III) or ^{111}In(III) in the bloodstream, thus improving the tumor or abscess image and decreasing exposure of the patient to unnecessary radiation.[6,9]

Recognizing that microbes produce a hexadentate ligand, enterochelin[10] (enterobactin[11]), capable of effectively sequestering ferric ion,[12] a design concept was conceived to modify the hydrolytically unstable ester-linked backbone of enterobactin while preserving the ligand's inherent specificity for ferric ion as well as its stability.[13,14] Subsequent modifications have been to sulfonate[15] or carboxylate[16] the catechol rings to increase water solubility and decrease oxidation of the ligand by oxygen. Another modification seeks to increase the lipophilicity of the ligand by attaching organic moieities to the amide nitrogen.[17] This should change the tissue distribution of the metal complex in vivo. Indeed, the derivative having an *n*-octyl chain attached to the amide nitrogen crosses the blood/brain barrier,[18] which is seldom pen-

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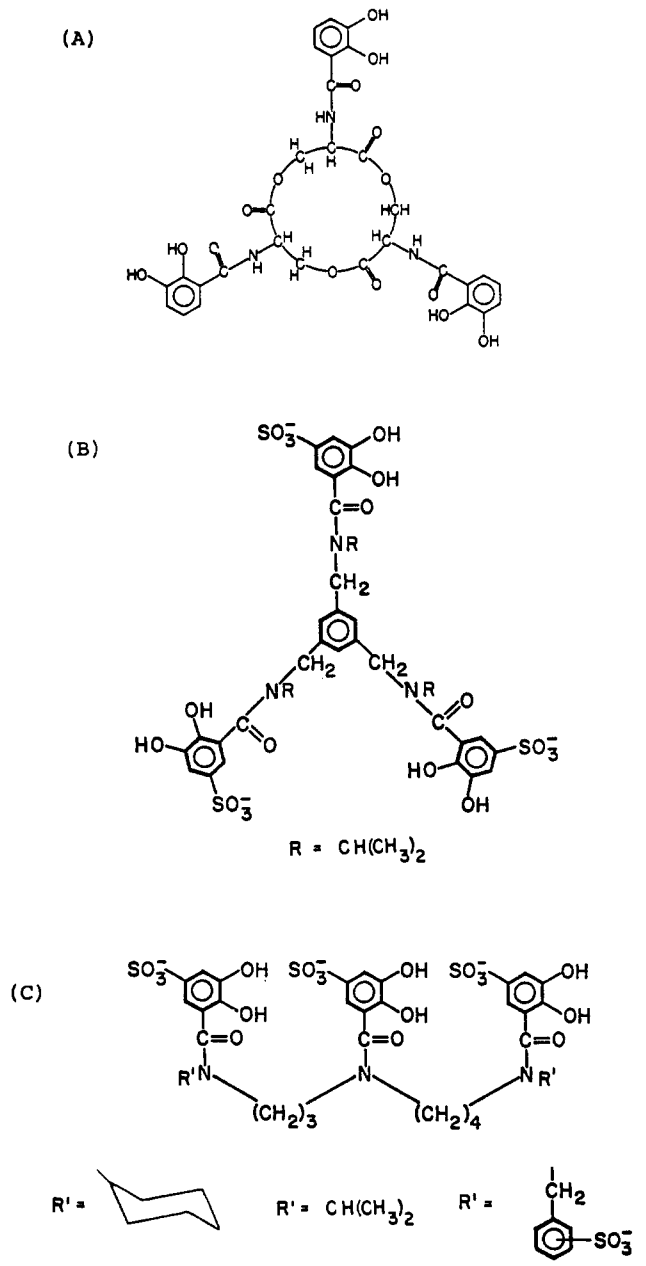

Figure 1. Structural formulas for enterobactin and the N-substituted tricatechoylamide analogues: (A) enterobactin; (B) the MECAMS derivative TiP-MECAMS (R = $CH(CH_3)_2$); (C) the LICAMS derivatives DC-3,4-LICAMS (R′ = $CH(CH_2)_5$), DiP-3,4-LICAMS (R′ = $CH(CH_3)_2$), and DB-3,4-LICAMS (R′ = $CH_2C_6H_4SO_3^-$).

etrated by foreign substances.[19] The N-substituted ligands have also been used to probe a hypothesized mechanism for iron release from enterobactin which requires a peptidase to cleave the amide bond of the ligand, thus raising the redox potential of ferric enterobactin (−750 mV at pH 7) to ranges accessible by natural reductants.[20] It has been shown that these ligands do supply iron to the growth medium of several different bacteria and that this may be related to the reductase system used rather than to peptidase activity.[21,22]

To be suitable chelating agents for Fe(III), Ga(III), or In(III), the ligands must be specific in their binding to the metal and complex the metal strongly and kinetically fast enough so that it can be removed from the transport protein transferrin, the accessible source of Fe(III), Ga(III), and In(III) in vivo.[23-28]

Reported here are the stability constants and protonation equilibria of Fe(III) and Ga(III) with the following four N-substituted catechoylamide ligands: *N,N″*-diisopropyl-*N,N′,N″*-tris(5-sulfonato-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (DiP-3,4-LICAMS); *N,N″*-dibenzyl-*N,N′,N″*-tris(5-sulfonato-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (DB-3,4-LICAMS); *N,N″*-dicyclohexyl-*N,N′,N″*-tris(5-sulfonato-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (DC-3,4-LICAMS); *N,N′,N″*-triisopropyl-*N,N′,N″*-tris(5-sulfonato-2,3-dihydroxybenzoyl)-1,3,5-tris(aminomethyl)benzene (TiP-MECAMS). Structural formulas are shown in Figure 1.

Experimental Section

Potentiometric Measurements. A detailed account of the apparatus used and the procedure followed for potentiometric titrations has been given previously.[14] In short, measurements were made with a Corning 130 digital pH meter equipped with a Corning Glass and saturated calomel electrodes. The meter was calibrated with standard acetate and nitric acid solns. to read hydrogen ion concentration, not activity. Solutions (40 mL of ~0.1 mM ligand) were kept under argon and were maintained at 25.0 ± 0.05 °C by a circulating water bath. The ionic strength was maintained at 0.1 M with KNO_3 for Fe^{3+} titrations or KCl for Ga^{3+} titrations. Carbonate-free 0.1 M KOH was prepared from Baker Dilut-It ampules with freshly boiled, doubly distilled H_2O. Back-titrations with HNO_3 were also performed with each ligand, and the resulting titration curves were compared with those obtained by titrating with base to ensure that there was no hysteresis of the curves. Potentiometric data were refined with use of a weighted nonlinear least-squares analysis in which log β's were varied to minimize the sum of the squared differences between the observed and calculated pH at each point in the titration curve.[29]

Spectrophotometric Measurements. Spectrophotometric titrations were recorded on a Hewlett-Packard 8450A UV/vis spectrophotometer. The visible spectra of ~0.2 mM ferric ion–ligand complexes (0.1 M KNO_3) were monitored as a function of pH. After each small addition of ~3 M HNO_3, the pH was measured, an aliquot was removed, the spectra were recorded, and the sample was returned to the solution.

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$$\Delta \log \beta = (\mathbf{D}^T\mathbf{D})^{-1}\mathbf{D}^T\mathbf{r}$$

The weighting factor, $1/\sigma_i$, was based on the estimated uncertainty in the pH reading at each point in the titration curve. This uncertainty has two components: the precision of the pH meter itself and the precision of titrant delivery (volume V_T). Thus, the weight was calculated as

$$\sigma_i^2 = \sigma_{meter}^2 + \left(\frac{\partial pH}{\partial V_T}\right)_i^2 \sigma_{V_T}^2$$

where $\sigma_{meter} = 0.003$ pH unit, $\sigma_{V_T} = 0.002$ mL, and $\partial pH/\partial V_T$ is the slope of the titration curve at each point of the titration. This weighting scheme emphasizes the more accurate data from buffer regions and minimizes the relatively inaccurate pH readings from steep inflections. The general least-squares procedure utilizes the program ORGLS. For a description of the procedure and program see the publication: Busing, W. R.; Levy, H. A. *Oak Ridge Natl. Lab.*, [*Rep.*] *ORNL-TM (U.S.)* **1962**, *ORNL-TM-271*.

Absorbance measurements for the spectrophotometric competitions with Na_2EDTA were taken on a Cary 118 UV/vis spectrophotometer. For the Fe(III)–catechoylamide competitions, 12 replicates were made for each of the ligands with varying amounts of ferric ion, ligand, and Na_2EDTA. The pH range studied was from 5.5 to 7.0. Equilibrium was approached from both directions: i.e., ferric EDTA plus free catechoylamide and ferric catechoylamide plus Na_2EDTA. The absorbance was checked after 72 h and after 144 h to ensure that equilibrium had been attained. The Ga(III)–catechoylamide competitions were carried out as previously described,[30] using the Fe(III)–catechoylamide complex as the spectral probe. These competitions were allowed to equilibrate for as long as 4 weeks to ensure that equilibrium had been reached. Details on this experimental procedure were explained previously.[30]

Metal Stock Solutions. The ~0.1 M $Fe(NO_3)_3$ solution was prepared by dissolving $Fe(NO_3)_3 \cdot 9H_2O$ (Mallinckrodt) in ~0.1 M HNO_3. The solution was standardized with disodium ethylenediaminetetraacetic acid (Na_2EDTA) and Eriochrome Black T indicator by back-titrating with standardized Mn(II) as described elsewhere.[31]

The ~0.1 M $GaCl_3$ solution was prepared by dissolving gallium metal in ~0.2 M HCl. The solution was then standardized by direct titration with Na_2EDTA using Pyrocatechol Violet as the indicator.[31]

The hydrogen ion concentration of both solutions was determined by potentiometric titration of the EDTA complex.

Syntheses. The syntheses of the unsulfonated derivatives DiP-3,4-LICAM, DB-3,4-LICAM, and DC-3,4-LICAM are reported in detail elsewhere.[17] The sulfonation procedure for these catechoylamide ligands is also documented elsewhere.[15] Briefly, the unsulfonated derivative is added to 30% fuming H_2SO_4 in ice. The solution is brought to neutral pH by addition of NaOH. Excess Na_2SO_4 is removed by extractions with MeOH–H_2O mixtures. This yields the hygroscopic white trisodium salt of the ligand. Titration of the free ligand yields an effective molecular weight. These determined molecular weights agree within 1% with those obtained by elemental analysis. Elemental analyses were performed by Analytical Services, Chemistry Department, University of California, Berkeley, CA. The ^1H NMR spectra in Me_2SO-d_6 were recorded on a 90-MHz JEOL FX90Q Fourier transform NMR spectrometer at Lawrence Berkeley Laboratory or on a 250-MHz Fourier transform NMR spectrometer in the Chemistry Department at the University of California, Berkeley, CA.

DiP-3,4-LICAMS. Anal. Calcd for $C_{34}H_{40}N_3O_{18}S_3Na_3 \cdot 4H_2O$: C, 40.20; H, 4.73; N, 4.14. Found: C, 40.13; H, 4.62; N, 4.04. ^1H NMR at 74 °C: δ 1.1 (doublet, 12 H, $-CH(CH_3)_2$); δ 1.3–1.9 (b, 6 H, $-NCH_2CH_2-$); δ 3.5–2.9 (b, 10 H, $-NCH(CH_3)_2$ and $-NCH_2-$); δ 6.82 (quartet, 3 H, 4-H on sulfonated catechol); δ 7.10 (triplet, 3 H, 6-H on sulfonated catechol).

DC-3,4-LICAMS. Anal. Calcd for $C_{40}H_{48}N_3O_{18}S_3Na_3 \cdot 6H_2O$: C, 42.44; H, 5.30; N, 3.71. Found: C, 42.37; H, 4.94; N, 3.69. ^1H NMR at 22 °C: δ 2.0–0.75 (b, $-NCH_2CH_2-$); δ 4.5–2.6 (b, $N-CH_2-$); δ 6.75 and 7.10 (doublet of doublets, Ar*H*).

DB-3,4-LICAMS. Anal. Calcd for $C_{42}H_{38}N_3O_{24}S_5Na_5 \cdot Na_2SO_4 \cdot 2CH_3OH \cdot 3H_2O$: C, 35.18; H, 3.46; N, 2.80. Found: C, 35.17; H, 3.46; N, 2.86. ^1H NMR at 22 °C: δ 1.9–1.2 (b, $-NCH_2CH_2-$); δ 3.4 (b, CH_3OH); δ 3.5–2.8 (b, $-NCH_2-$); δ 4.7 (b, $SO_3^-C_6H_4-CH_2-N-$); δ 6.8 (b, 4 H, 2.6-H on 3-sulfobenzyl); δ 7.5–7.1 (t, 6 H, 4,6-H on 5-sulfocatechol); δ 7.55 (b, 4 H, 3,4-H on 3-sulfobenzyl).

TiP-MECAMS. Synthesis of this ligand followed routes similar to those used in the synthesis of the other N-substituted ligands;[17] 200 mmol of isopropylamine (Aldrich Chemical Co., 99%) was added to a (room temperature, water bath cooled) mixture of 30 mmol of 1,3,5-benzenetricarboxylic acid chloride (Aldrich, 98%) in 150 mL of THF (previously distilled over CaH_2). The solution was stirred overnight under a Drierite tube. The white precipitate was washed well with THF and EtOH (to remove unreacted i-$PrNH_2 \cdot HCl$). The triamide was a single spot on silica gel TLC with eluent of 93 mL of THF, 7 mL of cyclohexane, and 5 mL of H_2O, after illumination by UV (R_f 0.69). The product was dried at 100 °C in an open-air oven to get 85% yield. Reduction of 8.0 g of the triamide (24 mmol) was completed by mixing it with 220 mmol of 1 N BH_3 in THF and refluxing overnight at 65 °C. The reaction was quenched by addition of 25 mL of 6 N HCl with stirring for 3 h. Evaporation of the solution to a residue followed by several coevaporations with MeOH yielded a white solid. This solid was dissolved in $CHCl_3$ and dried with $MgSO_4$. The resulting filtered solution was clear and colorless and was added dropwise to stirred Et_2O to get a white solid. This was washed with EtOH and dried in a vacuum oven at 60 °C overnight. This triisopropyltriamine backbone was then used to couple via an amide linkage to 2,3-dimethoxybenzoyl chloride as described previously in the synthesis of MECAM.[13] The tricatechoylamide was then synthesized by deprotection of the 2,3-dimethoxybenzoyl moieties with a solution of CH_2Cl_2–BBr_3 also as described in the MECAM synthesis.[13] Sulfonation of TiP-MECAM (vide supra) yielded a beige powder. Anal. Calcd for $C_{39}H_{42}N_3O_{18}S_3Na_3 \cdot Na_2SO_4 \cdot 2CH_3OH \cdot 3H_2O$: C, 38.89; H, 4.43; N, 3.32. Found: C, 38.74; H, 4.18; N, 3.32. ^1H NMR at 22 °C: δ 1.1 (b, $-CH(CH_3)_2$); δ 3.4 (b, CH_3OH); δ 4.7 (b, $Ar-CH_2-$); δ 6.90 (s, 3 H, Ar*H*); δ 7.4–7.15 (t, 6 H, 4,6-H on 5-sulfocatechol).

Table I. Ligand Protonation Constants[a]

ligand	log K^H_4	log K^H_5	log K^H_6	log K^H_{av}
DiP-3,4-LICAMS	8.50 (3)	7.78 (1)	7.13 (1)	7.8
DB-3,4-LICAMS	8.51 (1)	7.84 (1)	7.03 (3)	7.8
DC-3,4-LICAMS	8.49 (5)	7.77 (1)	7.05 (3)	7.8
TiP-MECAMS	8.61 (2)	7.85 (1)	6.60 (2)	7.7
$(Me)_3$MECAMS[b]	8.52 (2)	7.57 (2)	6.72 (2)	7.6
MECAMS[c]	7.26	6.44	5.88	6.5
3,4-LICAMS[c]	8.28	7.07	6.11	7.2

[a] $K^H_n = [H_nL]/[H^+][H_{n-1}L]$. [b] Reference 34. [c] Reference 32.

Results and Discussion

Ligand Protonation Constants. The ligand protonation constants obtained by potentiometric titration for the N-substituted ligands are shown in Table I. Those constants represented (log K^H_{4-6}) are the protonation constants of the phenolic oxygen ortho to the carbonyl group. The protons of the phenolic oxygen meta to the carbonyl dissociate at high pH in a range inaccessible by potentiometric methods.

Comparison of the protonation constants of the N-substituted 3,4-LICAMS derivatives to the protonation constants of 3,4-LICAMS[32] indicates that the N-substituted derivatives are considerably less acidic. The lower acidity of the N-substituted derivatives is due to the presence of the alkyl groups on the amide nitrogen that donate electron density to the carbonyl group, thus lessening the inductive effect of the carbonyl on the aromatic ring. A similar phenomenon can be observed by comparing the pK_a's of salicylaldehyde ($pK_a \approx 8.22$) to 2-acetylphenol ($pK_a \approx 9.94$),[33] in which case the alkyl group is directly attached to the carbonyl and the effect is greater. Likewise, comparison of the protonation constants of MECAMS,[32] $(Me)_3$MECAMS (MECAMS[34] that has methyl groups on the amide nitrogen), and TiP-MECAMS (Table I) shows a gradual decrease in acidity as the alkyl chain gets larger.

The protonation constants of the phenolic oxygens meta to the phenol have been estimated to be log $K^H_{1,3} = 11.7$ for the 3,4-LICAMS N-substituted derivatives and log $k^H_{1,3} = 11.8$ for TiP-MECAMS.[34] These estimates are based on structurally related compounds with known protonation constants.[33,35]

Fe(III) Equilibria. The ferric complexes of the N-substituted catechoylamides were investigated by both spectrophotometric and potentiometric methods.

The potentiometric titration curves of the Fe(III) complexes are shown in Figure 2.[36] All ligands form tris(catechol) complexes with concomitant release of six protons. The titration curves of the Fe(III) complexes of TiP-MECAMS, DiP-3,4-LICAMS, and DC-3,4-LICAMS all show distinct inflections at $a = 4$, indicating that the tris(catecholato)iron(III) complexes form at pH values greater than 5.

The spectra of the tris(catecholato)iron(III) complexes display a λ_{max} at 485 nm, characteristic of a ligand-to-metal charge-transfer band of Fe(III) tris(catecholates),[32] with extinction coefficients of 4900 M^{-1} cm^{-1} for DiP-3,4-LICAMS and DC-

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Table II. Ferric Complex Protonation Constants[a]

ligand	log K_{MHL}		log K_{MH_2L}		log K_{MH_3L}	
	potentiometric	spectral	potentiometric	spectral	potentiometric	spectral
DiP-3,4-LICAMS[b]			12.66 (4)	12.9 (1)		
DB-3,4-LICAMS	6.48 (10)	6.27 (11)[c]	4.65 (8)	4.72 (13)[c]	3.41 (7)	...
DC-3,4-LICAMS	6.68 (4)	6.85 (7)	6.02 (2)	5.78 (8)	3.62 (3)	...
TiP-MECAMS	7.85 (8)	7.89 (7)	6.07 (8)	6.06 (8)	3.84 (8)	3.81 (7)
$(Me)_3$MECAMS[d]	...	6.80 (3)	6.61 (5)	...	5.80 (5)	...
MECAMS[e]	5.74 (2)	5.19 (3)	4.10 (4)	...	3.46 (5)	...
3,4-LICAMS[e]	6.16 (6)	5.85 (2)	5.3 (1)	5.32 (7)	3.10 (4)	3.05 (4)

[a] $K_{MH_nL} = [MH_nL]/[MH_{n-1}L][H^+]$. [b] One two-proton step occurs, i.e. $K_{MH_2L} = [MH_2L]/[ML][H^+]^2$. [c] By nonlinear least-squares refinement (see text). [d] Reference 34. [e] Reference 32.

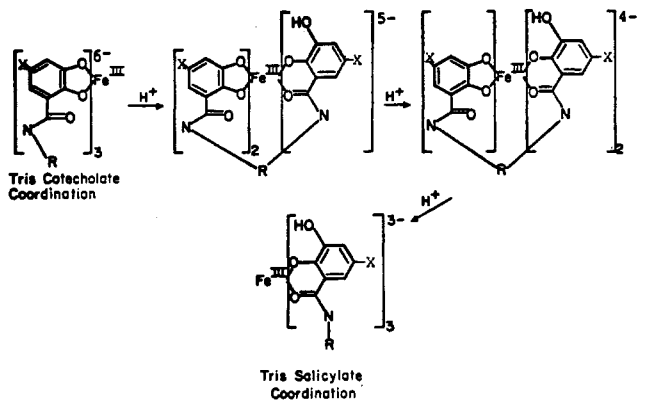

Figure 3. Schematic representation of the first three protonation steps of salicylate binding modes (X = SO_3^-).

3,4-LICAMS and 4350 M^{-1} cm^{-1} for DB-3,4-LICAMS and TiP-MECAMS. These extinction coefficients are considerably less than those observed for 3,4-LICAMS and MECAMS ($\epsilon \approx$ 6200 M^{-1} cm^{-1})[32] and just slightly less than that observed for $(Me)_3$MECAMS ($\epsilon \approx$ 5200 M^{-1} cm^{-1}).[34]

By monitoring the absorbance of the Fe(III) complexes as a function of pH, one can study the tris(catecholato) to bis(catecholato) equilibrium. Previous studies have shown that the ferric complexes of 3,4-LICAMS and MECAMS protonate via *two separate one-proton steps* rather than by a single two-proton step with concomitant dissociation of a catecholate arm.[32] The single-proton stoichiometry, which has been assigned as an Fe(III) tris(catecholato) complex protonation at the phenolic oxygen meta to the carbonyl (the least acidic position) is followed by a shift in coordination of the Fe(III) ion to the carbonyl oxygen adjacent to the catecholate ring. Figure 3 illustrates the first three protonation steps, reminiscent of salicylate bonding. The ferric complexes of DB-3,4-LICAMS, DC-3,4-LICAMS, and TiP-MECAMS all show this same protonation behavior. The spectra taken at varying pHs of these complexes contain isosbestic points at 532, 540, and 542 nm, respectively. The spectra of ferric TiP-MECAMS and DC-3,4-LICAMS retained this isosbestic point over a range in pH that can be correlated to the potentiometric titration curves, indicating that 1 equiv of acid has been added and that only two species are in solution. A graphical method developed by Schwarzenbach[37] in which the observed absorbance (A_{obsd}) at any particular wavelength and pH is plotted vs. $(A_0 - A_{obsd})/[H^+]^n$, where A_0 is the initial absorbance of the tris(catecholato) complex at high pH and n is the proton stoichiometry of the reaction, will be linear if n is chosen correctly. The slope of this curve is $1/K_{MH_nL}$, where K_{MH_nL} is defined as $[ML][H^+]^n/[MH_nL]$. Thus, the full equation is

$$A_{obsd} = \epsilon_{MH_nL}C_T + \frac{A_0 - A_{obsd}}{[H^+]^n}\left(\frac{1}{K_{MH_nL}}\right) \quad (1)$$

where C_T is the total concentration of absorbing species in solution and ϵ_{MH_nL} is the extinction coefficient of the protonated species. These plots were linear for $n = 1$ in the case of ferric TiP-MECAMS and ferric DC-3,4-LICAMS, indicating bis(catecholato), mono(salicylato) coordination about the Fe(III). The values of the protonation constants, K_{MHL} and K_{MH_2L}, obtained from this technique agree well with those obtained in potentiometric measurements and are shown in Table II.

Although the spectra of the ferric complexes of DB-3,4-LICAMS contained an isosbestic point at 532 nm, this isosbestic point was not retained over a 1-equiv change in pH. In the region $a = 6$–4 several equilibria overlap strongly:

$$Fe(DB\text{-}3,4\text{-}LICAMS)^{6-} + H^+ \xrightleftharpoons{K_{MHL}} Fe(HDB\text{-}3,4\text{-}LICAMS)^{5-} \quad (2)$$

$$Fe(HDB\text{-}3,4\text{-}LICAMS)^{5-} + H^+ \xrightleftharpoons{K_{MH_2L}} Fe(H_2DB\text{-}3,4\text{-}LICAMS)^{4-} \quad (3)$$

Since the method developed by Schwarzenbach does not apply to this more complex equilibria scheme, a nonlinear least-squares refinement was used to calculate the extinction coefficients and protonation constants by minimizing the sum of the squared differences between the observed and calculated absorbances.[32] At each pH, the absorbances were recorded at 490, 520, 560, and 590 nm. At any wavelength the absorbance is given by eq 4. The

$$A^\lambda = [ML]\epsilon_{ML}{}^\lambda\,\epsilon_{ML}{}^\lambda = K_{MHL}[H^+]\epsilon_{MHL} + K_{MHL}K_{MH_2L}[H^+]^2\epsilon_{MH_2L} \quad (4)$$

values of [ML] can be calculated from mass balance by using an initial set of equilibrium constants

$$[Fe]_{total} = [ML](1 + K_{MHL}[H^+] + K_{MHL}K_{MH_2L}[H^+]^2) \quad (5)$$

The value of $\epsilon_{ML}{}^{\lambda_{1-4}}$ is obtained directly from the spectra at high pH. Thus there are a total of 10 variables: $\epsilon_{MHL}{}^{\lambda_{1-4}}$, $\epsilon_{MH_2L}{}^{\lambda_{1-4}}$, K_{MHL}, K_{MH_2L}. These were refined simultaneously with use of 52 data points at 13 pH values between pH 8.5 and 4.7. The final values for the equilibrium constants are shown in Table II, and they agree well with the potentiometric values.

The protonation equilibria of the ferric complex of DiP-3,4-LICAMS differ from those of the other N-substituted ligands. Figure 4[36] shows the visible spectra from $a = 5.9$ to $a = 4.5$. The isosbestic point at 551 nm indicates that probably only two species are in solution over this pH range. This would imply that the protonation behavior of Fe^{III}(DiP-3,4-LICAMS)$^{6-}$ differs from that of all other synthetic catechoylamides that have carbonyl groups adjacent to the catechol ring and that it dissociates via *on two*-proton step. Indeed, the Schwarzenbach plot over this pH ranges is linear only for $n = 2$ (Figure 5). It is not clear why the ferric complex of DiP-3,4-LICAMS should protonate in this manner.

The overall formation constants of the Fe(III) complexes of tris(catecholato) ligands cannot be determined directly because the iron binding is so strong that the complexes are not appreciably dissociated into free ligand and free iron above pH 2. Therefore, proton-dependent stability cosntants have been determined spectrophotometrically by competition with EDTA.[32] Because the three largest ligand protonation constants are unknown, it is not possible to calculate standard formation constants, i.e., those

(37) Schwarzenbach, G.; Schwarzenbach, K. *Helv. Chim. Acta* **1963**, *46*, 1390.

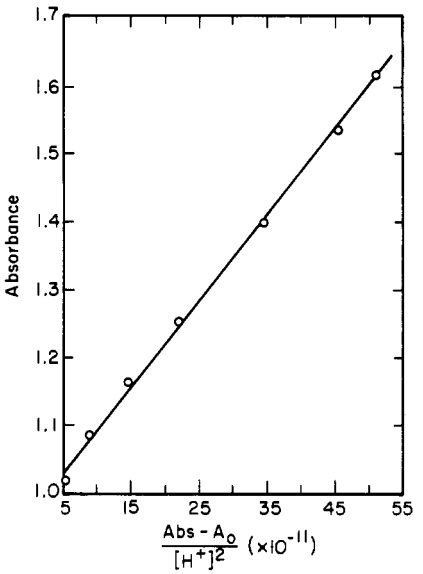

Figure 5. Schwarzenbach plot of Fe(III)–DiP-3,4-LICAMS indicating one two-proton-step stoichiometry.

written in terms of the fully deprotonated form of the ligand. Instead, the equilibria are expressed in terms of the H_3L species:

$$Fe^{3+} + H_3L \overset{K^*}{\rightleftharpoons} FeL + 3H^+ \quad (6)$$

$$K^* = \frac{[FeL][H^+]^3}{[Fe^{3+}][H_3L]} \quad (7)$$

Values of K^* can be obtained from a competition constant, K_{comp}, by using the stability constant for FeEDTA ($\log \beta_{110}^{FeEDTA} = 25.0$).[33]

$$K_{comp} = \frac{[FeL][H^+]^3[EDTA]}{[FeEDTA][H_3L]} = \frac{K^*}{\beta_{110}^{FeEDTA}} \quad (8)$$

The concentrations of all the species in eq 8 can be calculated by using the ligand protonation constants of EDTA and the tris(catecholate), the metal chelate protonation constants of FeEDTA and tris(catecholato)iron, the pH, the absorbance at 484 nm, and the mass balance equations. Over the pH range studied the pertinent equations are

$$A^{484} = [FeL](\epsilon_{FeL} + K_{FeHL}[H^+]\epsilon_{FeHL} + K_{FeHL}K_{FeH_2L}[H^+]^2\epsilon_{FeH_2L}) \quad (9)$$

$$[Fe]_{total} = [FeL] + [FeHL] + [FeH_2L] + [FeEDTA] + [FeOHEDTA] \quad (10)$$

$$[L]_{total} = [H_3L](1 + K^H_4[H^+] + K^H_4K^H_5[H^+]^2 + K^H_4K^H_5K^H_6[H^+]^3) + [FeL] + [FeHL] + [FeH_2L] \quad (11)$$

$$[EDTA]_{total} = [EDTA](1 + K^H_1[H^+] + K^H_1K^H_2[H^+]^2) + [FeEDTA] + [FeOHEDTA] \quad (12)$$

The log K^* values and the log β_{110} estimates for the ferric complexes of the N-substituted ligands are shown in Table III. The log β_{110} constants are estimated on the basis of the high protonation constants from literature values for simple substituted catechols.[33,35] The N-substituted 3,4-LICAMS derivatives have estimated log $K^H_{1-3} = 11.7$, and TiP-MECAMS has an estimated log $K^H_{1-3} = 11.8$.[34] These values are higher than those values estimated previously because log K_{1-3}(av) is higher for the N-substituted ligands.

The ferric complexes of the N-substituted tricatechoylamides, appear to be of comparable stability to the unsubstituted derivatives.[32] However, at physiological pH hydrogen ion is in sufficiently high concentration that it competes with iron for the tricatechoylamide anion, such that the complexing form of the ligand represented in β_{110}^{FeL}, the fully deprotonated form, is virtually nonexistent at pH 7. To facilitate comparison under

Table III. pH-Dependent Equilibrium Constants and Normal Formation Constants of a Series of Ferric Complexes

ligand	log K^* [a]	log β_{110} [b]
DiP-3,4-LICAMS	5.36 (13)	40
DB-3,4-LICAMS	7.0 (4)	42
DC-3,4-LICAMS	4.85 (15)	40
TiP-MECAMS	4.15 (15)	40
$(Me)_3$MECAMS[c]	5.21 (3)	41
MECAMS[d]	6.57 (10)	41
3,4-LICAMS[d]	6.40 (9)	41

[a] $K^* = [FeL][H^+]^3/[H_3L][Fe]$. [b] $\beta_{mlh} = [M_mL_lH_h]/[M]^m[L]^l[H]^h$ is defined as the overall formation constant for $M_mL_lH_h$, where M = metal ion, L = ligand in its deprotonated (i.e., complexing) form, and H = hydrogen ion. [c] Reference 34. [d] Reference 32.

Table IV. pM[a] Values

ligand	pM	
	Fe(III)	Ga(III)
DiP-3,4-LICAMS	26.8	22.3
DB-3,4-LICAMS	28.4	22.4
DC-3,4-LICAMS	26.3	21.3
TiP-MECAMS	26.2	21.0
$(Me)_3$MECAMS	26.9[b]	
MECAMS	29.4[c]	26.3[g]
3,4-LICAMS	28.5[c]	26.0[g]
enterobactin	35.5[d]	
ferrioxamine B	26.6[e]	
transferrin	23.6[f]	21.3[h]
EDTA	22.2[e]	21.6[e]

[a] Conditions: $[L]_{total} = 10^{-5}$ M; $[M]_{total} = 10^{-6}$ M; pH 7.4. [b] Reference 34. [c] Reference 32. [d] Reference 12. [e] Calculated from stability constants in ref 33. [f] Calculated from stability constants in ref 38. [g] Reference 30. [h] Harris, W. R.; Pecoraro, V. L. *Biochemistry* **1983**, *22*, 292.

biologically reasonable conditions, a pM scale has been introduced, where pM = $-\log [M(H_2O)_6^{n+}]$ under conditions of $[M]_{total} = 10^{-6}$ M, $[L]_{total} = 10^{-5}$ M, and pH 7.4. The pM values for a number of synthetic tritricatechoylamide ligands as well as for several other ligands are listed in Table IV. From the pM values it is apparent that the less acidic N-substituted ligands are not as effective in sequestering Fe(III) as are MECAMS or 3,4-LICAMS.[32] In particular, DB-3,4-LICAMS, which has the highest value for log K^*, actually has a pM comparable to that of 3,4-LICAMS because of its less acidic protons. However, all the lipophilic derivatives do have pM values that are higher than the pM value for the iron-transport protein transferrin.[38] This is important since the most likely mechanism for successful iron removal is to administer chelating agents that will remove the iron from transferrin and facilitate iron excretion from the body, then allowing the apotransferrin to mobilize the less accessible iron stores.

Ga(III) Equilibria. Potentiometric titrations and spectrophotometric competitions with Na_2EDTA were performed to assess the stabilities of the complexes of Ga(III) with the N-substituted catechoylamides.

Figure 6[36] shows the potentiometric titration curves of the Ga(III) complexes. All the ligands form a tris(catecholato) complex with Ga(III) with concomitant release of six protons. Refinement of the buffer regions using a weighted nonlinear least-squares analysis[29] yielded the complex protonation constants found in Table V. The complex protonation constants for Ga(III) with DiP-3,4-LICAMS and DC-3,4-LICAMS were refined successfully only with use of a model assuming one two-proton-step equilibrium; i.e., no mono(salicylato)bis(catecholato)gallium(III) complex is formed. This was observed previously in the analysis of the Ga(III)–3,4-LICAMS equilibrium.[30] Refinement of the data assuming a model with two sequential one-proton steps yielded two identical constants (e.g., for Ga–DiP-3,4-LICAMS

(38) Aasa, R.; Malmström, B. G.; Saltman, P. Vänngard, T. *Biochim. Biophys. Acta* **1963**, *75*, 203.

Table V. Thermodynamic Data of Ga(III) N-Substituted Catechoylamide Complexes

ligand	log K_{MHL}[a]	log K_{MH_2L}	log K_{MH_3L}	log β_{110}[b]
DiP-3,4-LICAMS		12.1 (1)[c]		36
DB-3,4-LICAMS	6.6 (1)	4.8 (1)	3.3 (1)	36
DC-3,4-LICAMS		12.0 (1)[c]		35
TiP-MECAMS	7.20 (2)	5.8 (1)	3.1 (1)	35
MECAMS[d]	5.7	4.9 (2)		38
3,4-LICAMS[d]		10.2 (1)[c]		38

[a] $K_{MH_nL} = [MH_nL]/[H^+][MH_{n-1}L]$. [b] $\beta_{110} = [GaL]/[Ga][L]$; see footnote *b* in Table III. [c] Refined only as one two-proton step; $K_{MH_2L} = [MH_2L]/[ML][H^+]^2$. [d] Reference 30.

log K_{MH_2L} = 12.1 for one two-proton step and log K_{MHL} = log K_{MH_2L} = 6.05 for two one-proton steps). The refinements indicate that the complexes with DB-3,4-LICAMS and TiP-MECAMS do protonate via one-proton steps. This protonation behavior may be due to the presence of a tertiary carbon which is attached to the amide nitrogen in DC-3,4-LICAMS and DiP-3,4-LICAMS, hindering the rotation about the amide bond that is required to bring the carbonyl group in from its normal position distal from the metal and thus not allowing the formation of a mono(salicylate) complex. Although TiP-MECAMS also has a tertiary carbon attached to the amide nitrogen, the nature of the organic backbone of the ligand does not allow this hindrance of rotation to occur. This, however, does not explain the behavior of the Ga(III) 3,4-LICAMS complex, which protonates via one two-proton step.[30]

The spectrophotometric competitions with EDTA were performed with use of the absorbance of the Fe–catechoylamide at 484 nm as a spectral probe. In these experiments exchange of GaEDTA with Fe–catechoylamide (or FeEDTA with Ga–catechoylamide was allowed to occur:

$$\text{GaEDTA} + \text{FeL} \rightleftharpoons \text{GaL} + \text{FeEDTA} \tag{13}$$

$$K_{comp} = \frac{[GaL][FeEDTA]}{[FeL][GaEDTA]} \tag{14}$$

Knowledge of the formation constants of GaEDTA,[33] FeEDTA, and Fe–catechoylamide, along with mass balance, absorbance, and pH measurements, allows calculation of a formation constant for Ga–catechoylamide:

$$K_{comp} = \frac{\dfrac{[GaL]}{[Ga][L]}\dfrac{[FeEDTA]}{[Fe][EDTA]}}{\dfrac{[GaEDTA]}{[Ga][EDTA]}\dfrac{[FeL]}{[Fe][L]}} = \frac{\beta_{110}^{GaL}\beta_{110}^{FeEDTA}}{\beta_{110}^{GaEDTA}\beta_{110}^{FeL}} \tag{15}$$

$$\beta_{110}^{GaL} = \frac{K_{comp}\beta_{110}^{GaEDTA}\beta_{110}^{FeL}}{\beta_{110}^{FeEDTA}} \tag{16}$$

The details of this calculation have been described previously.[30] The formation constants of the Ga(III) complexes are shown in Table V. Earlier estimates of the constants of Ga(III) with DiP-3,4-LICAMS and TiP-MECAMS[6] we regard as inaccurate. Unlike the Fe(III) complexes, the Ga(III) formation constants of the N-substituted ligands are all significantly lower than the formation constants of Ga(III) with MECAMS and 3,4-LICAMS.[30]

Table IV contains the pM values for the Ga(III) complexes calculated from the formation constants. These values indicate a significant decrease in affinity for Ga(III) at physiological pH for the lipophilic derivatives compared to 3,4-LICAMS and MECAMS. In fact, the relative pM values indicate that TiP-MECAMS and DC-3,4-LICAMS may not be able to remove Ga(III) from transferrin efficiently. Biological studies of TiP-MECAMS and DiP-3,4-LICAMS with Ga(III) and In(III) have indicated that TiP-MECAMS is less effective in removing excess ^{67}Ga from the blood than is DiP-3,4-LICAMS.[6] This difference may well be due to the limited ability of TiP-MECAMS to remove Ga(III) from transferrin. In vivo testing of DB-3,4-LICAMS with ^{67}Ga is currently in progress.

Summary. The lipophilic analogues of enterobactin form stable complexes with ferric ion, thermodynamically capable of removing Fe(III) from the iron transport protein transferrin. One ligand, DiP-3,4-LICAMS, forms a tris(catecholate) complex with Fe(III), which protonates via one two-proton step. Of all ligands studied, this is the only synthetic tricatechoylamide with a carbonyl adjacent to the ring that does not form a mono(salicylate) bis(catecholate) complex with Fe(III).

The Ga(III) complexes of the N-substituted ligands are not as stable as those formed with unsubstituted sulfonated tricatechoylamides. TiP-MECAMS and DC-3,4-LICAMS may not be thermodynamically capable of efficiently removing Ga(III) from transferrin. However, DiP-3,4-LICAMS has been shown to be effective for in vivo sequestering of ^{67}Ga.

It appears that increasing liophilicity of the ligand in this manner does lower the affinity of the ligand for the target metal. However, the lower stability of the Fe(III) chelate may be offset by an altered tissue distribution for in vivo studies. The complexes with Ga(III) are of borderline stability if the ligands are to be capable of removing Ga(III) from transferrin.

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Registry No. DiP-3,4-LICAMS, 96649-34-2; DB-3,4-LICAMS, 96666-27-2; DC-3,4-LICAMS, 96649-35-3; TiP-MECAMS, 96649-36-4; Fe, 7439-89-6; enterobactin, 28384-96-5.

Supplementary Material Available: pH profiles of the potentiometric titrations (Figures 2, 4, and 6) and tabulations of additional titration details (4 pages). Ordering information is given on any current masthead page.