Solution Thermodynamics of the Ferric Complexes of New Desferrioxamine Siderophores Obtained by Directed Fermentation

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Abstract: Thirteen new desferrioxamine-type siderophores, designated X_1-X_6, Et_1-Et_3, Te_1-Te_3, and P_1, were obtained from cultures of *Streptomyces olivaceus* Tü 2718 by supplementing the production medium with ornithine or 1,4-diaminobutane, 1,6-diaminohexane, bis(2-aminoethyl) ether, *S*-(2-aminoethyl)cysteine, and *N*-glycylethylenediamine. The stability constants of the ferric complexes of the trihydroxamate ligands, X_1-X_4, Et_1-Et_3, and Te_1-Te_3, were determined by EDTA competition reactions and are in the range log β_{110} = 29.7-31.8 (β_{110} = [FeL]/([Fe^{3+}][L^{3-}], 25 °C, 0.1 M KNO_3). The stability of the complexes decreases monotonically as the structure of the ligand differs from that of desferrioxamine E (β_{110} = 32.21, 25 °C, 0.1 M KNO_3), the main siderophore of *S. olivaceus* Tü 2718 under natural growth conditions. The dihydroxamic acid desferrioxamine X_5 forms two types of ferric complexes, depending on pH. The predominant species above pH 6.4 is a dimer of formulation Fe_2L_3, which dissociates into a monomeric cation, FeL^+, at lower pH. The stability constants of these two species determined by spectrophotometric titration are log β_{230} = 55.35 and log β_{110} = 19.18 (25 °C, 0.1 M KNO_3). The relative effectiveness of the new ferrioxamines as mediators of iron uptake via the hydroxamate ferric ion transport system of *Staphylococcus aureus* DSM 799 differs widely, however, without correlating with the complex stability.

Introduction

Under iron-deficient conditions most aerobic organisms secrete low molecular weight, highly specific iron(III) chelating compounds (siderophores), which solubilize ferric ions and subsequently actively transport them into the cells via specific receptors in the microbial membranes.[1,2] The pathogenicity of certain microorganisms has been associated with their ability to utilize iron from high-affinity proteins, such as transferrin and lactoferrin, in physiological systems.[3]

The desferrioxamines are an important class of hydroxamate-type siderophores, which are produced by most of the actinomycetes and various other bacteria.[4-7] They consist of units of acetate, succinate, and ω-amino-α-(*N*-hydroxyamino)alkane, which are combined into linear or cyclic molecules of about 500-600 Da. In general they are hexadentate ligands, containing three hydroxamate groups, which chelate iron(III) very specifically. Exceptions are desferrioxamine H,[8] bisucaberin,[9] and alcaligin,[10] which are dihydroxamic acids. The linear desferrioxamine B (Desferal) is produced industrially by fermentation of *Streptomyces pilosus* and used clinically for the treatment of a variety of disorders related to iron and aluminum overload in man.[11,12] Since the application of this drug is associated with certain problems, efforts continue to obtain better natural or synthetic substitutes for Desferal.

Our approach to obtain new siderophores was the directed fermentation of a *Streptomyces olivaceus* strain by adding suitable precursors to the culture medium. Under iron-limited conditions this strain produces high amounts of desferrioxamine E,[13] which is identical with the antibiotic nocardamine (Figure 1).[14] Since decarboxylation of lysine to form 1,5-diaminopentane is the first step of desferioxamine E biosynthesis, as shown for *Streptomyces glaucescens*,[15] diamino acids and diamines seemed to be suitable candidates for culture additives. Modified desferrioxamines are expected to exhibit different properties with regard to their pharmacological behavior, their specificity toward various metal ions (especially Fe^{3+} and Al^{3+}), and their uptake by microorganisms. Furthermore, the antitumor activity reported for the dihydroxamic acid bisucaberin[16] increases the interest in this class of compounds.

Here, we report on the iron(III) chelating properties of 10 new desferrioxamines, X_1-X_6, Et_1-Et_3, Te_1-Te_3, and P_1, obtained by feeding ornithine or 1,4-diaminobutane, 1,6-diaminohexane, bis(2-aminoethyl) ether, *S*-(2-aminoethyl)cysteine, and *N*-glycylethylenediamine to cultures of *S. olivaceus* Tü 2718.

Experimental Section

Thin-Layer Chromatography. TLC was performed on precoated silica gel 60 F_{254} plates (Merck, Darmstadt, FRG) using either chloroform/methanol/water (64:24:4) (I) or chloroform/methanol/acetic acid/water (65:24:3:4) (II) as the solvent system. Compounds were detected by spraying with ninhydrin, chlorine/4,4′-bis(dimethylamino)diphenylmethane, or $FeCl_3$ solution.

Isolation and Characterization of Desferrioxamines. The desferriox-

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Table I. Precursors and Structural Components of the New Desferrioxamines Isolated from *S. olivaceus* Tü 2718 (For Comparison Purposes, the Known Desferrioxamines E and D_2 Are Included)

precursor	desferrioxamine	A	B	C	D	E	X	Y	Z
none	E	CH_2	CH_2	*a*	CH_2	*a*	OH	OH	OH
	D_2	CH_2	CH_2	*a*	*a*	*a*	OH	OH	OH
ornithine or 1,4-diaminobutane	X_1	CH_2	*a*	*a*	*a*	*a*	OH	OH	OH
	X_2	*a*	*a*	*a*	*a*	*a*	OH	OH	OH
1,6-diaminohexane	X_3	CH_2	CH_2	CH_2	CH_2	*a*	OH	OH	OH
	X_4	CH_2	CH_2	CH_2	CH_2	CH_2	OH	OH	OH
	X_5	CH_2	CH_2	CH_2	CH_2	*a*	OH	OH	H
	X_6	CH_2	CH_2	CH_2	CH_2	CH_2	OH	OH	H
bis(2-aminoethyl) ether	Et_1	CH_2	CH_2	*a*	O	*a*	OH	OH	OH
	Et_2	CH_2	O	*a*	O	*a*	OH	OH	OH
	Et_3	O	O	*a*	O	*a*	OH	OH	OH
S-(2-aminoethyl)cysteine	Te_1	CH_2	CH_2	*a*	S	*a*	OH	OH	OH
	Te_2	CH_2	S	*a*	S	*a*	OH	OH	OH
	Te_3	S	S	*a*	S	*a*	OH	OH	OH
N-glycylethylenediamine	P_1[b]	CH_2	CH_2	*a*	C(O)	NH	OH	OH	OH

[a] Structural components which do not exist in the molecule. [b] Molecule P_1 does not contain position 8''; see Figure 1.

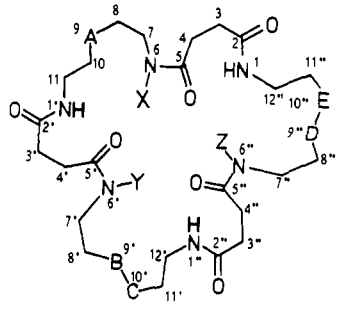

Figure 1. Structures of the desferrioxamines produced by *S. olivaceus* Tü 2718. Structural components are given in Table I.

amines were produced by fermentation of the wild-type strain *S. olivaceus* Tü 2718 under iron-limiting conditions and simultaneous feeding of one of the precursors: ornithine, 1,4-diaminobutane, 1,6-diaminohexane, bis(2-aminoethyl) ether, *S*-(2-aminoethyl)cysteine, or *N*-glycylethylenediamine.[13] The presence of new desferrioxamines in the culture supernatants was detected by reversed-phase HPLC of the ferric complexes combined with photodiode array detection.[17] The desferrioxamines were isolated by adsorption on the resin Amberlite XAD-16 (Rohm & Haas). After the column was washed with distilled water and $MeOH/H_2O$ (1:9), desorption was carried out with $MeOH/H_2O$ (8:2). After evaporation, the resulting brown material was further purified by flash chromatography on silica gel 60 using $CHCl_3/MeOH/H_2O$ (65:25:4) as the solvent system (column 25 × 5 cm i.d.; loaded with maximum 4 g of mixture). The Fe^{3+}-positive fractions were combined and evaporated to dryness. Separation of the desferrioxamine homologues was performed by preparative HPLC on a Nucleosil-100 C18 column (10 μm; 250 × 32 mm i.d., precolumn 20 × 16 mm i.d.; Grom, Ammerbuch, FRG) with $MeOH/H_2O$ (4:6) as eluent (flow rate 60 mL/min; detection at 240 nm). The apparatus and column were cleansed of Fe^{3+} by chromatographing a desferrioxamine E solution prior to use. Each fraction of pure desferrioxamine was concentrated to dryness and recrystallized from hot methanol. Amounts of 0.5–1 g of each compound were isolated (with the exception of the desferrioxamines X_4, X_6, and P_1, which were obtained only as crude material in very low yield (~5 mg)). The new compounds were identified by FAB and FD mass spectroscopy, by ^1H and ^{13}C NMR, and by GC–MS analysis of their acid hydrolysates.[18] Purity of the desferrioxamines was confirmed by TLC and elemental analysis. For comparison purposes, the known desferrioxamines E and D_2 were also isolated and included in this study.

E: $R_F(I) = 0.62$; $R_F(II) = 0.76$. Mp: 182–184 °C dec. Anal. Calcd (found) for $C_{27}H_{48}N_6O_9$: C, 53.99 (53.81); H, 8.05 (8.05); N, 13.99 (13.94). FAB(+)-MS: 601 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

D_2: $R_F(I) = 0.57$; $R_F(II) = 0.70$. Mp: 178–180 °C dec. Anal. Calcd (found) for $C_{26}H_{46}N_6O_9$: C, 53.23 (53.07); H, 7.90 (8.00); N, 14.32 (14.31). FAB(+)-MS: 587 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

X_1: $R_F(I) = 0.52$; $R_F(II) = 0.65$. Mp: 207–208 °C dec. Anal. Calcd (found) for $C_{25}H_{44}N_6O_9$: C, 52.43 (52.34); H, 7.74 (7.88); N, 14.68 (14.75). FAB(+)-MS: 573 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

X_2: $R_F(I) = 0.46$; $R_F(II) = 0.58$. Mp: 212–214 °C dec. Anal. Calcd (found) for $C_{24}H_{42}N_6O_9$: C, 51.16 (51.58); H, 7.58 (7.75); N, 15.04 (15.11). FD-MS: 559 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

X_3: $R_F(I) = 0.64$; $R_F(II) = 0.79$. Mp: 166–169 °C dec. Anal. Calcd (found) for $C_{28}H_{50}N_6O_9$: C, 54.71 (54.51); H, 8.20 (8.33); N, 13.67 (13.60). FAB(+)-MS: 615 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

X_4: $R_F(I) = 0.66$; $R_F(II) = 0.81$. $C_{29}H_{52}N_6O_9$. FAB(+)-MS: 629 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

X_5: $R_F(I) = 0.61$; $R_F(II) = 0.75$. Mp: 190–194 °C dec. Anal. Calcd (found) for $C_{28}H_{50}N_6O_8$: C, 56.17 (56.18); H, 8.42 (8.43); N, 14.04 (14.25). FAB(+)-MS: 599 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

X_6: $R_F(I) = 0.63$; $R_F(II) = 0.77$. $C_{29}H_{52}N_6O_8$. FAB(+)-MS: 613 ($M + H^+$).

Et_1: $R_F(I) = 0.57$; $R_F(II) = 0.67$. Mp: 178–181 °C dec. Anal. Calcd (found) for $C_{26}H_{46}N_6O_{10}$: C, 51.82 (51.69); H, 7.69 (7.74); N, 13.94 (13.77). FAB(+)-MS: 603 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

Et_2: $R_F(I) = 0.54$; $R_F(II) = 0.63$. Mp: 179–181 °C dec. Anal. Calcd (found) for $C_{25}H_{44}N_6O_{11}$: C, 49.66 (49.15); H, 7.33 (7.24); N, 13.90 (13.70). FAB(+)-MS: 605 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

Et_3: $R_F(I) = 0.51$; $R_F(II) = 0.59$. Mp: 158–163 °C dec. Anal. Calcd (found) for $C_{24}H_{42}N_6O_{12}$: C, 47.52 (47.61); H, 6.98 (7.04); N, 13.85 (13.95). FAB(+)-MS: 607 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

Te_1: $R_F(I) = 0.57$; $R_F(II) = 0.68$. Mp: 176–178 °C dec. Anal. Calcd (found) for $C_{26}H_{46}N_6O_9S$: C, 50.47 (49.75); H, 7.49 (7.53); N, 13.58 (13.81); S, 5.18 (5.37). FAB(+)-MS: 619 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

Te_2: $R_F(I) = 0.56$; $R_F(II) = 0.68$. Mp: 175–179 °C dec. Anal. Calcd (found) for $C_{25}H_{44}N_6O_9S_2$: C, 47.15 (46.90); H, 6.96 (6.94); N, 13.20 (13.21); S, 10.07 (10.04). FAB(+)-MS: 637 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

Te_3: $R_F(I) = 0.56$; $R_F(II) = 0.65$. Mp: 166–173 °C dec. Anal. Calcd (found) for $C_{24}H_{42}N_6O_9S_3$: C, 44.02 (44.02); H, 6.47 (6.34); N, 12.83 (12.67); S, 14.69 (14.48). FAB(+)-MS: 655 ($M + H^+$). ^1H and ^{13}C NMR (DMSO-d_6).[18]

P_1: $R_F(I) = 0.53$; $R_F(II) = 0.58$. $C_{26}H_{45}N_7O_{10}$. FD-MS: 616 ($M + H^+$). ^1H and ^{13}C NMR (D_2O).[18]

Solution Thermodynamics. Water used for solution thermodynamic experiments was deionized, distilled, and further purified by a Millipore cartridge system. It was degassed and kept under an argon atmosphere. Potassium nitrate was purified by extraction of an aqueous solution with 8-hydroxyquinoline in benzene/CCl_4 (3:1) and recrystallized from hot water. Ferric ion stock solutions were made from $Fe(NO_3)_3 \cdot 9H_2O$/0.1 M HNO_3 ($[Fe^{3+}] = 0.04738$ M; $[H^+] = 0.1056$ M) and $FeCl_3 \cdot 6H_2O$/0.1 M HCl ($[Fe^{3+}] = 0.002$ M) and standardized by titrating with a standard EDTA solution (Na_2EDTA, Aldrich, Gold label) using variamine blue as indicator.[19] Titrant solutions were prepared from Bak-

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Table II. Ligand Protonation Constants, Ferric Complex Stability Constants, and Relative Affinities of the Ferric Complexes of the Desferrioxamines to the Hydroxamate Iron Transport System of *S. aureus* DSM 799

desferrioxamine	log K_1[a]	log K_2[a]	log K_3[a]	log β_{110}[b]	log K_L*[c]	pM[d]	rel affinity[e]
E	10.03 (4)[f]	9.29 (3)	8.65 (2)	32.21 (4)	4.24	27.27	100
D_2	10.02 (4)	9.23 (3)	8.60 (2)	31.40 (7)	3.56	26.69	100
X_1	10.00 (5)	9.24 (3)	8.58 (3)	30.88 (3)	3.06	26.19	7
X_2	09.98 (2)	9.20 (1)	8.53 (1)	29.80 (5)	2.10	25.22	2
X_3	10.02 (2)	9.28 (8)	8.64 (1)	31.29 (3)	3.35	26.48	464
Et_1	10.02 (4)	9.23 (4)	8.61 (4)	31.78 (2)	3.92	27.05	100
Et_2	10.11 (9)	9.25 (2)	8.59 (3)	31.65 (5)	3.70	26.83	60
Et_3	10.12 (4)	9.22 (4)	8.60 (3)	31.35 (5)	3.41	26.54	14
Te_1	10.00 (6)	9.19 (5)	8.57 (6)	31.23 (3)	3.47	26.60	100
Te_2	10.02 (6)	9.14 (1)	8.53 (4)	30.53 (3)	2.84	25.96	100
Te_3	09.99	9.10	8.49	29.70 (4)	2.12	25.24	100
X_5	09.63 (3)	8.79 (8)		19.18[g]		17.78	5
				55.35[h]			

[a] $K_n = [H_nL^{(m-l)-}]/[H^+][H_{n-1}L^{m-}]$. [b] $\beta_{110} = [FeL]/[Fe^{3+}][L^{3-}]$. [c] $K_L^* = [FeL][H^+]^3/[Fe^{3+}][H_3L]$. [d] pM = −log $[Fe(H_2O)_6^{3+}]$, when $[L]_T = 10$ μM, $[M]_T = 1$ μM, pH 7.4. [e] Relative affinities were estimated from the antagonistic activities toward the antibiotic ferrimycin. The value found for ferrixamine E was set at 100%. [f] Parentheses indicate uncertainty in the last digit and correspond to the doubled standard deviation. [g] $\beta_{110} = [FeL^+]/[Fe^{3+}][L^{2-}]$. [h] Value corresponds to log β_{230}; $\beta_{230} = [Fe_2L_3]/[Fe^{3+}]^2[L^{2-}]^3$.

er-Dilut-it Concentrates and standardized. For final purification and subsequent handling of the desferrioxamines, only glassware which had been treated with 10 mM EDTA for at least 12 h and carefully rinsed with double-distilled water was used.

Potentiometric pH Titrations. The computer-controlled titration apparatus consisted of a Brinkman 655 Dosimat automatic buret, a Fisher 825 MP Accument pH meter connected with a Orion Ross combination pH electrode, a specifically designed water-jacketed titration cell (50 mL), which was maintained at 25.0 °C with a constant-temperature bath, and an IBM personal computer. The system was kept under oxygen and carbon dioxide free argon. The program TITRATE[23] was used to run automated titrations. The pH electrode was calibrated in concentration units by titrating standard HNO_3, diluted with 0.100 M KNO_3, with standardized KOH. The titration data were analyzed by a Marquardt nonlinear least-squares program.[20] The program calculates a calibration line as well as the dissociation constant of H_2O (pK_w), which was included in the refinement of the ligand titration data. The calibration of the electrode was repeated before every ligand titration.

For determination of the protonation constants of the desferrioxamines, ca. 0.001 M ligand solutions in 0.100 M KNO_3 were titrated from low to high pH in the range of pH 6–10.5 using a volume increment of 0.010 or 0.015 mL. In order to check for reversibility of deprotonation, one titration from high to low pH was performed for each ligand. In each case no hysteresis was found. The protonation constants were refined from the 150–200 titration points by means of the nonlinear least-squares refinement program BETA, which has been described elsewhere.[21,22] Three titrations were performed for each ligand, except desferrioxamine Te_3, which was titrated only once.

Potentiometric titrations of Fe(III)/desferrioxamine X_5 solutions were similarly carried out and analyzed.

Spectrophotometric Titrations. The apparatus and controlling computer program used for the automated spectrophotometric pH titration of Fe(III)/desferrioxamine X_5 have been described previously.[23,24] A solution of ligand (1.192×10^{-4} M) and Fe(III) (4.409×10^{-5} M; $Fe(NO_3)_3$ stock solution) was titrated from pH 2.5 to 8. The ionic strength was adjusted to 0.100 M (KNO_3), and the temperature was maintained at 25.0 °C. The experiment was performed under a water-saturated argon atmosphere. The electrode was calibrated in concentration units with degassed solutions of pH 2.301 (HNO_3) and pH 11.478 (KOH) at ionic strength 0.100 M (KNO_3) and 25 °C. Data analysis was performed on an IBM-AT computer using REFSPEC,[25] a spectral componentization and least-squares refinement program. A set of 29 spectra in the range 410–550 nm was used for the calculation of the proton-independent complex formation constants.

Spectrophotometric pH titrations of the ferric complexes of the trihydroxamic acids were carried out discontinuously. Aqueous solutions of ligand (0.5 mM) and Fe(III) (0.4 mM; $FeCl_3$ stock solution) were titrated with 0.1 M NaOH starting from pH 1. After the desired pH was achieved, an aliquot of the solution was transferred into a 1-cm quartz cuvette and a spectrum was recorded from 300 to 600 nm (LAMBDA 5 spectrophotometer, Perkin-Elmer). A similar procedure was applied to the titrations of desferrioxamine X_5 (0.9 mM at pH 7; 0.6 mM at pH 1) with Fe(III) ($FeCl_3$ stock solution). The pH was adjusted either with 0.1 M HCl or with 0.1 M NaOH. The absorbance was measured at 470 nm (pH 1) and 428 nm (pH 7).

Competition Studies. The proton-dependent ferric complex formation constants of the trihydroxamic acids were determined spectrophotometrically by EDTA competition experiments as previously described.[26] For each desferrioxamine, six solutions were prepared containing ligand (~0.64 mM), EDTA (~6 mM), and Fe(III) (0.4–0.5 mM; $Fe(NO_3)_3$ stock solution). The pH was adjusted to 5–7 and the ionic strength to 0.100 M (KNO_3). Excess EDTA served to buffer the solutions. Equilibrium was approached from both directions: Free EDTA was added to a ferrioxamine solution, or alternatively, free desferrioxamine was added to a solution of ferric EDTA. The solutions were maintained at 25 °C until no further change in the visible spectrum was seen, which took 4–25 days depending upon the ligand. After equilibrium was attained, the final pH and UV/vis spectrum (HP 8450A spectrometer; 1-cm cell) of each solution were measured at 25 °C. The electrode was calibrated in concentration units by the two-point method as described above. For calculation of the ferrioxamine equilibrium concentrations, absorbances in the region 460–480 nm were used, where ferric EDTA does not absorb significantly.

Ferrimycin Antagonism Test. The antagonism between ferrioxamines and ferrimycin was investigated according to the agar diffusion method described by Zähner et al.[27] using *Staphylococcus aureus* DSM 799 (from Deutsche Stammsammlung für Mikroorganismen, Braunschweig, FRG) as the test strain. Growth conditions and experimental details of this test were identical with those previously reported.[28]

Results and Discussion

Directed Fermentation and Isolation of Desferrioxamines. Under natural growth conditions the bacterial strain *S. olivaceus* Tü 2718 produces the known siderophores desferrioxamine E (80%) and desferrioxamine D_2 (20%) (Figure 1). In order to obtain new, modified desferrioxamines, the culture medium was supplemented with a series of unnatural diamines and diamino acids.[13] In Table I the precursors are listed which lead to the formation of new siderophores. Isolation of the desferrioxamines from the culture filtrates was achieved by adsorption of the iron-free ligands to the adsorber resin Amberlite XAD-16, flash chromatography on silica gel, and isocractic preparative reversed-phase HPLC.

Ligand Protonation Constants. The protonation constants (log K_a) of the di- and trihydroxamic acids X_5, E, D_2, X_1–X_3, Et_1–Et_3,

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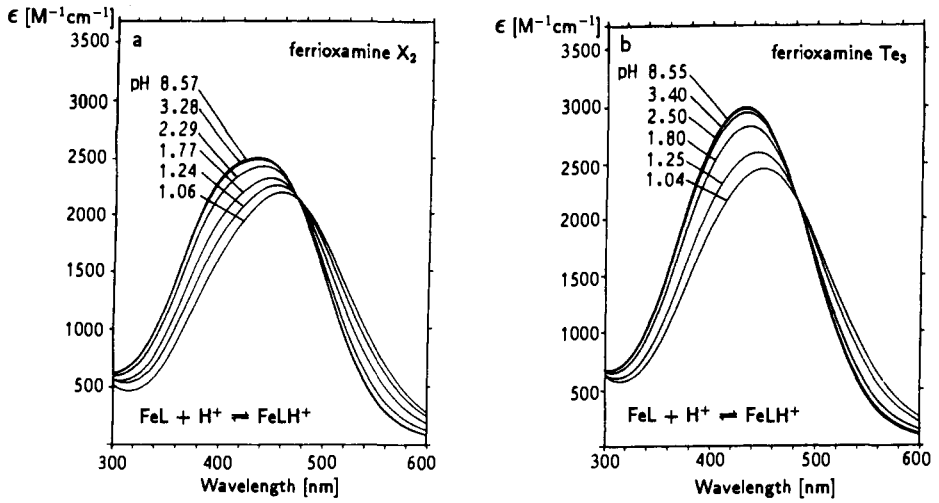

Figure 2. Extinction coefficient spectra of (a) ferrioxamine X_2 and (b) ferrioxamine Te_3 as a function of pH.

Table III. Summary of the Spectrophotometric pH Titration Results for the Trihydroxamic Acid Ferric Complexes (0.1 M KNO_3; 25 °C)

ferri-oxamine	shifts observed: pH 7–3 λ_{max}, nm (ϵ, M^{-1} cm^{-1})	pH 3–1 $\Delta\lambda_{max}$	pH 3–1 $\Delta\epsilon$	isosbestic point, nm
X_2	432 (2670)	28	−390	469[a]
X_1	432 (2730)	16	−270	476[a]
D_2	432 (2840)	8	−180	
E	432 (2890)	4	−90	
X_3	429 (2900)	12	−330	479[a]
Et_1	431 (2890)	5	−150	
Et_2	431 (2850)	7	−150	
Et_3	431 (2910)	6	−180	
Te_1	430 (2980)	7	−240	
Te_2	429 (3070)	14	−390	478[a]
Te_3	429 (3190)	18	−550	477[a]

[a] An isosbestic point is only observed at pH >1.0.

and Te_1–Te_3 were obtained by potentiometric titrations in the region pH 6–10.5 at 25 °C and 0.1 M ionic strength (KNO_3). The values obtained on the basis of a two- or three-proton model, respectively, are presented in Table II. Refinements resulted in GOF's[29] from 0.71 to 3.35. The constants determined for the various ligands are very similar. In the series E, D_2, X_1, X_2 the log K_a's tend to decrease somewhat. However, no significant shift of the protonation constants was found for the oxygen-containing Et-desferrioxamines, as might be expected from the negative inductive effect of the additional oxygen atoms. The protonation constants of desferrioxamine E were determined in 1963 by Schwarzenbach et al.[30] The reported log K_a's of 9.98, 9.42, and 8.65 (μ = 0.1 M; 20 °C) are very close to those found here.

Visible Spectra of the Trihydroxamic Acid Ferric Complexes. The visible spectra of the trihydroxamic acid ferric complexes (FeL; spectra only shown for ferrioxamine X_2 and Te_3; Figure 2) exhibit the typical metal–ligand charge-transfer band at about 430 nm, which does not change over a wide range of pH. Only when the pH is lowered below 2–3 does λ_{max} start to shift to higher values and an isosbestic point is observed, indicating the formation of a protonated species $FeLH^+$ (Table III). A low-pH dependence was found for the ferric complexes of the desferrioxamines (ferrioxamines) E, D_2, and Et_1–Et_3, a high-pH dependence for the ferrioxamines X_2 and Te_3. In general, protonation of the FeL complex is easier the more the structure of the ligand differs from that of desferrioxamine E. The ferrioxamines X_2 and Te_3 already begin to dissociate into free ligand and free Fe(III) at about pH 1.0, as indicated by a decreasing absorbance at the isosbestic point (Figure 2). Absorbance maxima and extinction coefficients of the ferrioxamines are presented in Table III. The values for ferrioxamine E were redetermined. They are somewhat different from those previously reported[30] (λ_{max} = 430 nm, ϵ_{max} = 2620 M^{-1} cm^{-1}, aqueous solution).

Stability Constants of the Trihydroxamic Acid Ferric Complexes. The proton-independent formation constants β_{110} of the trihydroxamic acid ferric complexes are defined by the following equations:

$$Fe^{3+} + L^{3-} \rightleftharpoons FeL \quad (1)$$

$$\beta_{110} = \frac{[FeL]}{[Fe^{3+}][L^{3-}]} \quad (2)$$

Since there is no significant metal–ligand dissociation in the pH region accessible to titrations, as can be seen from the pH-dependent electronic spectra of the ferrioxamines (Table III), no direct measurement of the metal–ligand equilibrium is possible. For this reason the proton-dependent formation constants K_L^* were determined by EDTA competition experiments. The complexation equilibria of ferric ion (Fe^{3+}), desferrioxamine (H_3L), and Na_2EDTA (H_2Y^{2-}) are expressed by the following equations

$$H_3L + FeY^- \rightleftharpoons FeL + H_2Y^{2-} + H^+ \quad (3)$$

$$K_{comp} = \frac{K_L^*}{K_Y^*} = \frac{[FeL][H^+][H_2Y^{2-}]}{[H_3L][FeY^-]} \quad (4)$$

where

$$K_L^* = \frac{[FeL][H^+]^3}{[Fe^{3+}][H_3L]} \quad (5)$$

These equilibria were monitored spectrophotometrically at pH values between 5 and 7. The period of time required for attaining equilibrium ranged from 4 to 25 days, increasing with pH and complex stability. The concentrations of all species in eq 4, and thus the competition equilibrium constants K_{comp}, were calculated from the protonation constants of the two ligands, the equilibrium pH and absorbances, and mass balance equations including the hydolyzed species $Fe(OH)Y^{2-}$ ($K_{Fe(OH)Y}$ = $[FeY^-]/[Fe(OH)Y^{2-}][H^+]$ = $10^{7.37}$). The proton-independent stability constants β_{110} of the ferrioxamines were obtained from the known formation constant of ferric EDTA (log β_{110} = 25.0)[31] and the ligand protonation constants (Table II).

(29) GOF (goodness of fit) = $[\sum w^2(pH_{obs} - pH_{calc})^2/(N_{obs} - N_{var})]$, where $(1/w)^2 = \sigma^2(pH) + (\delta(pH)/\delta(vol))^2\sigma^2(vol)$. σ denotes errors in pH measurement (0.003 pH unit) and titrant delivery (0.002 mL), $(\delta(pH)/\delta(vol))$ is the slope of the titration curve at each point. N_{obs} and N_{var} equal the number of observations and the number of independent variables, respectively.

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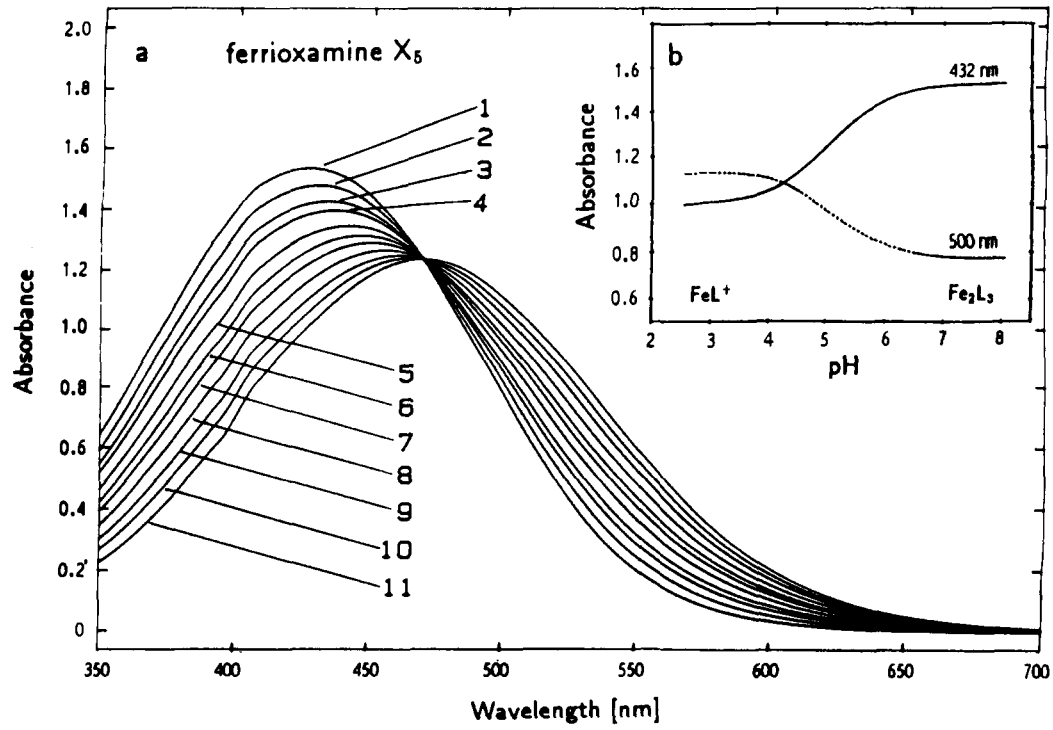

Figure 3. (a) Visible absorption spectra of ferrioxamine X_5 as a function of pH: (1) 8.06; (2) 6.21; (3) 5.82; (4) 5.64; (5) 5.35; (6) 5.16; (7) 5.00; (8) 4.79; (9) 4.55; (10) 4.33; (11) 2.91 ($[Fe]_T = 4.409 \times 10^{-5}$ M; $[L]_T = 1.192 \times 10^{-4}$ M). (b) Plot of the absorbances at 432 and 500 nm vs pH.

The highest formation constant was found for desferrioxamine E, the principal siderophore under natural growth conditions. Schwarzenbach et al.[30] reported in 1963 log $\beta_{110} = 32.43$ (μ = 0.1 M; 20 °C) for this ligand. This is in accordance with our observation that β_{110} decreases with increasing temperature, indicating that the formation of the FeL complex is an exothermic reaction. In the series of investigated desferrioxamines the stability of the ferric complexes decreases monotonically as the structure of the ligand deviates from that of desferrioxamine E, which agrees with the behavior found for metal–ligand complex protonation. The reduced ring size and the substitution of CH_2 groups by oxygen and sulfur atoms cause changes in the ligand molecules which make them less favorable for iron(III) complexation. However, all these desferrioxamines (with the exception of X_2 and Te_3) are better iron(III) chelators than desferrioxamine B (log $\beta_{110} = 30.5$; μ = 0.1 M; 20 °C),[30] which is the therapeutically used drug for iron(III) and aluminum(III) removal. In Table II are also included the pM values of the ferrioxamines which reflect the relative iron binding ability of the ligands under physiological conditions[2] (see Table II for definition). The higher the pM the more effective is the ligand. Since the protonation constants of all desferrioxamines are almost identical, the pM differences observed originate primarily from the differences in the proton-independent complex formation constants of the ferrioxamines.

Stability Constants of the Desferrioxamine X_5 Ferric Complexes. Spectrophotometric titration of ferrioxamine X_5 from pH 2.5 to 8.0 revealed strong pH dependence of the spectra (Figure 3a). As the pH is raised, λ_{max} starts to shift, from 472 nm at pH 3.2 to 428 nm at pH $\geq$ 6.4, with a sharp color change from red to orange at about pH 5. The isosbestic point observed at 470 nm indicates the presence of two absorbing species in solution. The titration curves (Figure 3b) exhibit two well-separated plateaus between pH 3 and 8. At pH $\leq$3 the absorbance starts to decrease slightly, indicating the onset of dissociation of the Fe(III)/desferrioxamine X_5 complex present at pH 3. This was confirmed by an additional investigation of the region pH 1–3 (spectra not shown), where λ_{max} of the metal–ligand charge-transfer band is constant and the absorbance decreases over the whole range of wavelengths. From the position of λ_{max} and the molar extinction coefficients determined for the two absorbing species (428 nm (3040 M^{-1} cm^{-1}) and 470 nm (2500 M^{-1} cm^{-1}); 0.1 M KNO_3, 25 °C; extinction coefficients are calculated per Fe^{3+} ion), it was concluded that two hydroxamate groups per ferric ion are bound at low pH and three at high pH.[32] This led to the formulation of a 1:1 complex FeL^+ at low and a 2:3 complex Fe_2L_3 at high pHs, as has been described for the dihydroxamic acid siderophore rhodotorulic acid, which exhibits a buffer region at pH 2–4.[33]

The stoichiometry of the two complex species was determined by titrating desferrioxamine X_5 with Fe^{3+} at pH 1 and pH 7 (Figure 4). A sharp break in the titration curves at 1 equiv of Fe^{3+} at pH 1 and 0.66 equiv at pH 7 confirmed the above formulation. Moreover, potentiometric titrations of Fe(III)/desferrioxamine X_5 at several metal to ligand ratios revealed a sharp break in each titration curve at 3 equiv of base/mol of Fe^{3+} (Figure 5), indicating that each ferric ion is being coordinated by three hydroxamate groups with the concomitant release of three protons. That confirms the formation of a dimeric Fe_2L_3 complex at higher pH. Thus, the Fe(III)/desferrioxamine X_5 system is described by a pH-dependent monomer/dimer equilibrium (K_2 = stepwise stability constant):

$$2FeL^+ + L^{2-} \rightleftharpoons Fe_2L_3 \quad (6)$$

$$K_2 = \frac{[Fe_2L_3]}{[FeL^+][L^{2-}]} \quad (7)$$

The overall stability constants of the two Fe(III)/desferrioxamine X_5 complexes, β_{110} and β_{230}, are defined by the following equations:

$$Fe^{3+} + L^{2-} \rightleftharpoons FeL^+ \quad (8)$$

$$\beta_{110} = K_1 = \frac{[FeL^+]}{[Fe^{3+}][L^{3-}]} \quad (9)$$

$$2Fe^{3+} + 3L^{2-} \rightleftharpoons Fe_2L_3 \quad (10)$$

$$\beta_{230} = \frac{[Fe_2L_3]}{[Fe^{3+}][L^{3-}]^3} = K_1^2K_2 \quad (11)$$

The constants β_{110} and β_{230} were determined from the spectrophotometric titration data (Figure 3) using the componentization

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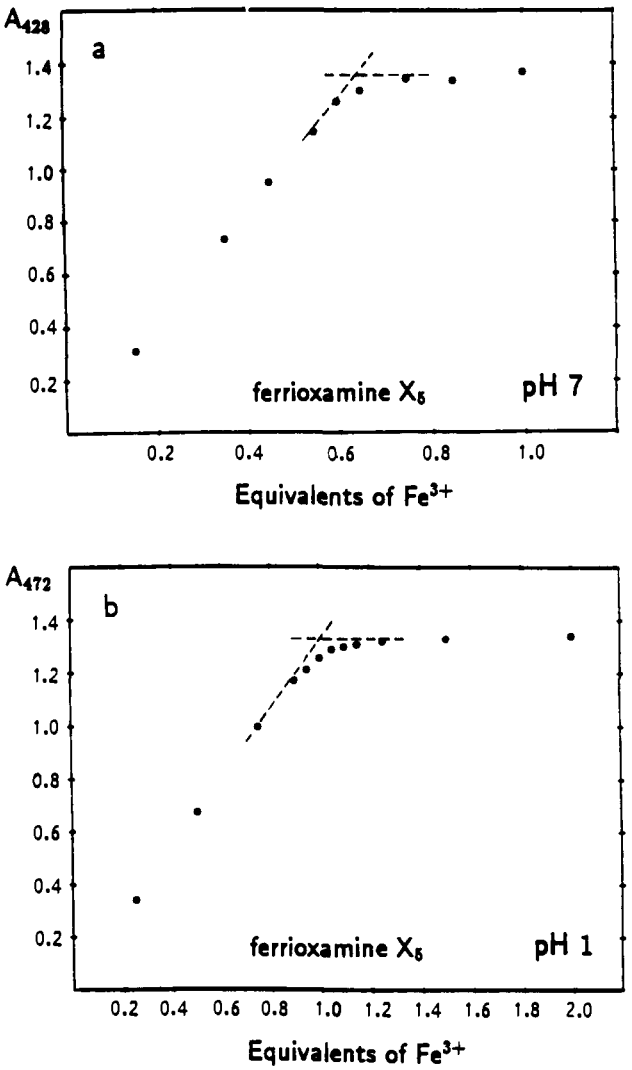

Figure 4. Spectrophotometric titration of desferrioxamine X_5 with Fe^{3+} at (a) pH 7 and (b) pH 1. Absorbance values are normalized by the concentration of desferrioxamine X_5 (0.9 mM at pH 7; 0.6 mM at pH 1).

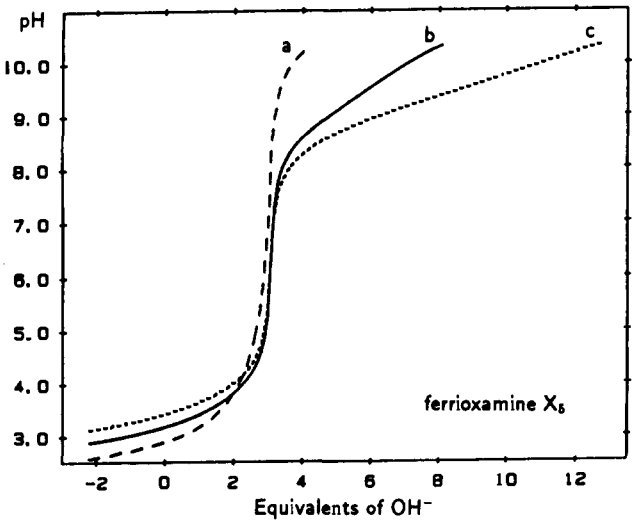

Figure 5. Potentiometric titration curves for the ferrioxamine X_5 metal–ligand system: (a) 0.65 mM $[Fe^{3+}]$, L/M = 1.53; (b) 0.30 mM $[Fe^{3+}]$, L/M = 3.61; (c) 0.17 mM $[Fe^{3+}]$, L/M = 6.00.

and least-squares refinement program REFSPEC.[25] The spectral componentization gave two absorbing species, which agrees with the model established above. The refinement of the complex formation constants yielded log β_{110} = 19.18 and log β_{230} = 55.35

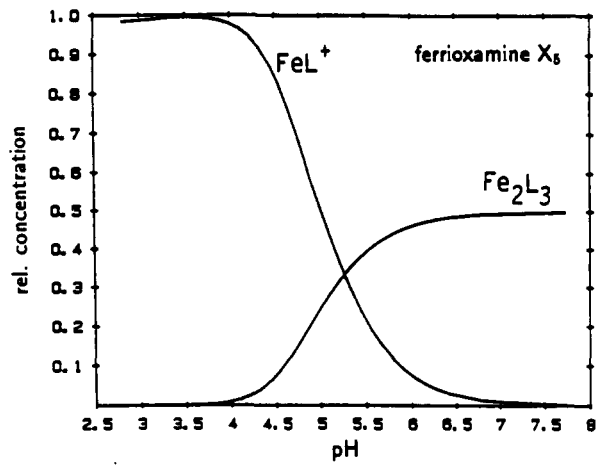

Figure 6. Species distribution of the ferrioxamine X_5 metal–ligand system. Total $[Fe^{3+}] = 5.26 \times 10^{-5}$ M, total $[L] = 1.12 \times 10^{-4}$ M.

(0.1 M KNO_3, 25 °C) with refinement factor R = 1.3%.[34] Thus, the stepwise stability constant log K_2 = 16.99. Comparison of the stability of the ferrioxamine X_5 Fe_2L_3 complex with the monomeric ferric complexes of the trihydroxamic acids is possible, if the formation constant per ferric ion is taken into consideration. The overall stability constant of the $FeL_{1.5}$ complex (log $\beta_{230/2}$ = 27.68) is lower than any formation constant determined for the trihydroxamate desferrioxamines. Figure 6 shows the species distribution calculated for the ferrioxamine X_5 metal–ligand system.

The relatively low stability of the Fe_2L_3 complex of desferrioxamine X_5 is not a consequence of the lower denticity of this ligand compared to the hexadentate trihydroxamic acids, since the FeL_3 complex, formed by the bidentate acethydroxamic acid ligand, is already more stable (log β_{130} = 28.3; μ = 0.1 M; 20 °C)[35] than the $FeL_{1.5}$ complex of desferrioxamine X_5. Moreover, the corresponding formation constants of the tetradentate rhodotorulic acid are significantly higher (log β_{110} = 21.55; log β_{230} = 62.2; μ = 0.1 M; 25 °C).[33] Therefore, steric interactions seem responsible for the low stability of the ferrioxamine X_5 complexes. In the case of the dimer, there are three bulky macrocycles which have to be placed around two ferric ion centers. Theoretically there are two structures possible: singly bridged and triply bridged. The latter is considered more likely in case of the smaller rhodoturulic acid, as derived from crystal structures determined for a series of synthetic model complexes.[2,36,37] However, the significant structural difference between the siderophores lessens the certainty of the prediction of this structure for the Fe_2L_3 complex of ferrioxamine X_5. In any case, the dimer Fe_2L_3 is the predominant ferrioxamine X_5 species at physiological pH and thus acts as a microbial iron transport agent.

Biological Activity. The transport properties of the new ferrioxamines were studied by an agar diffusion antagonism test using *S. aureus* DSM 799 as a test strain and the antibiotic ferrimycine as an antagonist. The latter is taken up by the cells via the ferrioxamine transport system.[38] All of the new ferrioxamines are able to antagonize ferrimycine competitively, and thus they are recognized by the same receptor. This agrees with the previous observation that in the case of *S. pilosus* the various natural ferrioxamines utilize the same transport system.[39] However, estimations of the antagonistic activities of the ferrioxamines towards ferrimycin (relative affinities) revealed considerable differences in complex recognition by the trihydroxamate receptor

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of *S. aureus* (Table II). The relative affinities do not correlate with the complex stability, as can be seen from the data in Table II.

Conclusion

We have shown, for the first time, that directed fermentation of siderophore-producing microorganisms is a powerful biosynthetic approach to "unnatural", structurally modified siderophores. By feeding suitable diamino acid and diamine precursors to cultures of *S. olivaceus* Tü 2718, 13 new desferrioxamine siderophores have been isolated by a three-step chromatographic procedure. The new siderophores are analogues of the "natural", macrocyclic trihydroxamic acid desferrioxamine E, from which they differ in ring size and by substitution of CH_2 groups of the diaminopentane moieties by oxygen or sulfur atoms or by amide bonds. Two of the new desferrioxamines are dihydroxamic acids lacking one of the *N*-hydroxyl groups.

Spectrophotometric titrations and EDTA competition experiments performed with the trihydroxamic acid desferrioxamines have shown that the stability of the complexes decreases steadily as the structure of the ligand deviates from that of desferrioxamine E. By far the lowest complex stability is found for the structurally most deviant dihydroxamic acid, desferrioxamine X_5. It is demonstrated that desferrioxamine X_5 forms two types of ferric complexes, a monomer FeL^+ at low pH and a dimer Fe_2L_3 at high pH. All of these desferrioxamines are effective iron transport agents. There is no correlation between the complex stability and biological activity.

Acknowledgment. This investigation was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 323) and U.S. National Institutes of Health (AI11744). Doctorate stipends from the Studienstiftung des Deutschen Volkes (S.K.-R.) and the DE-CHEMA (J.M.) are gratefully acknowledged.

Highly Oxidized Iron Complexes of *N*-Methyltetra-*p*-tolylporphyrin

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Abstract: Two different, highly oxidized iron complexes of *N*-methyltetra-*p*-tolylporphyrin (*N*-MeTTPH) have been detected by ^1H and ^2H NMR spectroscopy after oxidation with *m*-chloroperoxybenzoic acid. In CH_2Cl_2/CH_3OH (4:1, v/v) in the presence of sodium methoxide (*N*-MeTTP)$Fe^{II}Cl$ is converted to (*N*-MeTTP)$Fe^{II}(OCH_3)$ and then to (CH_3O)(*N*-MeTTP)Fe^{IV}=O upon addition of the peroxyacid. The latter is observable over the temperature range −90 to 0 °C, has a magnetic susceptibility of 2.9 μ_B ($S = 1$), and is reduced by phenyldimethylphosphine to (*N*-MeTTP)$Fe^{II}(OCH_3)$. In the absence of sodium methoxide, (*N*-MeTTP)$Fe^{II}Cl$ is oxidized by the peroxyacid to form a very unstable species observable from −90 to −75 °C whose ^1H NMR spectrum shows large hyperfine shifts for the meso aryl substituents that are consistent with the presence of a π-*N*-methylporphyrin radical in the product.

Introduction

N-Alkylated[1] porphyrins are formed during the inactivation of cytochrome P-450 by a variety of chemical agents.[2] Inactivation during the epoxidation of terminal olefins produces adducts in which the terminal carbon of the olefin is bound to a pyrrole nitrogen and a hydroxyl group is bound to the internal carbon.[3]

Considerable study of the cytochrome P-450 cycle in the enzyme itself and in model systems have led to the hypothesis that the active oxidant is a highly oxidized iron porphyrin generally described as a ferryl $(Fe^{IV}=O)^{2+}$ ion coordinated to an oxidized porphyrin in its π-radical form (ie., $P^{\bullet}Fe^{IV}=O$).[4,5] This intermediate, which cannot be directly observed during the operation of cytochrome P-450, is felt to be at the same level of oxidation as the observable intermediate compound I that is present during turnover of horseradish peroxidase.[6]

Synthetic meso-tetraarylporphyrins that are capable of catalyzing the epoxidation of olefins with nonphysiological oxidants, iodosoarenes, or hypochlorite also can undergo N-alkylation during the catalytic cycle.[7-12] It has been noted, however, that in these cases N-alkylation does not completely inhibit epoxidation activity.[10,11] That is, the N-alkylated porphyrin iron complex retained some lesser activity as a epoxidation catalyst. This suggests that highly oxidized iron complexes of these N-substituted porphyrins may exist. Here we describe efforts to directly detect such species. Our efforts have concentrated on examining the reactivity of the high-spin, five-coordinate Fe^{II} complex **1** toward another non-

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‡ The University of Wrocław.

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