respective B/B′ dihedral angles are 71 and 87°, a significant difference which can apparently be attributed only to the presence in the structure of the different anions.[57]

In regard to the perturbation of a DNA structure upon binding of the *cis*-$(NH_3)_2Pt^{II}$ moiety in an intrastrand cross-linking mode for regions of high Guo-Cyd content,[5–6,8,15–16] the above deductions are suggestive of varying degrees of stereochemical interference with the native motif of the DNA duplex. As noted in the Introduction, various modes of intrastrand cross-linking may be envisioned. All of these demand, of course, some degree of local denaturation or premelting in order to accommodate the formation of the cross-link. The degree of local denaturation of the duplex to accommodate a G[N(7)]–Pt–G[N(7)] mode may, however, be slight in comparison to that demanded by a linkage of the type C[N(3)]–Pt–C[N(3)], although the occurrence of the latter may be rare as its formation is mitigated against by strong interbase repulsion. Finally, if one accepts the notion[58] that cancer cells are deficient in their ability to excise defects from strands of DNA, then such cells may find defects imposed by cross-links of the type C[N(3)]–Pt–C[N(3)] particularly difficult to repair.[59]

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Supplementary Material Available: Tables of nonhydrogen atom anisotropic thermal parameters and of parameters for the hydrogen atoms and a list of calculated and observed structure factor amplitudes (46 pages). Ordering information is given on any current masthead page.

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Ferric Ion-Specific Sequestering Agents. 7. Synthesis, Iron-Exchange Kinetics, and Stability Constants of N-Substituted, Sulfonated Catechoylamide Analogues of Enterobactin[1]

Vincent L. Pecoraro, Frederick L. Weitl,[2] and Kenneth N. Raymond*[3]

Contribution from the Department of Chemistry and Materials and Molecular Research Division, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720.
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Abstract: Two analogues of enterobactin are reported which exhibit (i) stability to base-catalyzed hydrolysis of the central ring, (ii) water solubility, and (iii) N-substitution to block peptidase hydrolysis of the amide bonds. The first compound 1,3,5-tris(*N*-methyl-*N*-(2,3-dihydroxysulfobenzoyl)aminomethyl)benzene ($Me_3MECAMS$) was prepared, via the amide of trimesoyl chloride and $MeNH_2$, in four steps and 6% overall yield. The proton-dependent formation constant ($\log K^* = \log([FeL^{6-}][H^+]^3)/([Fe^{3+}][H_3L^{6-}])$) is 5.21, which gives an equilibrium concentration of $[Fe^{3+}]$ at pH 7.4 of 1×10^{-27} M for 10^{-5} M $Me_3MECAMS$ and 10^{-6} M total Fe^{3+}. The estimated formation constant ($\log \beta_{110}$) is 41. At low pH the FeL^{6-} complex undergoes a series of one-proton reactions which probably gives a tris-"salicylate" complex formed by the carbonyl and ortho-catechol oxygens of the 2,3-dihydroxybenzoyl units. After 6 h, in the presence of 6 mM ascorbate ($T = 37$ °C, $\mu = 0.05$ M), $Me_3MECAMS$ (6.0 mM) removed 3.7% of the ferric ion initially sequestered by the iron-storage protein ferritin. The human iron-transport protein transferrin releases iron to $Me_3MECAMS$ with a pseudo-first-order rate constant of 1.9×10^{-3} min^{-1} (ligand concentration 2×10^{-4} M, $T = 25$ °C, $\mu = 0.10$ M). This rate is comparable to that of enterobactin and other catechoyl amide sequestering agents and greatly exceeds that of desferrioxamine B (Desferal), the current drug of choice in treating iron overload. Two related compounds have been prepared in which the catechol ring is attached to the amide nitrogen through a methylene group, with amide formation with an acetyl group. In 1,3,5-tris(*N*-acetyl-*N*-(2,3-dihydroxysulfobenzyl)aminomethyl)benzene [NAcMECAMS] and its unsulfonated precursor, the amide linkage of the catechoyl amides such as $Me_3MECAMS$ has been shifted from an endo position relative to the benzene and catechol rings to an exo position in which the amide carbonyl is not conjugated with the catechol ring and cannot form a stable chelate ring in conjunction with a catechol oxygen. In comparison with $Me_3MECAMS$, the protonation of NAcMECAMS proceeds by an initial two-proton step in contrast to the one-proton reactions typical of the catechoyl amides, which can form a "salicylate" mode of coordination involving the amide carbonyl group. Also as a result of the removal of the carbonyl group from conjugation with the catechol ring, the acidity of NAcMECAMS is less than $Me_3MECAMS$. While the estimated $\log \beta_{110} = 40$ is approximately the same as for $Me_3MECAMS$, the effective formation constant ($\log K^*$) and pM ($-\log [Fe_{aq}^{3+}]$) values are lower (4.0 and 25.0, respectively).

It is established that virtually all organisms need iron.[4] Human beings maintain a total inventory of ca. 5 g in the adult through a complex process of iron transport and storage.[5] In this light, it is a well documented fact that, in excess, Fe^{3+} is very toxic. Indeed, acute iron overload (primarily from ingestion of iron supplement preparations by infants) is one of the most common

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(2) Materials and Molecular Research Division, Lawrence Berkeley Laboratory, Berkeley, CA 94720.

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forms of poisoning in the United States.[6,7] In addition, long-term (i.e., chronic) iron overload can result from the transfusion therapy of Cooley's anemia (β-thalassemia major),[8] a blood disorder affecting approximately 3 million people worldwide, and is the major cause of death associated with this disease.

The present treatment for transfusional iron overload in the β-thalassemic patient is either subcutaneous or intravenous slow infusion of Desferal, the mesylate salt of desferrioxamine B. This therapy has exhibited efficacies as low as 10%, presumably due to the instability of the drug in the body and its rapid excretion.[9] The pool of labile, low-molecular-weight iron, the iron-storage protein ferritin, and the iron-transport protein transferrin are three possible sources for removable iron. Desferal presumably sequesters ferric ion from the labile iron pool and, with the coadministration of ascorbate, from ferritin. Although Desferal is thermodynamically capable of removing iron from transferrin, the process has been shown to be kinetically slow.

We are actively involved in the synthesis and characterization of new iron sequestering compounds which are intended to be potential therapeutic agents in the treatment of iron overload. These ligands are analogues of the catechol-containing siderophore, enterobactin, which has been shown to be both thermodynamically and kinetically able to remove iron from transferrin.[10] Unfortunately, enterobactin is unsuitable for clinical use due to its low aqueous solubility, extreme oxidative and hydrolytic lability, and its capacity to cause toxic bacteremia in iron-overloaded mice.[11] Preliminary animal experiments on hypertransfused rats have shown that sulfonated catechoylamide sequestering agents are capable of mobilizing iron in vivo. No acute toxic effects were reported; however, after 10 days the animals died due to toxic bacteremia.[12] Bacterial proliferation is presumably due to mobilization of iron from biological stores which are normally unavailable for bacterial growth. It seems possible that the bacteria are in some way removing iron from the metal complex. A reduction mechanism for iron release at pH 7.4 in aqueous solution seems unlikely since $Fe(Me_3MECAMS)^{6-}$, $Fe(NAcMECAMS)^{6-}$, and other tricatecholate compounds have estimated reduction potentials of approximately −750 mV vs. the normal hydrogen electrode at this pH. As with ferric enterobactin, this redox potential is too negative to be affected by physiological reductants. Another possible scenario for extraction of iron would be protease cleavage of the amide bond, which would raise the redox potential within the physiological range.[13] The size, charge, and hydrophobicity of the substituents of the peptide bond can dramatically alter protease activity.[14] Based upon hydrophobicity and steric considerations, we have attached an alkyl group to the amide nitrogens of MECAMS in order to alter enzyme–ligand interactions. Thus, we hope to test the validity of this mechanism for iron aquisition by microbes.

The title compounds have been designed to avoid the drawbacks of enterobactin. Sulfonation enhances the water solubility and phenolic acidity of the ligand. The chelate effect imparted to enterobactin via the central triester ring is retained by use of a benzenoid platform. Alkylation of the amide nitrogen still allows the formation of the FeL^{6-} complex and, moreover, may make the free ligand and iron complex resistant to microbial peptidase activity. We report here the synthesis and stability constants of 1,3,5-tris(*N*-methyl-*N*-(2,3-dihydroxysulfobenzoyl)aminomethyl)benzene [Me_3MECAMS (**6**)] and 1,3,5-tris(*N*-acetyl-*N*-(2,3-dihydroxysulfobenzyl)aminomethyl)benzene [NAcMECAMS (**11**)]. In addition, the kinetics of iron removal from the mammalian-transport and -storage proteins by Me_3MECAMS have been determined.

Materials and Methods

Potentiometric Measurements. We have previously given a detailed account of the apparatus used and the procedure followed for potentiometric titrations.[15] Briefly, measurements were made with a Corning 130 digital pH meter equipped with Corning Glass and saturated calomel electrodes. The meter was calibrated with standard acetate and nitric acid solutions to read hydrogen ion concentration, not activity. Solutions (40 mL) were kept under inert atmosphere (argon) and were maintained at 25 ± 0.05 °C by a circulating water bath. The ionic strength was maintained at 0.1 M with KNO_3. Carbonate-free 0.1 M KOH was prepared from Baker Dilut-It ampules by using freshly boiled, doubly distilled H_2O. Potentiometric data were refined by using a nonlinear least-squares analysis described previously.[15]

Spectrophotometric Measurements. Spectra were recorded on a Cary 118 spectrophotometer. The visible spectra of ferric Me_3MECAMS as a function of pH were obtained from a single solution. After each adjustment of pH, an aliquot was removed, the spectrum recorded, and the sample returned to the original solution. Spectrophotometric competitions using Na_2H_2EDTA[16] were carried out as previously described.[15] Spectra were recorded 24 and 72 h after mixing to assure equilibrium had been achieved.

Kinetics. Apotransferrin (Sigma chemicals) was saturated by the procedure of Bates et al.[17] using 0.02 M $[Fe(NTA)_2]^{3-}$ at pH 7.4. The resulting complex was gel filtered on Sephadex G-25 resin equilibrated with 0.1 M Tris pH 7.4 buffer. The ratio $A_{280}/A_{466} = 11$ demonstrated greater than 95% saturation of transferrin. Kinetic studies were monitored at 520 nm in a Cary 118 UV/vis spectrophotometer equipped with a thermostated quartz cell (1-cm path length) maintained at 25.0 ± 0.05 °C. The time of mixing was taken as time zero. The concentration of ligand used was 0.2 mM and the ionic strength was maintained at 0.1 M with Tris HCl in order to make comparisons with previously published results.[10] Linear plots of $\ln [(A_t - A_\infty)/(A_0 - A_\infty)]$ vs. time were obtained for 1.5 half-lives. The values of k_{obsd} were obtained by linear least-squares analysis.

Horse spleen ferritin was purchased from United States Biochemical. The ferritin was purified by centrifugation for a period of 2 h followed by gel permeation chromatography on Sephadex G-25 by using 0.05 M Tris HCl[16] buffer at pH 7.4 as an eluant. Ferric ion concentration was determined by the absorption at 420 nm ($E^{1\%}_{1cm} = 100$).[18] The procedure of Lowry et al. was followed for the protein assay.[19] The L(+)-ascorbic acid (Matheson, Coleman and Bell) was used as received. Freshly prepared stock solutions of the ligand and ascorbic acid were used for all kinetic runs.

Kinetic studies were performed in a Cary 118 UV/vis spectrophotometer maintained at 37.0 ± 0.05 °C. Reaction mixtures were stored in a water bath at 37 °C in between the time of each absorbance measurement. Time zero was taken to be the time of addition of the stock ferritin solution to the remainder of the reaction mixture. Absorbance measurements at 487 nm were taken once every hour for a period of at least 12 h for all samples. Reaction mixtures were buffered at pH 7.4 (Tris HCl). No attempt was made to exclude oxygen from the sample vials.

The percent iron removed in 6 h was calculated by correcting the absorbance change at 487 nm for the base line absorbance due to ferritin alone. With use of a molar extinction coefficient of 5400 M^{-1} cm^{-1} for $[FeMe_3MECAMS]^{6-}$, the number of millimoles of iron removed was calculated. This value was compared to the initial concentration of ferritin-bound iron.

Syntheses. Experimental Methods. Melting points were taken on a Buchi apparatus in open capillaries and are uncorrected. ^1H NMR spectra were recorded on a Varian A-60 instrument by using Me_4Si (with organic solvents) or 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt

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(with D_2O) as internal standard. Infrared spectra were recorded on a Perkin-Elmer 283 instrument. Evaporations were accomplished in vacuo with a Buchi Rotovapor-RE at ≤55 °C. Thin-layer chromatography (TLC) was performed on precoated 60F-254 silica gel sheets which were developed in tetrahydrofuran (93 mL)/C_6H_{12} (7 mL)/H_2O (5 mL) and then visualized with UV, iodine, or Fe^{3+}/H_2O/EtOH spray. Column chromatography was performed by using 60-200 mesh silica in a 35 × 2.5 cm od column, and fractions were monitored with TLC. Microanalyses and mass spectra (EI-70 eV) were performed by Analytical Services, Chemistry Department, University of California, Berkeley. Trimesoyl chloride (**1**) was purchased from Aldrich Chemical Co., Milwaukee, WI. Compound **7** was prepared according to the literature method.[20]

***N,N′,N″*-Trimethyl-1,3,5-carboxamidobenzene (2).** To a vigorously stirred water-bath-cooled solution of trimesoyl chloride (**1**) (20 g, 75 mmol) in tetrahydrofuran (THF, 300 mL) was added excess methylamine via gas diffusion tube. The reaction mixture was stirred overnight under a Drierite tube and then was filtered. The resulting cake was washed well with H_2O to remove amine·HCl. The cake was then recrystallized from boiling aqueous ethanol to obtain **2** (16.0 g, 86%), mp 312–314 °C.

Anal. Calcd for $C_{12}H_{15}N_3O_3$: C, 57.82; H, 6.07; N, 16.86. Found: C, 57.89; H, 6.02; N, 16.91.

1,3,5-Tris[(*N*-methylamino)methyl]benzene Trihydrochloride (3). Under argon atmosphere, a slurry of triamide **2** (11.0 g, 44 mmol) in BH_3/THF (1 N, 30 mL) was slowly brought to reflux and maintained for 24 h. Then aqueous HCl (6 N, 50 mL) was added (*Caution*! H_2↑) dropwise and maintained at reflux for 60 h. Evaporation in vacuo, followed by coevaporation with methanol to volatilize the borates, gave crude, dry solid which, when crystallized from boiling methanol by the addition of ethanol (cooling and scratching), gave hygroscopic **3** (9.9 g, 70%, dried in vacuo at 70 °C, 6 h), mp 252–255 °C.

Anal. Calcd for $C_{12}H_{21}N_3 \cdot 3HCl$: C, 45.51; H, 7.64; N, 13.27; Cl, 33.58. Found: C, 45.19; H, 8.26; N, 12.93; Cl, 33.04.

1,3,5-Tris(*N*-methyl-*N*-(2,3-dimethoxybenzoyl)aminomethyl)benzene (4). To 2,3-dimethoxybenzoyl chloride (45 mmol) dissolved in THF (75 mL) was added solid **3** (4.75 g, 15 mmol) followed by NEt_3 (12.5 mL, 90 mmol). The reaction mixture was stirred vigorously in a stoppered flask (60 h). Filtration removed the NEt_3·HCl. Evaporation of the THF solution gave crude, oily product which was dissolved in CCl_4 and the mixture was washed well with aqueous NaOH and then entered onto a silica gel column. Elution with (0–5%, v/v) CH_3OH in $CHCl_3$ mixture provided pure product, TLC R_f 0.63. Coevaporation with CCl_4 to remove all other solvents and then Abderhalden drying at 60 °C and 48 h gave glassy **4** (8.3 g, 75%): mp 70–75 °C; IR (neat, NaCl) 2935, 2835 (CH), 1635 (*CO*NR_2), 1580, 1480, 1428, 1400, 1308, 1278, 1230, 1050, 1000, 795, 750 cm^{-1}; ^{1}H NMR (CCl_4) δ 2.72, 2.92 (two s, 9 H, CH_3N), 3.83 (s, 18 H, OCH_3), 4.32, 4.70 (two s, 6 H, $ArCH_2$), 6.7–7.4 (complex m, 12 H, ArH); mass spectrum, *m/e* (relative intensity), 699 (M, 11), 668 (M – OCH_3, 12), 577 (2), 549 (9), 534 [M – $C_6H_3(OCH_3)_2CO$, 10], 165 [$C_6H_3(OCH_3)_2CO$, 100].

Anal. Calcd for $C_{39}H_{45}N_3O_9 \cdot {}^1/_4CCl_4$: C, 63.84; H, 6.14; N, 5.69. Found: C, 63.50; H, 6.22; N, 5.59.

1,3,5-Tris(*N*-methyl-*N*-(2,3-dihydroxybenzoyl)aminomethyl)benzene (5). A solution of **4** (8 g, 10.8 mmol) under argon atmosphere in CH_2Cl_2 (75 mL) was added dropwise via addition funnel to a vigorously stirred solution of BBr_3 (8 mL, 80 mmol) in CH_2Cl_2 (200 mL) and cooled by a room-temperature water bath. An immediate precipitate formed and the slurry was stirred overnight. Dropwise addition (*Caution*! HBr↑) of water (50 mL) followed by aqueous NaOH (40 mL, 6 M) gave a (pH ~6) mixture which was filtered, and the cake was washed well with water. This crude light tan product was dissolved in ethanol and precipitated by the addition of large volumes of ethyl acetate. Filtration, ethyl acetate wash, and then drying in vacuo at 90 °C overnight provided amorphous powder **5** (4.6 g, 70%): mp 130–140 °C (glass); IR (KBr) 1610 (*CO*NR_2), 1585, 1470, 1408, 1280, 1100, 1070, 790, 750, 735 cm^{-1}; ^{1}H NMR (TFA) δ 3.38 (br s, 9 H, NCH_3), 5.22 (vbr s, 6 H, ArCH_2N), 7.0–7.5 (complex m, 12 H, Ar*H*); mass spectrum, *m/e* (relative intensity), 615 (M, 2), 479 [M – $C_6H_3(OH)_2CO$, 6], 343 (M – [$C_6H_3(OH)_2CO$)]$_2$, 5), 136 [$C_6H_3(OH)_2CO$, 26].

Anal. Calcd for $C_{33}H_{33}N_3O_9 \cdot 1{}^1/_2H_2O$: C, 61.67; H, 5.65; N, 6.54. Found: C, 61.38; H, 5.35; N, 6.46.

1,3,5-Tris(*N*-methyl-*N*-(2,3-dihydroxysulfobenzoyl)aminomethyl)benzene Trisodium Salt (6). Solid **5** (4.0 g, 6.0 mmol) was added in portions to vigorously stirred 30% SO_3/H_2SO_4 (36 mL) at room temperature, and stirring was continued in a stoppered flask for 20 h before quenching on excess ice. This solution was brought to pH 4 by slow addition of concentrated aqueous NaOH with external ice-bath cooling. The addition of an equal volume of CH_3OH, filtration of the boiling mixture, and washing of the Na_2SO_4 cake with hot, 1:1 CH_3OH/H_2O gave product-free cake (colorless Fe^{3+} test). The product solution and wash was concentrated in vacuo to a brown water solution. Initially CH_3OH, then EtOH, and finally acetone were sequentially added in small amounts to precipitate less soluble, colored impurities each time removed by filtration. Finally, several volumes of acetone were added to give white product **6**·6H_2O (4.3 g, 69%): mp 290 °C; IR (KBr) 3700–2500 (OH, CH), 1615 (*CO*NR_2), 1492, 1421, 1290, 1230–1170 (SO_3^-), 1108, 1046, 620 (SO_3^-) cm^{-1}; ^{1}H NMR (D_2O) δ 2.8–3.3 (br m, 9 H, CH_3N<), 4.4–4.8 [br m, 6 H, $CH_2N(CH_3)$], 7.2–7.7 (m, 9 H, ArH).

Anal. Calcd for $C_{33}H_{30}N_3O_{18}S_3Na_3 \cdot 6H_2O$: C, 38.49; H, 4.11; N, 4.08; S, 9.34. Found: C, 38.66; H, 3.76; N, 4.01; S, 8.66.

1,3,5-Tris(*N*-(2,3-dimethoxybenzyl)aminomethyl)benzene (8). To an argon-flushed system containing **7** (10.6 g, 16.1 mmol) was added at once, via syringe and septum, BH_3/THF solution (1 H, 150 mL), and then the mixture was heated in a 70 °C oil bath (60 h). Next, aqueous HCl (6 N, 25 mL) was added dropwise (*Caution*! H_2↑) and reflux continued overnight. Evaporation of the THF followed by $CHCl_3$ extraction gave crude aqueous **8**·3HCl solution. Excess 6 N NaOH was added, and the strongly basic solution was extracted well with Et_2O, and then the organic layer was dried with $MgSO_4$ to give a solution of **8**: mass spectrum, *m/e* (relative intensity), 615 (M, 3), 478 [M – $C_6H_3(OCH_3)_2$, 34], 464 [M – $CH_2C_6H_3(OCH_3)_2$,49], 450 [M – $NHCHC_6H_3(OCH_3)_2$, 100]; IR (neat, KBr plate) 3310 (NH), 2940, 2815 (CH), 1585, 1475, 1270, 1220, 1075, 1005, 775, 750 cm^{-1}; ^{1}H NMR (TFA) δ 4.04, 4.12 (two s, 18 H, OCH_3), 4.6 (br, 12 H, $CH_2N^+H_2CH_2$), 7–7.5 (complex m, 9 H, ArH), 7.8–8.0 (complex m, ArH). The addition of HCl, via gas diffusion tube, caused the separation of a white semisolid. The Et_2O was decanted and the compound dissolved in CH_2Cl_2 which was evaporated to leave a white powder and further dried over P_2O_5/NaOH overnight to yield **8**·3HCl (10.6 g, 91%).

Anal. Calcd for $C_{36}H_{45}N_3O_6 \cdot 3HCl$: Cl, 14.67. Found: Cl, 15.20.

1,3,5-Tris(*N*-acetyl-*N*-(2,3-dimethoxybenzyl)aminomethyl)benzene (9). Acetylation of **8** was achieved as follows. **8**·3HCl (10.6 g, 15 mmol) and NEt_3 (6.2 mL, 45 mmol) were slurried in THF (100 mL) for 30 min before the sequential, dropwise addition of CH_3COCl (3.6 mL, 50 mmol), and NEt_3 (6.9 mL, 50 mmol) was dissolved in THF (50 mL). The resulting mixture was stirred overnight in a closed flask at 20–25 °C. Filtration removed NEt_3·HCl, and the THF solution was evaporated to residual oil. The latter was dissolved in CCl_4 and eluted from an aluminum oxide column (neutral, grade 1) sequentially with CCl_4, CH_2Cl_2, and $CHCl_3$ to obtain, in the first fractions, the pure oil **9** (5.0 g, 45%): TLC R_f 0.63; EI-mass spectrum, *m/e* (relative intensity), 741 (M, 10), 698 (M – $COCH_3$, 1), 590 [M – $CH_2C_6H_3(OCH_3)_2$, 5], 548 (23), 534 [M – $CH_3CONHCHC_6H_3(OCH_3)_2$, 10], 208 [$CH_3CONHCHC_6H_3(OCH_3)_2$, 54], 151 [$CH_2C_6H_3(OCH_3)_2$, 100]; IR (neat, KBr plate) 3100–2800 (CH), 1650 (CH_3CON<), 1480, 1420, 1270, 1220, 1080, 1062, 1005, 770, 750 cm^{-1}; ^{1}H HMR (CCl_4) δ 2.10 (br s, 9 H, CH_3CO), 3.73, 3.80 (two s, 18 H, CH_3O), 4.40 [br s, 12 H, $CH_2N(COCH_3)CH_2$], 6.6–7.2 (m, 12 H, ArH).

Anal. Calcd for $C_{42}H_{51}N_3O_9$: C, 68.00; H, 6.93; N, 5.66. Found: C, 67.79; H, 6.73; N, 5.45.

1,3,5-Tris(*N*-acetyl-*N*-(2,3-dihydroxybenzyl)aminomethyl)benzene (10). In an argon atmosphere, **9** (5.0 g, 6.7 mmol) dissolved in CH_2Cl_2 (50 mL) was added dropwise via addition funnel to a vigorously stirred solution of BBr_3 (5 mL, 50 mmol) in CH_2Cl_2 (200 mL) immersed in a 20–25 °C water bath. After the solution was stirred overnight, H_2O (50 mL) was added dropwise (*Caution*! HBr↑) followed by sufficient 6 N aqueous NaOH to maintain pH 4 in the aqueous layer. Solid was collected by filtration, washed well with H_2O, and then crystallized from hot aqueous EtOH upon overnight cooling. Filtration, water wash, and vacuum desiccator drying over P_2O_5/NaOH gave white powder **10**·1$^1/_2H_2O$ (3.0 g, 65%): mp 186–189 °C; IR (KBr) 3600–2700 (OH, CH), 1615 (CH_3CON<), 1590, 1485, 1440, 1360, 1272, 743 cm^{-1}; ^{1}H NMR (TFA) δ 2.57, 2.97 (two s, 9 H, CH_3CO), 5.07 [br s, $CH_2N(COCH_3)CH_2$], 7.0–7.7 (br m, 12 H, ArH); mass spectrum, *m/e* (relative intensity), 658 (M, 19).

Anal. Calcd for $C_{36}H_{39}N_3O_9 \cdot 1{}^1/_2H_2O$: C, 63.15; H, 6.18; N, 6.14. Found: C, 63.27; H, 6.06; N, 6.14.

1,3,5-Tris(*N*-acetyl-*N*-(2,3-dihydroxysulfobenzyl)aminomethyl)benzene Trisodium Salt (11). Compound **10** (2.6 g, 3.8 mmol) was dissolved in SO_3/H_2SO_4 (30%, 25 mL) vigorously stirred, at ambient temperature, and allowed to react overnight in a stoppered flask. It was then poured onto ice and neutralized to pH 4 by the slow addition of 6 N NaOH with vigorous stirring and ice-bath cooling. Slow addition of an equal volume of MeOH to the boiling solution and filtration and evaporation of the filtrate gave crude product. The latter was dissolved in a small amount of H_2O and adjusted to turbidity by successive addition of CH_3OH, EtOH, and Et_2O; each time filtering out the inorganic and colored impurities. Finally, several volumes of Et_2O precipitated white

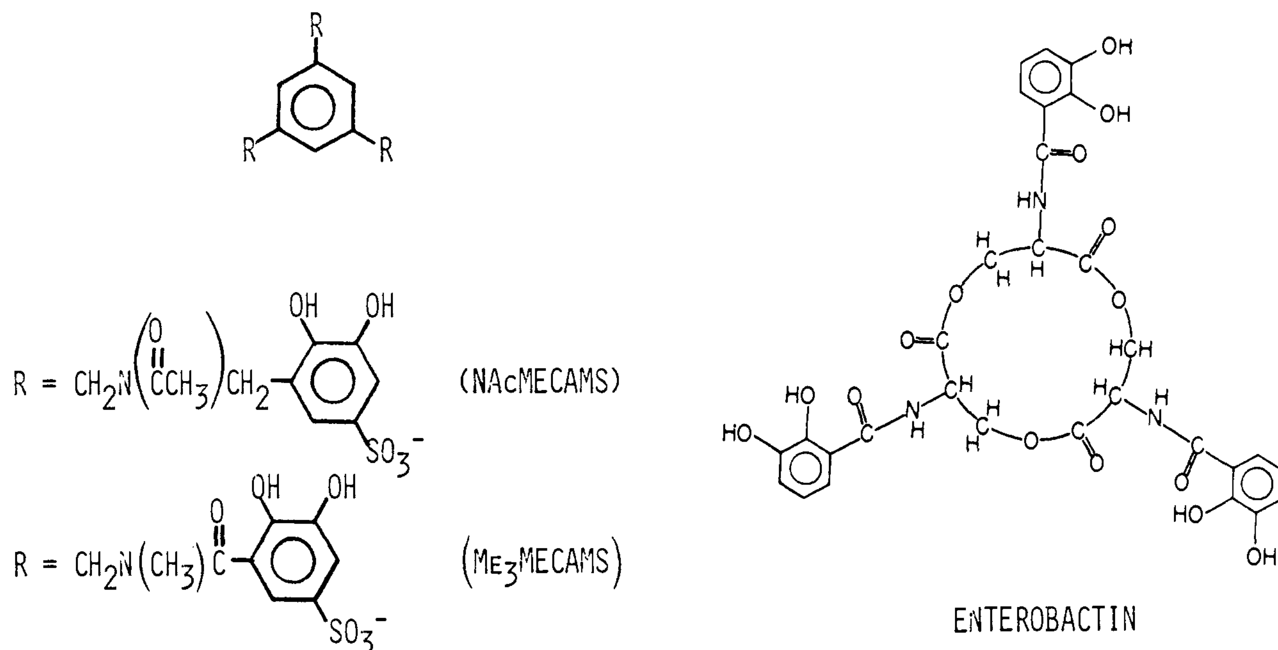

Figure 1. (a) Enterobactin; (b) 1,3,5-tris[(methyl(2,3-dihydroxysulfobenzoyl)amino)methyl]benzene [Me_3MECAMS]; (c) 1,3,5-tris[(acetyl(2,3-dihydroxysulfobenzyl)amino)methyl]benzene [NAcMECAMS].

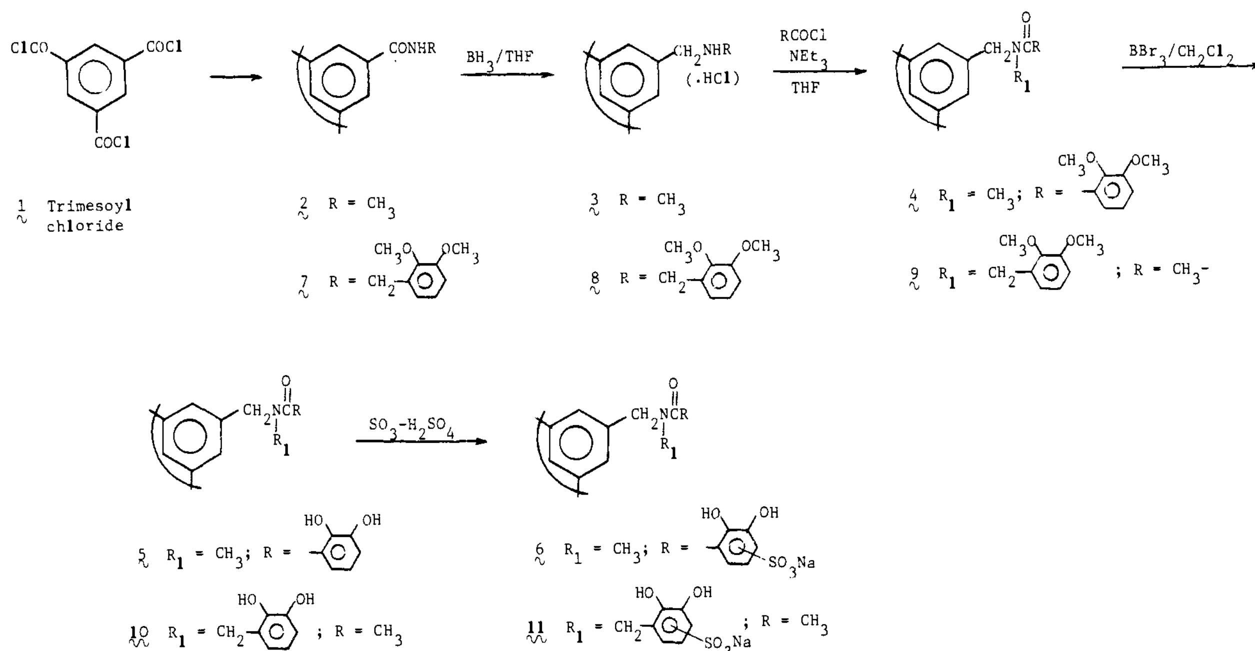

Figure 2. Synthesis of symmetrical 1,3,5-trisubstituted benzene tertiary catechoylamides.

product from colorless filtrate. The product was redissolved in H_2O and evaporated to dryness to remove organic solvents and then dried over P_2O_5, in vacuo, at 20–25 °C to obtain hygroscopic **11**·$6H_2O$ (3.3 g, 80%): mp 260–262 °C dec; IR (KBr) 3700–2500 (OH, CH), 1615 (CH_3CON<), 1490, 1435, 1360, 1300–1150 (SO_3), 1108, 1048, 630 cm^{-1}; ^1H NMR (D_2O) δ 2.20, 2.37 (two s, 9 H, CH_3CO), 4.3–5.0 [br, 12 H, $CH_2N(COCH_3)CH_2$], 6.6–7.5 (m, 6 H, ArH).

Anal. Calcd for $C_{36}H_{36}N_3O_{18}S_3Na_3 \cdot 6H_2O$: C, 40.34; H, 4.51; N, 3.92, S, 8.97. Found: C, 40.17; H, 4.02; N, 3.81; S, 9.45.

Note: Since there is no unambiguous proof of the position of sulfonation, we have not specified this for compounds **6** and **11** (Figure 2). However, previous work with similar compounds showed this occurs exclusively in the 5 position of the catechol ring.

Results and Discussion

Thermodynamics. Me_3MECAMS. The potentiometric equilibrium curves (pH vs. OH^- added) for deprotonation of Me_3MECAMS (**6**) and complexation of Fe(III) are shown in Figure 3. Since the individual catechol groups of Me_3MECAMS are essentially the same as the monocatechol ligand *N,N*-dimethyl-2,3-dihydroxy-5-sulfobenzamide (**12**), the protonation constants of **12** (log K_1 = 11.5, log K_2 = 7.26)[1] should be very similar to the average values for Me_3MECAMS. Indeed, the six protonation reactions of Me_3MECAMS fall into two groups of three protons each. The first (more basic) protonations occur at pH >11 with no evidence of appreciable dissociation below this pH. No attempt was made to determine directly log K_n^H for n = 1–3. An estimate for these constants, based on the protonation of **12**, is log $K_n^{H(av)}$ = 11.8 for n = 1–3 for Me_3MECAMS. The three more acidic protons have log $K_{4-6}^{H(av)}$ = 7.6 (see Table II). The break at a = 3 is consistent with these results. These protons are much more acidic than those of enterobactin and previously synthesized unsulfonated catechoylamide sequestering agents.

The potentiometric titration of the ferric complex (curve b in Figure 3) shows that initial complexation is quite strong, as is typical for iron(III) catecholate complexes. There is a two-proton buffer region between pH 5.5 and 7.3. Least-squares refinement

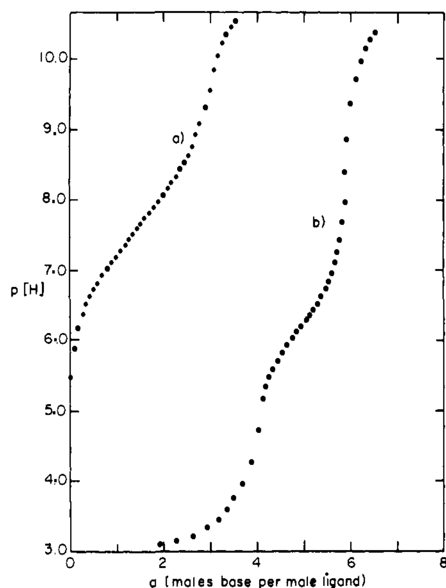

Figure 3. Potentiometric equilibrium curves for $Me_3MECAMS$. (a) 1.5 × 10^{-3} M $Me_3MECAMS$; (b) $Me_3MECAMS + Fe^{3+}$, 1:1, 1.3 × 10^{-3} M.

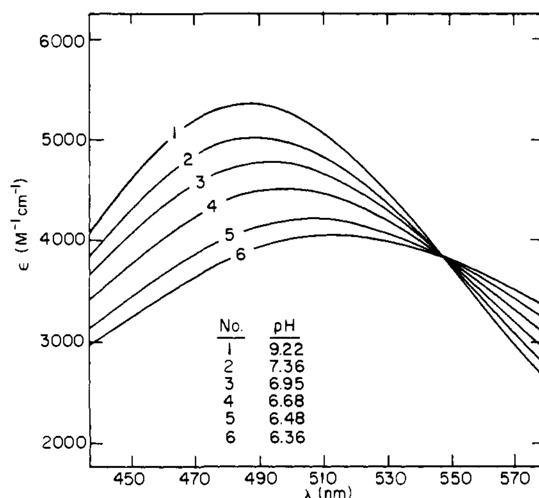

Figure 4. Visible spectrophotometric titration of $[FeMe_3MECAMS]^{6-} + H^+ \rightarrow [HFeMe_3MECAMS]^{5-}$ from pH 6 to 9.5 ($[FeMe_3MECAMS]$ = 2 × 10^{-4} M; μ = 0.10 (KNO_3); T = 25 °C).

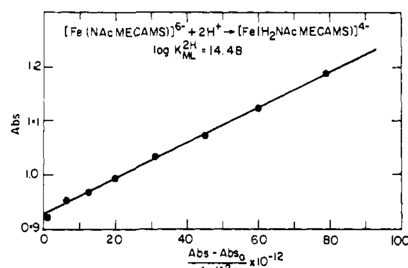

Figure 5. Schwarzenbach plot for the first chelate protonation reaction of $[FeMe_3MECAMS]^{6-}$. Conditions are given in Figure 4.

of these data gives the protonation constants listed in Table II. The visible spectra for the first protonation equilibrium are shown in Figure 4. The λ_{max} for the tris complex is 487 nm (ϵ = 5390 M^{-1} cm^{-1}). Upon protonation, the peak shifts to lower energy with a maximum near 512 nm. The first metal protonation constant was also determined spectrophotometrically by the method of Schwarzenbach in which ϵ vs. $[(\epsilon - \epsilon_0)/[H^+]^n]$ is plotted.[21] A

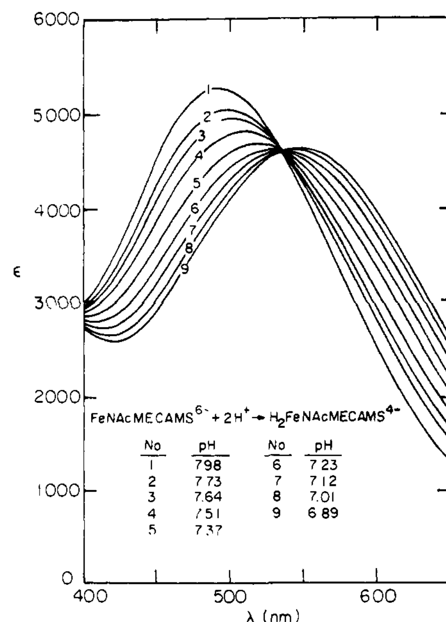

Figure 6. Visible spectrophotometric titration of $[FeNAcMECAMS]^{6-} + 2H^+ \rightarrow [H_2FeNAcMECAMS]^{4-}$ from pH 7 to 8 ([FeNAcMECAMS] = 1.9 × 10^{-4} M; μ = 0.10 (KNO_3); T = 25 °C).

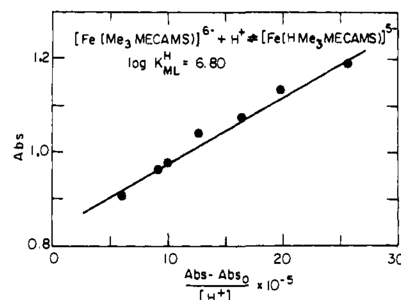

Figure 7. Schwarzenbach plot for the first chelate protonation reaction of $[FeNAcMECAMS]^{6-}$. Conditions are given in Figure 6.

linear plot (Figure 5) is obtained by choosing the appropriate value for n, which is simply the proton stoichiometry for the reaction. The slope of the line directly gives K^H_{ML}. The multiple, overlapping equilibria made it impossible to use this method to refine the lower constants. The proton-dependent formation constant (K^*, Table II) was calculated from competition experiments with Na_2H_2EDTA at pH ~6.8. This value was corrected for metal–ligand protonation and hydrolysis equilibria observed in this pH regime.

NAcMECAMS. The phenolic protons of NAcMECAMS are much more basic than for $Me_3MECAMS$ as can be seen in Table I. The decreased acidity is a direct result of the relocation of the amide carbonyl to a position where it can no longer be an electron-withdrawing substitutent of the catechol ring. As with $Me_3MECAMS$ the higher protonation constants have been estimated by using DMBS[16] **12** as a model; however, due to the slightly higher pK's of the acidic protons of NAcMECAMS, a value of 12.1 for the basic protons has been used.

At high pH, the ferric complex of NAcMECAMS is similar to other catecholate ligands investigated with ϵ = 5400 M^{-1} cm^{-1} at 487 nm for the tris complex. Figure 6 illustrates the change in the visible absorption spectra as the pH is lowered. Unlike $Me_3MECAMS$ and other tricatecholate ligands with carbonyl oxygens α to the ring, NAcMECAMS undergoes a single, two-proton step to yield a ferric bis catecholate complex (Figure 7). This protonation scheme is similar to that observed for TRIMCAMS and DMBS.[1] Competition experiments (as above) with Na_2H_2EDTA were again used to determine K^*.

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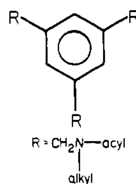
Table I. Symmetrical 1,3,5-Trisubstituted Benzene Catechoylamides (3°)



no.	alkyl	acyl	% yield	mp, °C	M^+ (rel int)	ν_{CO} (KBr), cm^{-1}	^{1}H NMR δ[a] $CH_3N<$	^{1}H NMR δ[a] $CH_3CON<$
4	CH_3	2,3-dimethoxybenzoyl	75	70-75	699 (11)	1635	2.72, 2.92 (CCl_4)	
9	2,3-dimethoxybenzyl	acetyl	45	oil	741 (10)	1650		2.10 (CCl_4)
5	CH_3	2,3-dihydroxybenzoyl	70	130-140	615 (2)	1610	3.38 (TFA)	
10	2,3-dihydroxybenzyl	acetyl	65	186-189	658 (19)	1615		2.57, 2.97 (TFA)
6	CH_3	(2,3-dihydroxybenzoyl) sodium sulfonate	69	290 dec		1615	2.8, 3.3 (D_2O)	
11	(2,3-dihydroxybenzyl) sodium sulfonate	acetyl	80	260-262 dec		1615		2.20, 2.37 (D_2O)

[a] Broad/overlapping double resonance due to hindered rotation about the amide bond.

Table II. Physical Data for $Me_3MECAMS$ and NAcMECAMS with Fe(III)

	$Me_3MECAMS$	
$\log K_{1-3}{}^{av} = 11.8$[a]	$\log \beta_{013}{}^{f} = 35.4$[a]	$\log K^{H}_{ML} = 6.80$ (3)[b]
$\log K_4 = 8.52$ (2)	$\log \beta_{014} = 43.9$ (8)[a]	$\log K^{H}_{ML} = 6.61$ (5)[c]
$\log K_5 = 7.57$ (2)	$\log \beta_{015} = 51.5$ (8)[a]	$\log K^{H}_{MLH} = 5.80$ (5)[c]
$\log K_6 = 6.72$ (2)	$\log \beta_{016} = 58.2$ (8)[a]	
$\log K^* = 5.21$ (3)[g]	$\log \beta_{110} = 40.6$ (8)[a]	pM 26.9[d]
iron removed from horse spleen ferritin after 6 h = 3.7%[e]		
	NAcMECAMS	
$\log K_{1-3}{}^{av} = 12.1$[a]	$\log \beta_{013} = 36.3$ (3)[a]	$\log K^* = 4.00$ (5)[g]
$\log K_4 = 9.3$ (1)	$\log \beta_{014} = 45.6$ (8)[a]	$\log \beta_{110} = 40.3$ (8)[f]
$\log K_5 = 8.4$ (1)	$\log \beta_{015} = 54.0$ (8)[a]	$\log K^{2H}_{ML} = 14.48$ (3)[b]
$\log K_6 = 7.7$ (1)	$\log \beta_{016} = 61.7$ (8)[a]	pM 25.0[d]

[a] Based on estimate for high protonation constant using N,N-dimethyl-2,3-dihydroxy-5-sulfobenzamide. [b] Determined from spectrophotometric titration. [c] Determined from potentiometric titration. [d] Conditions are pH 7.4, 10 μM total ligand, 1 μM total Fe^{3+}. [e] [$Me_3MECAMS$] = 6.0 mM; [ascorbate] = 6.0 mM. [f] The formation constant, β_{mlh}, for the reaction $mM + lL + hH^+ \rightarrow M_mL_lH_h$. [g] $K^* = ([H^+]^3[FeL^{6-}])/([H_3L^{6-}][Fe^{3+}])$.

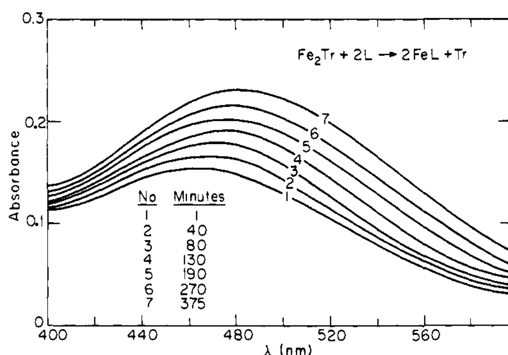


Figure 8. Spectral changes associated with exchange of transferrin-bound iron with $Me_3MECAMS$ ([$Me_3MECAMS$] = 2.0×10^{-4} M; [Fe_2Tr] = 2.9×10^{-5} M; pH 7.4; T = 25 °C).

Kinetics. The visible spectra for the reaction of $Me_3MECAMS$ with diferric transferrin is shown in Figure 8. The absorbance maximum shifts from 466 nm, indicative of an iron transferrin complex, to the 487-nm peak of the tris catecholate species. A plot of ln $[(A_t - A_\infty)/(A_0 - A_\infty)]$ vs. time, gave a first-order rate constant, k_{obsd}, for the reaction (Figure 9). Under the conditions previously used,[9] $k_{obsd} = 1.9 \times 10^{-3}$ min^{-1} with a half-life of 6 h. After 30 min 4% of the iron had been removed from transferrin.

Stability Constants. Table II lists all relevant stability constants. A direct effect of sulfonating the catecholate ring is the increased acidity of the phenolate oxygens. The final three protonations of $Me_3MECAMS$ occur at pH values much lower than observed

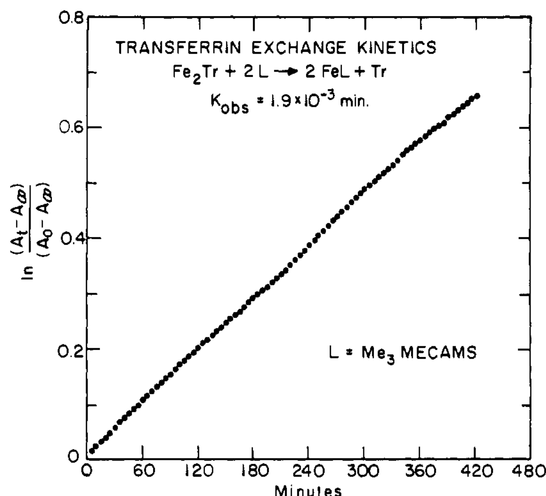


Figure 9. Plot in ln $[(A_t - A_\infty)/(A_0 - A_\infty)]$ vs. time for the removal of iron from transferrin. The slope of this line gives the observed rate constant, k_{obsd}.

for enterobactin or other unsulfonated catechoylamides (9.2, 8.4, and 7.6 for log K_n^{ent} where n = 4-6, respectively). Although NAcMECAMS is sulfonated, the log K_{1-3}^{av} for this ligand is comparable to that of enterobactin. Since the carbonyl group is no longer conjugated to the catechol ring, its electron-withdrawing capacity has been replaced by a slightly electron-donating methylene group. In essence, the increased electron-withdrawing

Table III. pM Values of Selected Fe(III) Sequestering Agents

ligand	pM^a ($-\log [Fe_{aq}^{3+}]$)	ref[b]
enterobactin	35.5	27
HBED[c]	31.0	28
MECAM	29.4	29
MECAMS	29.1	1
3,4-LICAMS	28.5	1
$Me_3MECAMS$	26.9	
ferrioxamine B	26.6	1
EHPG[d]	26.4	28
TRIMCAMS	25.1	1
NAcMECAMS	25.0	
NTPA[e]	24.7	27
transferrin	23.6	30, 31
EDTA[f]	22.2	28
tiron[g]	19.5	32

[a] Calculated for 10 μM ligand, 1 μM Fe^{3+}, pH 7.4. [b] pM values based on thermodynamic data from these references. [c] *N,N*-bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid. [d] Ethylene-1,2-bis(2-hydroxyphenylglycine). [e] Diethylenetriaminepentaacetic acid. [f] Ethylenediaminetetraacetic acid. [g] 1,2-Dihydroxy-3,5-disulfobenzene.

ability of the sulfonyl group is canceled by the loss of the α-carbonyl and is reflected in the ligand protonation constants. In general, however, sulfonated ligands, as discussed below, become more effective chelating agents at physiological pH.

Since the corresponding trialkyl-substituted ligand 1,3,5-tris-(*N*-isopropyl-*N*-(2,3-dihydroxy-5-sulfobenzoyl)aminomethyl)-benzene [$(i\text{-Pr})_3$MECAMS][23] does not form a tris complex with Fe(III) below pH 10, there was some concern that the methyl groups on the amide nitrogen of $Me_3MECAMS$ might similarly hinder the formation of a tris catecholato complex until high pH. However, by pH 9, both $Me_3MECAMS$ and NAcMECAMS apparently form tris complexes with Fe(III). The visible spectra ($\lambda_{max} = 487$, $\epsilon = 5390$), potentiometric titration curve (break at $a = 6$), and magnitude of the formation constant (vide infra) are all consistent with a tris species.

The metal chelate protonation equilibria for $Me_3MECAMS$ are similar to those of enterobactin, MECAM, and MECAMS. Upon protonation, a shift from a catecholate to a salicylate mode of bonding (via the amide carbonyl and ortho phenolate oxygens) has been proposed for these three ligands.[15] The visible spectra and proton stoichiometry determined from a Schwarzenbach plot (Figure 6) for $Fe(Me_3MECAMS)^{6-}$ are consistent with those of the previously studied ligands and would suggest that this complex also undergoes a shift to salicylate coordination.

In contrast to ligands with a carbonyl group α to the catechol ring, the low pH mode of bonding in NAcMECAMS is via bis catecholate coordination. This formulation is supported by a break at $a = 4$ in the potentiometric titration curve. The visible spectra of the deprotonated species (Figure 5) is similar to that of $Fe(cat)_2^-$. Moreover, an analysis of the visible titration, using a Schwarzenbach plot, demonstrates that this reaction proceeds via a single two-proton step (Figure 7).

For NAcMECAMS, bis catecholate coordination of ferric ion is preferable to a salicylate bonding mode since the formation of a complex via the *o*-phenol and carbonyl oxygens would require an eight-membered chelate ring, whereas salicylate coordination in $Me_3MECAMS$ is achieved by a stable five-membered ring.

The usual proton-independent formation constant, $\log \beta_{110}$ ($Fe^{3+} + L^{9-} \rightarrow FeL^{6-}$), is estimated to be 40.6 and 40.3 for $Me_3MECAMS$ and NAcMECAMS, respectively. This value is nearly 13 orders of magnitude lower than the value reported for enterobactin and 6 orders of magnitude less than values for the unsulfonated derivatives MECAM or LICAM.[15] However, these constants are strictly valid only at pH 14, where the reacting species is a fully deprotonated L^{9-} ligand. Below this pH, ligand and metal chelate protonation equilibria have a profound effect. For this reason we have used pM, defined as $-\log [M^{n+}]$, as a basis for comparison of relative ligand complexing strength at pH 7.4 and a defined metal and ligand concentration, thus affording a direct evaluation of sequestering ability under physiologically relevant conditions. The pM value for $Me_3MECAMS$ is within 2.5 log units of MECAM under the conditions listed in Table III, even though MECAM has a much greater formation constant. More importantly, $Me_3MECAMS$ has a pM which is 3 log units greater than transferrin. Thus, $Me_3MECAMS$ is thermodynamically capable of removing iron from transferrin under physiological conditions.

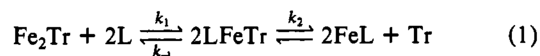
(23) Harris, W. R., personal communication.

Table IV. Kinetic Ability of Ligands to Remove Transferrin-Bound Iron

ligand	$10^3 k_{obsd}$,[a] min^{-1}	% Fe removed (30 min) 40:1[b]	1:1
enterobactin	2.2		6
MECAM	3.4		13
3,4-LICAMS	2.2	50	6
$Me_3MECAMS$	1.9		4[c]
3,4-LICAMC[d]	2.1		6[c]
desferal		5[e]	
EDTA		37[f]	
DMBS		36	0

[a] Ligand concentration 0.2 mM. [b] Ratio of ligand to transferrin. [c] 7:1 ratio. [d] Synthesis and characterization to be reported separately.[22] [e] 100:1 ratio. [f] 2500:1 ratio.

Kinetics. Carrano and Raymond[10] have investigated the ability of catecholate chelating agents to remove iron from transferrin. The mechanism of iron removal is believed to involve a ternary complex as is shown in eq 1. The results indicated that both



$$Fe_2Tr + 2L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2LFeTr \overset{k_2}{\rightleftharpoons} 2FeL + Tr \quad (1)$$

natural and synthetic catechoylamides were capable of kinetically affecting exchange of transferrin-bound iron. The rate constant reported for $Me_3MECAMS$ (Table IV) is consistent with these results. Although iron exchange with $Me_3MECAMS$ is slightly slower than with other catechoylamides, it is still able to sequester 4% of transferrin-bound iron in 30 min at a 7:1 ligand to transferrin ratio. In contrast, Desferal, at a 100:1 ligand to transferrin ratio, can remove only 5% of the transferrin-bound iron. These results suggest that $Me_3MECAMS$ is both thermodynamically and kinetically able to remove iron from transferrin.

Mobilization of storage-type ferric ion from ferritin, through mediation of ascorbate, is one mechanism by which Desferal is believed to sequester in vivo iron.[8] For this reason, we investigated the ability of $Me_3MECAMS$ to remove iron from ferritin. The ascorbate dependence and mechanism of iron exchange are discussed more fully in a separate paper.[24] Under the conditions of 6 mM $Me_3MECAMS$ and 6 mM ascorbate, 3.7% of total ferritin-bound iron was removed in 6 h at pH 7.4. This value is equal to or better than that reported for ferrioxamine B,[24,25] suggesting that the sulfonated catechol derivative would as effectively sequester storage iron forms.

Experiments performed by Dr. Rowe Byers' group at the University of Mississippi have shown that $FeMe_3MECAMS$ acts as a growth factor for *B. subtilis*.[26] Thus, it is unlikely that

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cleavage at the amide bond is a mechanism for iron release in synthetic catechoylamide sequestering agents.

Summary

We have described here a new ligand, Me_3MECAMS, which has a high affinity for ferric ion at physiological pH and can remove iron from transferrin at a significant rate. Moreover, Me_3MECAMS can mobilize iron from ferritin when ascorbate is present. Sulfonation of the ligand affords a high water solubility, as well as lowering the ligand protonation constants. The solution chemistry of Me_3MECAMS is analogous to enterobactin, in that it forms a tris catechol complex at high pH and most likely shifts to a salicylate mode of bonding upon protonation. A second compound, NAcMECAMS, forms a tris(catecholato)iron(III) complex at high pH but undergoes a 2 H^+ step which causes dissociation of an arm to form a bis catecholate coordination geometry. Unlike previously prepared catechoylamides, Me_3MECAMS and NAcMECAMS contain only tertiary amide nitrogens. Although N-substitution presumably does not affect microbial acquisition of iron from $FeMe_3$MECAMS, this modification may be important for future design of orally efficacious drugs which could encounter nonspecific peptidase activity.

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High-Resolution Solid-State ^{13}C NMR. Conformational Studies of NADH and NAD^+ Model Systems

Gary R. Hays,*,† Rob Huis,† Brian Coleman,*,† Derek Clague,† Jan W. Verhoeven,‡ and Frank Rob‡

Contribution from the Koninkliyke/Shell-Laboratorium, Amsterdam (Shell Research B.V.), Badhuisweg 3, 1031 CM Amsterdam, The Netherlands, and the Laboratory for Organic Chemistry, University of Amsterdam, 1018 WS Amsterdam, The Netherlands.
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Abstract: High-resolution solid-state ^{13}C NMR shows that, in the solid form, the Hantzsch ester [3,5-bis(ethoxycarbonyl)-2,6-dimethyl-1,4-dihydropyridine] adopts an asymmetric conformation. A full analysis of the solid-state spectrum was possible by comparison with the available X-ray data. The asymmetric conformation can be rationalized in terms of intramolecular electronic interactions, and similar arguments have allowed us to deduce conformational information from the solid-state spectra of three other symmetrically substituted 1,4-dihydropyridines and their parent pyridinium ions, for which no X-ray data are presently available. All three dihydropyridines display an asymmetric conformation while their pyridinium analogues are symmetrical. In the case of at least one of these [1-benzyl-3,5-bis(methylaminocarbonyl)pyridinium bromide] it is possible to show that both carbonyl groups point away from the ring nitrogen.

In the last couple of years, the combined techniques of high-power proton decoupling, cross-polarization (CP), and magic-angle sample spinning (MAS) have become more and more routinely used in order to obtain high-resolution ^{13}C NMR spectra of solids. Dipolar decoupling eliminates the dipolar interactions; MAS eliminates chemical shift anisotropy (CSA) if the speed of rotation is fast enough;[1] and a cross-polarization pulse sequence[2] enhances the sensitivity of the whole experiment.

Although the isotropic chemical shifts obtained from solids are generally close to those measured in solution, an interesting facet of such experiments on solids can be that dynamic averaging (which occurs readily in solution) is often absent. Hence the "freezing" of free rotation of bulky substituents leads to the formation of fixed conformations. This means that, whereas in solution motion occurs to give average chemical shifts for the various conformers, in the solid state these conformations are "locked" in position. This can create unique chemical environments for nominally equivalent carbon atoms. The chemical shift differences generated by these environments can be seen in the solid-state spectrum as additional line splittings.

Solid-state ^{13}C NMR has already shown this conformational isomerism in solid polymers in the glassy state,[1] examples being poly(phenylene oxide), polysulfone, and a variety of polycarbonates, as well as in crystalline polymers.[3] Less work has been published on small organic molecules. Crystalline 1,4-dimethoxybenzene exists in a single symmetric form rather than as a mixture of the two possible isomers. The preferred anti conformation is locked in position and produces two types of chemical environment for the protonated ring carbons. This difference has been detected by solid-state ^{13}C NMR: a doublet is seen for these carbons in the resultant spectrum.[4,5] 1,3,5-Trimethoxybenzene, on the other hand, produces a spectrum from which it is clear that all three protonated aromatic carbons are chemically nonequivalent in the solid state, giving rise to three lines of nearly equal intensity.[6] This result can only be consistent with the existence of a single asymmetric conformational isomer.

† Shell Research B.V.
‡ University of Amsterdam.

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