Origin of the Fe^{3+}-Binding and Conformational Properties of Enterobactin

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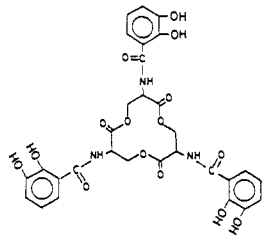
Abstract: Enterobactin (**1**) is one of the most efficient natural binders of ferric ions known to date. Structural analogues of enterobactin have been synthesized, including the tribenzamide (TBA) **7**, which differs from enterobactin only by lacking the catechol hydroxyl groups. The analogues have been studied by a combination of IR, NMR, and CD spectroscopy, X-ray diffraction, and empirical-force-field calculations. These studies elucidated the origin of enterobactin's unique binding properties and of its complex's right-handed chirality. TBA **7** in its most stable conformation, preferred in nonpolar solvents, possesses C_3 symmetry, its benzamide side chains are in axial positions, hydrogen bonds are formed between the amide hydrogen and the ring oxygen, and the phenyl rings are arranged in a right-handed (Δ) orientation. Uncomplexed enterobactin (**1**) is shown to resemble closely TBA **7**. The relation of the preferred Δ chirality of TBA **7** to the observed Δ chirality of $(Fe{\cdot}ent)^{3-}$ is discussed. A comparison between enterobactin and the hitherto best synthetic binder, a tricatecholamide derivative of mesitylene **3**, is presented. Enterobactin's superiority is partly due to its lower molecular strain upon binding and partly due to the lower conformational freedom of uncomplexed enterobactin. The binding strain of $(Fe{\cdot}ent)^{3-}$ resides more in the catecholamides than in the trilactone ring, while in the synthetic analogue **3** the mesitylene ring is more strained than the catecholamides.

Metal ions are essential for the maintenance of living systems. The alkali metal ions such as sodium control the transmission of nerve impulses, the alkaline earth metal ions such as calcium act as secondary messengers, and the transition-metal ions such as iron and copper are involved in enzymatic redox processes.[1] A large variety of ion carriers exist in nature to control the metal ion balance.[2] Carriers for the transition-metal ions are rare, with the exception of iron, for which protein and non-protein chelates exist. For iron, two families of low molecular weight carriers, or siderophores, are known: those utilizing hydroxamate groups and those utilizing catechol groups as binding sites.[3] Among the latter carriers, enterobactin (**1**) assumes a unique position.[4] Produced by enteric bacteria when grown in iron-deficient media, enterobactin is one of the most efficient binders and carriers known. It has a binding constant of log (K_{bind}) = 52 for the reaction

$$Fe^{3+} + ent^{6-} \rightarrow (Fe{\cdot}ent)^{3-}$$

where "ent^{6-}" is the sixfold deprotonated enterobactin.[3] Chemically, enterobactin is a tripodlike molecule. It consists of a trilactone ring, composed of three L-serine residues, each with an attached catechol ligand. Information on the conformation of enterobactin is limited to NMR studies in Me_2SO at elevated temperatures[5] or to IR studies in water.[6]



Enterobactin

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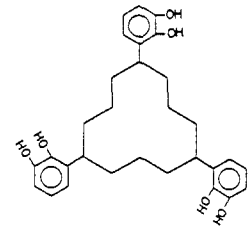
The outstanding properties of enterobactin stimulated extensive studies toward the synthesis of analogues with comparable ion binding properties.[7,8] Most of these studies concentrated on molecules with C_3-rotational symmetry, where the catechol binding groups are attached to mesitylene, aza,[7] or cyclododecane[8] rings as backbones. Examples of the compounds synthesized are given below (formulas **2–5**). Some of these compounds showed remarkably high binding constants for Fe^{3+}, reaching a value of log K_{bind} = 46 with the triamide **3**.[9] This value is, however, still six



E.J. Corey, 1977

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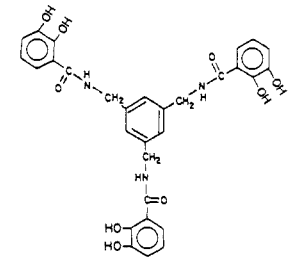

K.N. Raymond, 1979

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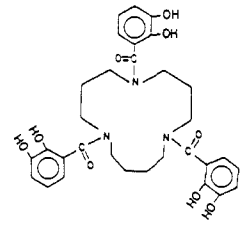

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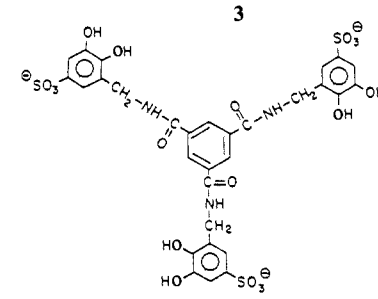

K.N. Raymond, 1983

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† Department of Organic Chemistry.
‡ Department of Chemical Physics.

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Table I. ^1H and ^{13}C NMR Spectra of Enterobactin and Model Compounds[a]

(a) ^1H NMR Spectra

compd	solvent	H^α	H^{β}_1	H^{β}_2	H^N	*o*-ArH	*p*-ArH	*m*-ArH
1[b]	Me_2SO-d_6	4.94 (m) J = 8.06, 4.19	4.66 (dd) J = 8.06, 10.8	4.41 (dd) J = 4.19, 10.8	9.06 J = 6.52	7.34	6.98	6.73
1b[b]	Me_2SO-d_6	5.12	5.22 J = 10.68	3.80 J = 10.68	11.72 J = 9.83	6.84	6.44	6.13
6	$CDCl_3$	3.46 (m)	3.46 (m)	4.05 (dd) J = 11, 11	2.63 (d) J = 9	7.4–7.6 (m)	7.2–7.35	
7	CD_2Cl_2/ Freon 11	4.99 (m) J = 7.8	4.96 (dd) J = 2.93, 11.40	4.54 (dd) J = 3.05, 11.40	7.35	7.67 (d) J = 7.15	7.51 (t) J = 7.5	7.28 (m)
7	$CDCl_3$	5.05 (m) J = 7.8	4.91 (dd) J = 3.1, 11.5	4.65 (dd) J = 3.3, 11.5	7.25	7.68 (d) J = 8.4	7.52 (t) J = 7.5	7.25 (m)
7	CD_3CN	4.85 (m)	4.85 (m) J = 4.05	4.47 (dd) J = 4.0, 12.2	7.78 (d) J = 6.6	7.67 (d) J = 7.2	7.54 (t) J = 7.5	7.3 (m)
7	Me_2SO-d_6, 25 °C	4.86 (m)	4.65 (dd) J = 7.60	4.38 (dd) J = 4.35, 10.89	8.88 (d) J = 7.07	7.85 (d) J = 7.04	7.58 (t) J = 7.27	7.47 (t) J = 7.63
7	Me_2SO-d_6, 85 °C	4.87 (m)	4.62 (dd) J = 8.02, 10.96	4.44 (dd) J = 4.24, 10.99	8.56 (d) J = 7.14	7.81 (d) J = 7.08	7.54 (t) J = 7.39	7.46 (t) J = 7.59
8[c]	$CDCl_3$	2.687 (t) J = 5.6	4.413 (t) J = 5.6					

^{13}C NMR Spectra

compd	solvent	C′O′O′	C′O′NH	C^α	C^β	*i*-Ar	*o*-Ar	*p*-Ar	*m*-Ar	c-Ar
1[b]	Me_2SO-d_6	169.4	169.0	51.6	63.8	118.9	116.3 148.5	118.5	118.5 146.3	
1b[b]	Me_2SO-d_6	170.2	168.8	52.1	65.3	114.8	112.5 158.6	113.7	113.3 155.0	
6	$CDCl_3$	172.39		54.47	66.67	145.32	128.02	128.67	126.6	71.36
7	CD_3CN	169.62	168.38	54.32	66.15	134.6	129.57	132.8	128.29	
8[c]	$CDCl_3$	171.55		35.23	61.73					

[a] The various model compounds are listed in the text. The top line for each entry gives the chemical shift (δ), ppm, and the second gives the coupling constants J in Hz. Only those coupling constants which can be deduced by first-order analysis are given. [b] Taken from ref 5. Compound **1b** is (enterobactin·Ga)$^{3-}$. Ga^{3+} was used since Fe^{3+} is paramagnetic; the ppm values of the complex refer to the pH range from 4.0 to 7.0. [c] Unsubstituted trilactone **8**.

orders of magnitude below that of the natural compound. Since the synthetic molecule[3] possesses the same binding sites as the natural one, it is plausible that its different behavior derives from stereochemical factors, as suggested by Raymond et al.[3] A better understanding of the steric and energetic properties of enterobactin and its analogues might therefore provide the basis for the design and synthesis of superior binders.

Enterobactin binds iron in an octahedral coordination by virtue of its catecholate binding sites. Its preferred coordination is of Δ-cis configuration, which forces the catechol side chains to tilt relative to the trilactones's main plane in a right-hand screw.[10] The formation of a Δ-cis Fe^{3+} complex from a tripodlike binder composed of L-amino acids (L-ser) is an exception among tripodlike siderophores. Ferrichromes and related binders composed of L-amino acids (L-ornithine) form Fe^{3+} complexes with a Λ-cis configuration.[3] Could the preference of the Δ-cis isomer of (Fe·ent)$^{3-}$, which is diastereometric to the Λ-cis isomer, be already imprinted in the free molecule? In order to examine this question, we looked for a model compound which would resemble natural enterobactin in its structural features. The tribenzamide analogue, TBA **7**, was the molecule of choice. It differs from enterobactin only by lacking the hydroxyls which hamper clear IR, UV, and CD spectroscopic analyses. Unlike enterobactin, TBA **7** is insensitive to oxygen and may readily be dissolved and studied in nonpolar, aprotic solvents.

The present study describes the synthesis of TBA **7** and of related compounds and examines their conformations by a combination of experimental (NMR, IR, CD, X-ray) and theoretical (empirical force field) methods. Particular emphasis will be placed on establishing the arrangement of the molecules' ligating side chains in relation to that in the enterobactin–Fe^{3+} complex and the forces that direct these arrangements.

Synthesis and Structure

Synthesis. The tribenzamide (TBA) **7** was synthesized by the same method applied earlier to the total synthesis of enterobactin itself.[11] The trilactone ring, i.e., the trityl derivative **6**, was generated by the organotin-induced cyclization of the β-lactone derived from serine. Removal of the protecting group and acylation with benzoylchloride provided the TBA **7**. The structures of the intermediate trityl derivative **6** and of the TBA **7** were confirmed by their ^1H and ^{13}C NMR and IR spectra (Tables I and II).

NMR Spectra. The ^1H and ^{13}C NMR spectra (Table I) were rather simple, as the three seryl residues, whether with *N*-trityl or with *N*-benzoyl groups, gave rise to single sets of signals. This observation suggests the presence of C_3-symmetric conformations or of rapidly interconverting conformations. The two molecules differed, however, in the orientation of their side chains when measured in $CDCl_3$. In the *N*-trityl-trilactone **6** the vicinal proton–proton coupling constant between the C^α-proton and one of the C^β-protons in $CDCl_3$ was large (J = 11 Hz). It indicated an axial–axial relationship[12] between the protons and implied equatorial orientation of the *N*-trityl groups. In the TBA derivative **7**, on the other hand, the corresponding vicinal coupling constants in $CDCl_3$ of H^α and each H^β were both small (J = 3.1 and 3.3 Hz). These values indicated equatorial–equatorial and axial–equatorial relationships between the vicinal protons and therefore an axial arrangement of the benzamide side chains. The parent

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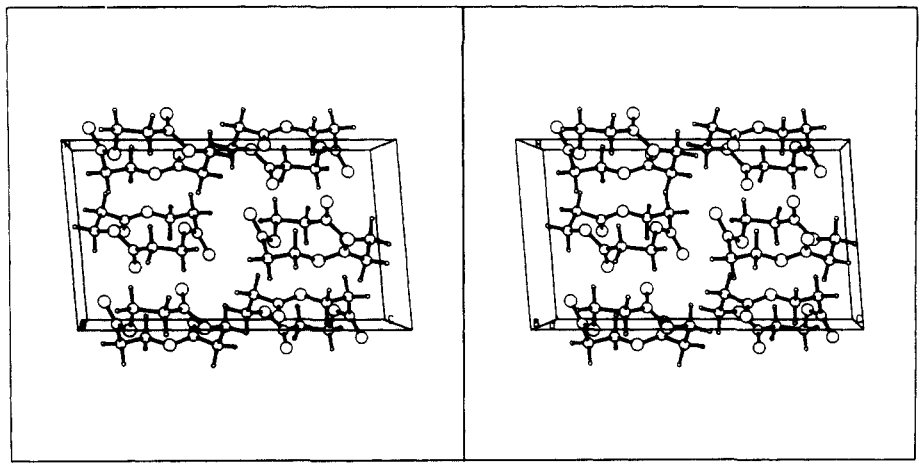


Figure 1. Stereoview of the crystal structure of trilactone **8**, showing two enantiomers in the unit cell.

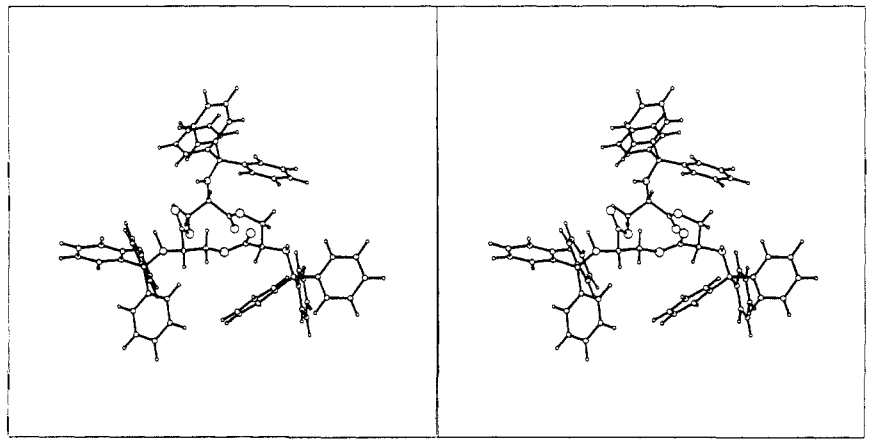
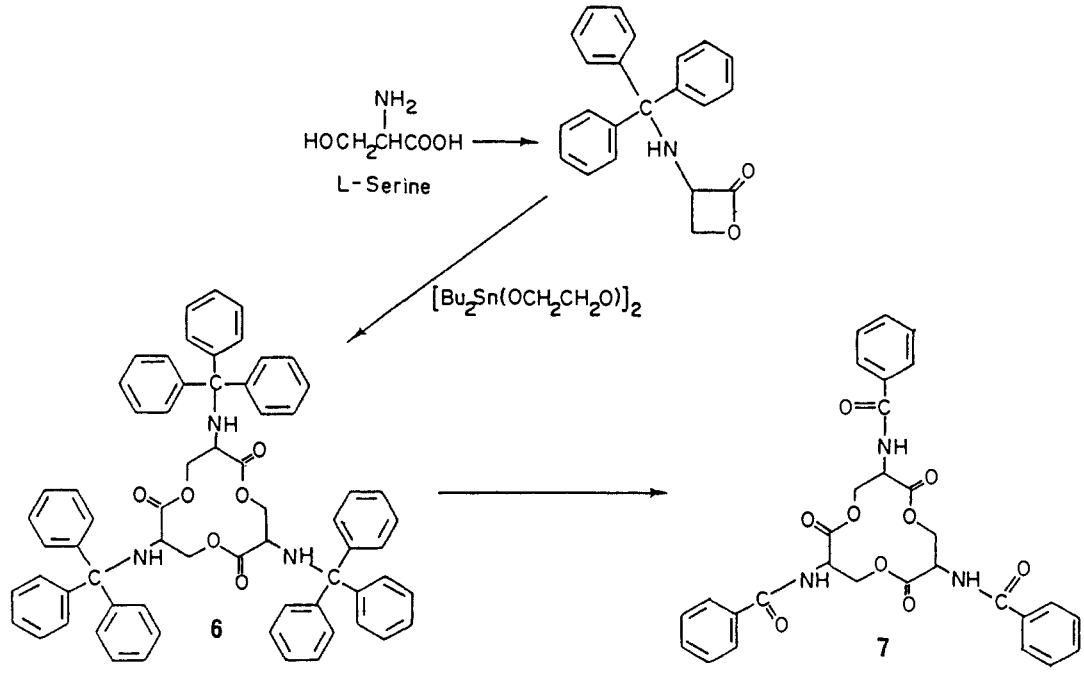


Figure 2. Stereoview of the crystal structure of *N*-trityl-trilactone **6**, showing two equatorial and one axial orientation of the side chains.

Scheme I



trilactone **8** cyclo (–C′O′–O–CH_2–CH_2–)$_3$,[13] showed a single signal for both H^{α}'s and H^{β}'s, with a vicinal coupling constant J = 5.6 Hz. This observation is compatible with fast interconversion of two enantiomeric conformations. The NMR characteristics of TBA **7** in solvents of varying polarities will be discussed below.

X-ray Diffraction. The conformations observed in single crystals supplement the NMR solution data. The parent trilactone **8** contains in its unit cell the two enantiomeric ring conformations of C_3 symmetry.[13] The *N*-trityl-trilactone **6** (Figure 2) assumes a nonsymmetric conformation with two of its side chains equatorial and one axial.[14] The nonsymmetry is likely to be due to the

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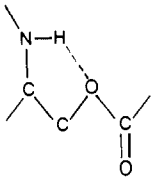
Table II. IR Carbonyl Stretching Frequencies (cm^{-1}) of Enterobactin and Model Compounds[a]

solvent	compound[a]							
	1[b]	**1a**[b]	**6**	**7**	**8**	**9**[c]	**10**[d]	**11**[e]
				(a) Amide Carbonyl				
CCl_4						1675		1688
$CHCl_3$				1662		1665		
$CHCl_3$ + 5% EtOH				1665				
CH_3CN				1667		1667		1676
				1651				
KBr	1640			1665		1650		
				1655				
				1645				
H_2O	1626	1600						
				(b) Ester Carbonyl				
CCl_4						1744	1749	
$CHCl_3$				1756	1739	1738	1742	
$CHCl_3$ + 5% EtOH				1755				
$CHCl_3$ + 10% EtOH				1755				
CH_3CN				1753	1744	1751	1745	
KBr	1750		1745	1770		1765		
				1750				
				1730				
D_2O	1740	1750						

[a] The various model compounds are listed in the text. [b] Taken from ref 6. Compound **1a** is $(Fe\cdot enterobactin)^{3-}$. [c] C_6H_5–C′O′–NH–CH_2–C′O′–O–C_2H_5. [d] Methyl acetate, taken from ref 17. [e] *N*-Methylacetamide, taken from ref 16.

bulkiness of the trityl groups. X-ray diffraction data of TBA and enterobactin are not yet available.

Infrared Spectra. The IR spectrum of TBA **7** (Table II) revealed shifts of the amide and lactone carbonyl frequencies which together helped to ascertain the existence of hydrogen bonds. Furthermore, these shifts were independent of the TBA **7** concentration, thus indicating that the hydrogen bonds were intramolecular. A ring of hydrogen bonds between the three amides is impossible, since in cis amides the N–H and C′=O′ bonds on adjacent amide groups are not oriented toward each other, and in trans amides they form a sharp angle of the order of 60°. Therefore, two types of intramolecular H bonds remain to be considered. In the first, more common type, the amide H^N hydrogen binds to the lactone carbonyl C′=O′. In the second type, H^N binds to the lactone oxygen –O–. Although the second type of H bonding is rare, it is not without precedence in the literature and has been documented for steroidal acetates.[15] Hydrogen bonds between the amide and lactone groups are expected to affect their mutual polarization, and consequently the IR frequencies of their carbonyls. The amide carbonyl oxygen becomes more negatively charged, its double bond character is diminished, its force constant is smaller, and its stretching frequency decreases. Similarly, the lactone carbonyl oxygen is also more negatively charged and its frequency decreases, if it is directly involved in an NH—O=C H bond of the first type. However, if the lactone carbonyl is indirectly involved in an H bond of the second type, namely according to the scheme



the lactone carbonyl oxygen becomes less negative, its double bond character is enhanced, its force constant is larger, and its frequency increases.

These considerations are fully reflected in the observed IR spectra (Figure 3 and Table 2) and are reinforced by comparing the carbonyl stretching frequencies of TBA **7** with an analogue of its monomer residue, *N*-benzoylglycyl ethyl ester **9** (C_6H_5CO-$NHCH_2COOC_2H_5$). This analogue forms an intramolecular H bond between the amide hydrogen and the ester carbonyl oxygen. Consider first the absorption of the amide carbonyl, taking as reference *N*-methylacetamide **11** in CCl_4 at low concentrations, i.e., in the absence of H bonds.[16] The amide carbonyl frequencies of TBA **7** as well as of its monomer residue analogue **9** in $CHCl_3$ are shifted to lower frequencies (1662 and 1665 cm^{-1}, respectively, vs. 1688 cm^{-1} in *N*-methylacetamide **11**),[16] indicating that the amide hydrogen is H bonded. Next consider the ester carbonyl, taking methyl acetate **10** as reference.[17] Here only TBA **7** has its ester (lactone) carbonyl frequency shifted toward higher values (1756 cm^{-1} in TBA **7** vs. 1742 cm^{-1} in methyl acetate **10**), since in TBA **7** the amide hydrogen is obliged to H bond to the lactone ring oxygen. In the analogue **9** the amide hydrogen prefers to H bond to the carbonyl oxygen of the ester, and the ester carbonyl frequency is shifted by 4 cm^{-1} in the opposite direction, to 1738 cm^{-1}. Note that TBA **7**'s lactone carbonyl frequency shift cannot be attributed to the nature of the trilactone ring, since the parent trilactone **8** absorbs at 1739 cm^{-1} in $CHCl_3$, indicating the presence of a strain-free ring system. Thus the analysis of the IR of the carbonyl frequencies in TBA **7** indicates that the amides of the side chains are H bonded to the lactone oxygens of the ring, and consequently that the benzamide side chains and the lactone carbonyls point in opposite directions.

The above considerations are further supported by the observation that both spectral shifts discussed above are diminished by reduction of the hydrogen bond strength upon addition of ethanol to the chloroform solutions, or upon replacing chloroform by acetonitrile. The intramolecular hydrogen bond is weakened; consequently the ester carbonyl frequencies of TBA **7** and of its analogue **9** reach almost identical values (1753 and 1751 cm^{-1}, respectively).

Circular Dichroism. The IR analysis did not resolve the question of whether the hydrogen bond involves the oxygen of the same seryl residue or the adjacent one. The NMR coupling (Table I) between H^α and H^N, implying by the Karplus equation a torsional angle of about ± 140° for H^α-H^N, was also consistent with both alternatives. This problem was resolved by the exciton chirality method. The first possibility would cause a right-handed chirality of the side chains and the second a left handed one. The exciton chirality method of coupled oscillators has been applied to a variety

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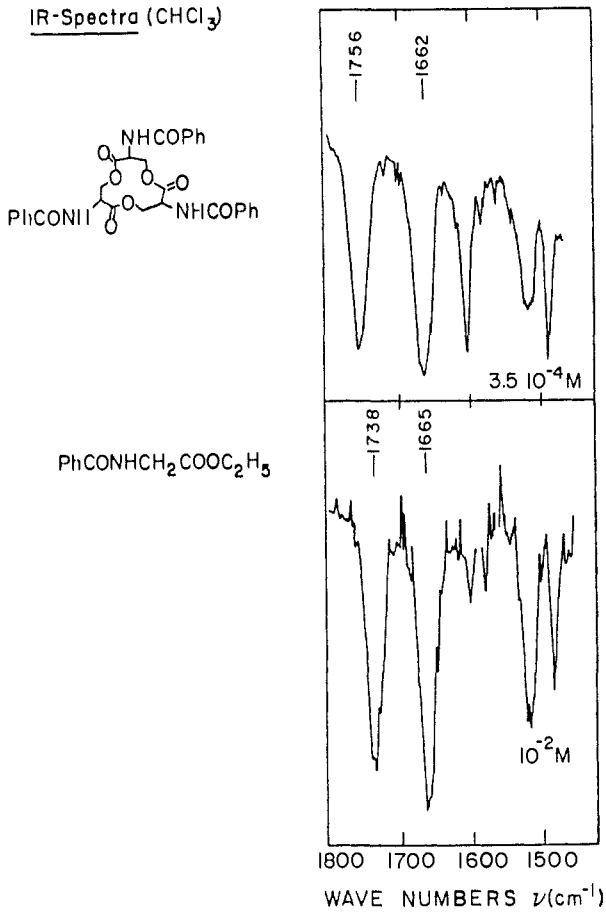

Figure 3. IR spectrum of tribenzamide **7** in $CHCl_3$ in comparison with *N*-benzoylglycyl ether ester **9** as an acyclic reference molecule.

Table III. UV and CD Spectral Extrema of TBA **7**

			UV		CD		
compd	solvent	concn ($\times 10^{-4}$ M)	λ_{max} (nm)	ϵ	λ (nm)	$\Delta\epsilon$	*A* value
7	CH_2Cl_2[a]	4.00	223	31 700	231	10.4	>10.4
					220	0.0	
7	CH_3CN	2.79	223	21 500	227.5	5.2	10.4
					218	0.0	
					205	-5.2	
7	EtOH	3.41	226	35 000	230	5.2	6.95
					225	0.0	
					213	-1.75	
12[b]	EtOH	10.3	226	13 000	230	1.75	
					217	0.0	
					203	1.4	

[a] The negative part of the CD cannot be determined because of the end absorption of the solvent. [b] Compound **12** is CH_3-O-C′O′-CH(-NH-C′O′-C_6H_5)-CH_2-OH.

of benzoates and related compounds.[18] It relies on the fact that two or more chromophores, when absorbing around the same wavelength, and when closely located in space, give rise to a Davidov-split CD curve, the sign of which reflects the chiral arrangement of the transition moments of the interacting chromophores. CD could thereby tell whether the aromatic residues of TBA **7** interact with each other and whether such interactions have a chiral preference.

The CD pattern in Figure 4 shows two Cotton effects, the first positive and the second negative, with the wavelength at the zero point close to the UV absorption maximum. This pattern indicates exciton coupling and a Δ arrangement of the benzamide chromophores of TBA **7**. This orientation has the same sense as that of the Δ-cis (Fe·enterobactin)$^{3-}$ complex.[10] Table III presents the

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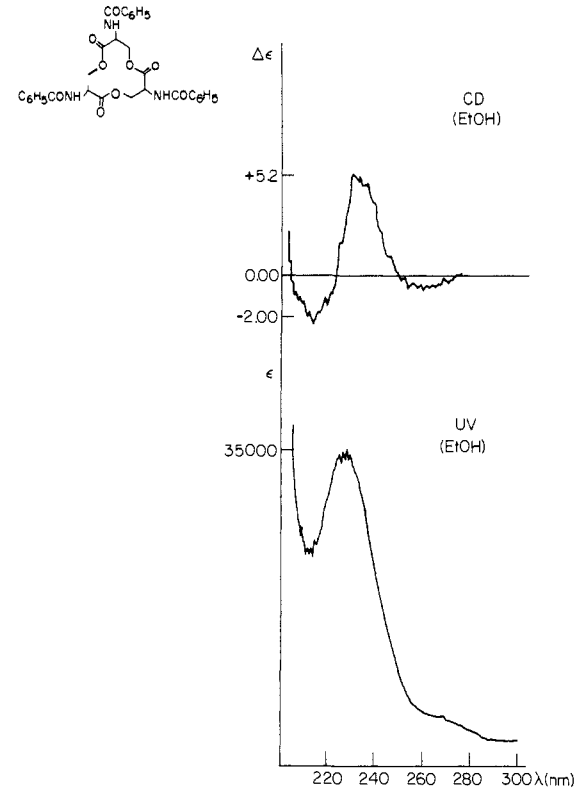

Figure 4. UV and CD spectra of tribenzamide **7** in EtOH (3.4×10^{-4} M), indicating exciton coupling.

CD spectrum of TBA **7** in other solvents. The *A* values (the sum of both absolute $\Delta\epsilon$ values) are larger in aprotic solvents (>10 in CH_2Cl_2) than in protic ones (7 in EtOH).

The exciton coupling observed by CD is relatively small, indicating that the deviation of the UV transition moments from their parallel position is small. However, it increases in nonpolar solvents where the amide–ester hydrogen bond is stronger (see the above discussion of IR).

The common origin of the NMR, IR, and CD observations is further evidenced by the consistency of their solvent dependence. In nonpolar solvents the NMR vicinal coupling constants are smaller (Table I), and the CD *A* values are higher (Table III) than in polar and protic solvents. These trends are consistent with an increased contribution of the axially oriented side chains, increased mutual interactions between the phenyl rings, and an enhanced right-handed chirality of their arrangement. The latter is compatible with the stronger hydrogen bonding in nonpolar solvents, as evidenced by the solvent dependence of the IR carbonyl frequencies (Table II).

In order to confirm the validity of using TBA **7** as a model for genuine enterobactin we determined its NMR spectrum under the conditions applicable and reported for enterobactin, namely Me_2SO at elevated temperatures.[5] The data for 25 and 85 °C are given in Table I. The closely resembling coupling constants between the vicinal protons for enterobactin and for TBA **7** demonstrate the conformational similarity of these two compounds. The higher vicinal coupling constant for TBA **7** in Me_2SO (J = 8 Hz) than in $CHCl_3$ (J = 3.1 Hz) indicates an increased contribution of equatorially positioned side chains brought about by the cleavage of the H bond in Me_2SO.

Theoretical Calculations

How do these findings relate to the conformational and binding mode of enterobactin? Experimental studies on the conformation of enterobactin are handicapped by the molecule's poor solubility and oxidative instability. CD spectra cannot be interpreted directly, because of the lack of information on the catechol chromophore's transition moments, and IR spectra are limited to protic solvents or solid KBr pellets, where inter- and intramolecular H bonds cannot be distinguished. NMR studies are limited to polar

Table IV. Calculated Conformations of the Tribenzamide (TBA) and Enterobactin (ent)

	axial Δ				axial Λ				equatorial		
	TBA	ent. a	ent. b	$(Fe{\cdot}ent)^{3-}$	TBA	ent. a	ent. b	$(Fe{\cdot}ent)^{3}$	TBA	ent. a	ent. b
figure no.	5			8	7			9	6		
					Relative Energy (kcal/mol)[a]						
E_{Tot}	0	0	0.3	0	4.1	4.0	6.1	0.5	2.1	4.4	4.2
E_{Coul}	0	0	−3.2	0	1.8	0.6	−0.2	−1.8	1.4	−1.3	−1.3
E_{LJ}	0	0	6.0	0	3.1	1.8	9.0	2.4	3.0	8.4	10.7
E_{Str}	0	0	−2.5	0	−0.8	1.6	−2.8	−0.1	−2.3	−2.7	−5.1
					Fe^{3+} and Amide Hydrogen Distances to Adjacent Oxygens (Å)						
Fe^{3+}–$O(C_2)$				2.02				2.02			
Fe^{3+}–$O(C_3)$				2.05				2.05			
$H(N_i)$–O_i	2.56	2.52	2.58								
$H(N_i)$–O_{i+1}					2.53	2.45	2.51				
H(N)–$O(C_2)$		1.83		1.59		1.82		1.58		1.83	
H(N)–O′									2.72	2.79	2.73
					Torsional Angles (deg)[b]						
ω_0	−175	−173	−176	174	179	177	180	−174	−174	−174	−176
χ_2	157	163	155	135	148	150	150	177	−118	−117	−119
χ_1, N	57	56	58	70	60	57	58	65	−175	−174	−175
χ_1, C′	−70	−72	−69	−58	−66	−67	−67	−65	61	62	61
ψ, N	−38	−41	−36	−30	−33	−32	−34	−60	122	121	122
ψ, C^{β}	86	82	87	97	92	94	91	67	−115	−116	−115
ϕ, C′	−56	−57	−51	−81	−168	−177	−173	−157	−157	−156	−155
ϕ, C^{β}	−179	180	−175	153	69	61	64	76	81	81	83
ω_N	170	175	170	157	−174	−177	−173	−158	180	180	180
ρ, C_2	1	−1	−177	3	−1	−3	176	−4	0	0	−179
$H^{\alpha}H^{\beta}_1$	54	53	55	68	58	58	57	60	61	62	62
$H^{\alpha}H^{\beta}_2$	−63	−65	−62	−49	−58	−60	−60	−58	180	−179	−179
$H^{\alpha}H^{N}$	−131	−133	−131	−167	139	130	137	161	141	143	143

[a] All zeros are reference values. [b] See figure in text for notation. Where necessary, the terminal atom involved in defining the torsional angle follows the angle notation, e.g., χ_2, N. The torsional angles of the unsubstituted trilactone ring **8** (ref 26), given for comparison purposes, are $\chi_2 = \mp 125°$, $\chi_1 = \pm 63°$, $\psi = \mp 110°$, and $\omega = \mp 176°$, where the upper sign refers to one conformation and the lower to its mirror image.

solvents at elevated temperatures.[5] Computer experiments on the other hand are not handicapped by such limitations and offer a detailed and reliable description of molecular conformations. The calculated equilibrium conformations of enterobactin and of its Fe^{3+} complex, as well as of the TBA **7** analogue, have therefore been used to examine the similarities and differences between them.

The Empirical-Force-Field (EFF) Method. The method and its applications to the study of ion-binding by ionophores have been discussed and reviewed in recent publications.[19-24] It involves the calculation of the energies and equilibrium conformations of the free ligands and their ion-complexes by elementary energy functions of bond lengths, bond angles, torsional angles, and interatomic distances. Both the strengths and limitations of the method derive from its simplifying assumptions and approximations, as only the main energy contributions to the binding are retained. These comprise the molecular conformational energy of the free ligand, the ion–ligand interaction energy, and the strain energy representing conformational change upon binding. Ignored are the solvent effects, entropy changes, electric polarization, and other secondary factors. Consequently, some computed results are faithful representations or predictions of reality while others are not. For example, the computed main features of the conformations of the free and complexed ligands, or the trends in binding of homologous series, can be trusted as good approximations, with only finer details being possibly in error. Other quantities, such as the binding constants and their variations with solvent and temperature, are at present either beyond reach by the EFF method or would require an excessive and hardly justifiable computational effort.

The energy parameters employed in the EFF calculations were taken from our previous studies,[21-23] except that new atoms or groups required some extensions. Thus equilibrium bond lengths and angles for the phenyl and catechol rings were adapted from the cambridge Structural Data Base;[25] the C′–C_1 bond connecting the amide and phenyl rings was attributed a partial double-bond character; and the Lennard–Jones parameters for Fe^{3+} were fixed in a manner analogous to that used for the alkali ions[21] as $r^* = 3.84$ Å and $\epsilon = 0.0192$ kcal/mol. The molecular conformations were constrained to C_3-rotational symmetry during the minimization process, except where otherwise noted.

Equilibrium conformations of TBA **7**, enterobactin **1**, and its Fe^{3+} complex were obtained by minimizing the total molecular energy. The initial conformation was that of the lowest energy C_3-symmetric conformation of the trilactone ring **8** given in ref 26, to which the benzamide or catechol amide side chains were attached.

Results of the EFF calculations on TBA **7**, free enterobactin, and its Fe^{3+} complex are presented in the stereoviews and in Table IV in a format suitable for comparisons. However, each of the three species deserves a separate analysis.

Tribenzamide (TBA). We have calculated a number of equilibrium conformations of TBA. The most stable conformation and two other preferred ones of low energy all possess C_3 sym-

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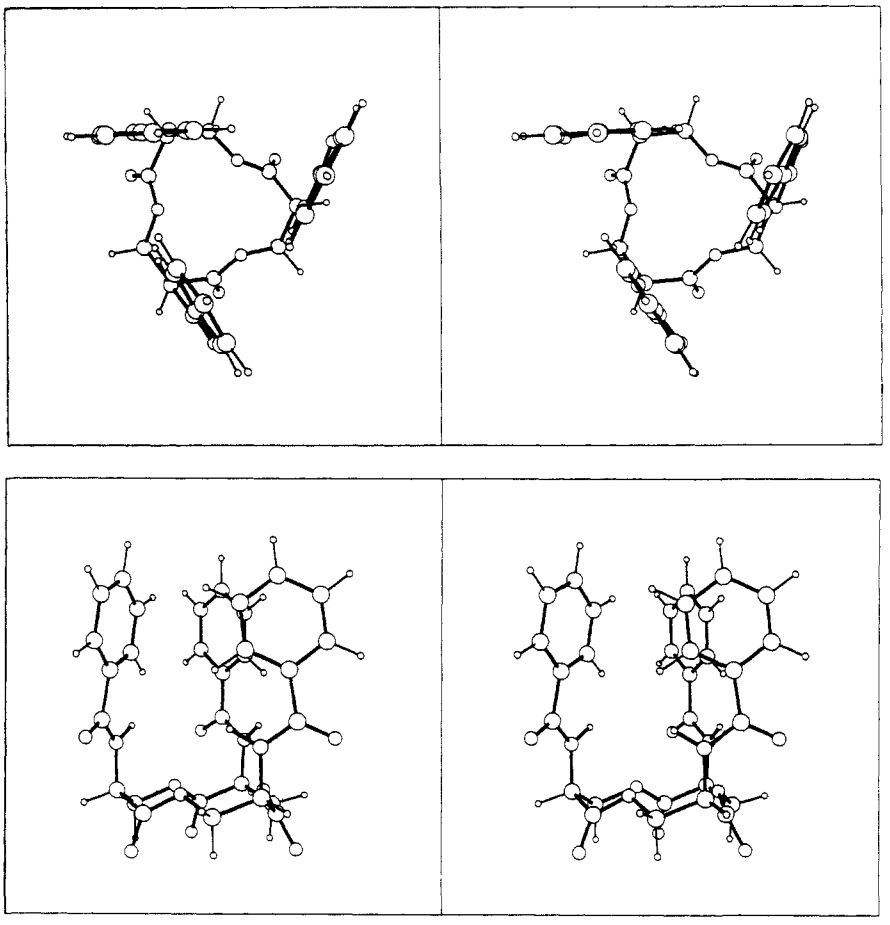


Figure 5. Stereoview of TBA 7 in its calculated most stable conformation, with its side chains in the axial Δ orientation.

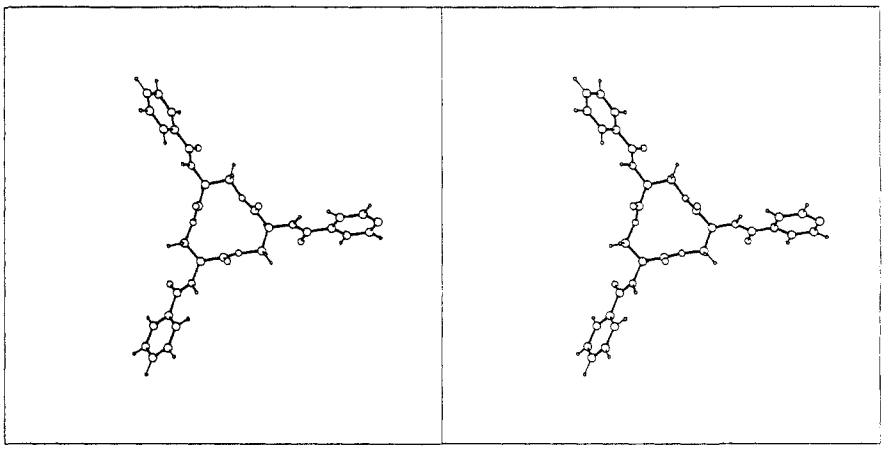
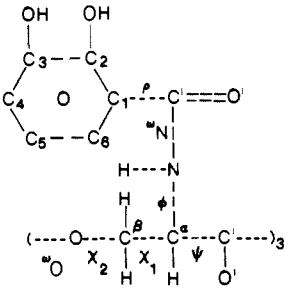


Figure 6. Stereoview of TBA 7 in the calculated equatorial orientation. Relative energy 2.1 kcal/mol.

metry. Some additional equilibrium conformations were found to be nonsymmetric. Three of them had low energies (0.2, 0.7, and 0.8 kcal/mol above that of the most stable conformation). They all resembled more or less the preferred conformation, in that one asymmetric unit shifted away while the two others changed relatively little. All other local minima that we obtained had too high energies to be of interest. All these conformations had very low energy barriers between them and were therefore easily interconvertible.

In the following we discuss only the C_3-symmetric conformations. Table IV presents their total energy E_{Tot} relative to that of the most stable conformation, and its three components: electrostatic or Coulomb (E_{Coul}), nonbonded or Lennard–Jones (E_{LJ}), and intramolecular strain (E_{Str}). Table IV lists also the torsional angles of the asymmetric unit (see the following diagram for notation). The torsional angles of the trilactone ring in all three conformations differ little from that of the unsubstituted trilactone ring[26] **8** (or its mirror image).



The most preferred conformation of TBA 7 (Figure 5), denoted as axial Δ, can be characterized as follows: First, the side chains

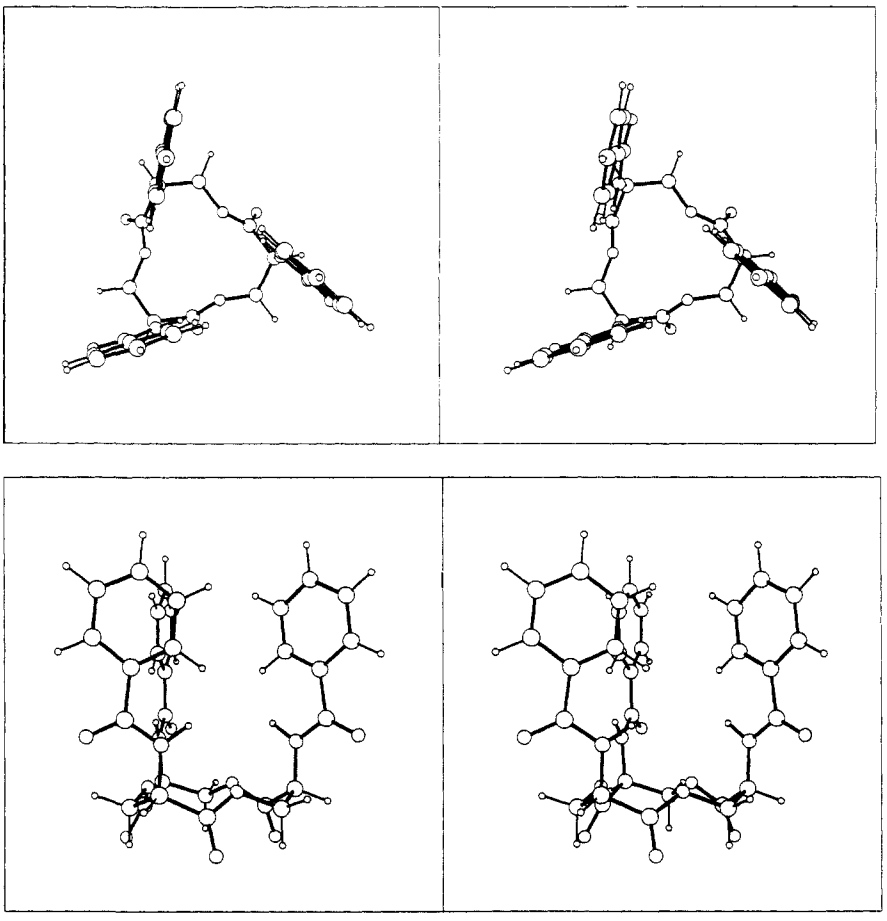


Figure 7. Stereoview of TBA 7 in the calculated axial Λ orientation. Relative energy 4.1 kcal/mol.

are axial. Second, the plane through C^{β}–C^{α}–N coincides with the plane through the phenyl ring. Third, the N–H bond is oriented toward the ring ester oxygen of the same seryl unit (the asymmetric unit in the diagram). This orientation defines the conformational chirality of the side chains such that an inward tilt of the phenyl rings creates a Δ configuration similar to that in the (Fe·enterobactin)$^{3-}$ complex.

In the second conformation (Figure 6) the side chains are equatorial, and the phenyl rings are widely separated. In the third conformation (Figure 7), denoted as axial Λ, which is the least stable of the three, the side chains are again axial, but their chirality is of opposite sense, and the N–H bond is oriented toward the ester oxygen of the next asymmetric unit.

Both the Δ and Λ conformations, having their phenyl groups in axial positions, are equally consistent with the NMR coupling constants for the ring and amide hydrogens (Table I). The Karplus equation gives approximately ±50° for the vicinal hydrogens H^{α}–$H^{\beta 1}$ and H^{α}–$H^{\beta}{}_2$, implying an axial orientation of the side chains. It also gives ±140° for H^{α}–H^{N}. These torsional angles compare well with those for the calculated axial conformations of TBA 7 in Table IV. The Δ conformation is calculated to be the more stable by 4 kcal/mol. This is consistent with the CD results described above, suggesting that the Δ configuration of the phenyl side chains should predominate.

So far, theory agrees with experiment. However, in both calculated axial conformations, the amide hydrogen is not close enough nor suitably oriented toward the ester oxygen (see Table IV and Figures 5 and 7) to form a stable hydrogen bond. Yet the experimental data supplied credible arguments for the existence of such a bond. In order to shorten the calculated H--O distance and improve the alignment of the N–H bond, the benzamide group must tilt toward the ester oxygen. Indeed, such a tilt is also implied by the CD results, which require that the transition dipole moments of the benzoyl groups, probably oriented along the C_1--C_4 line, be tilted relative to the molecular symmetry axis. However, the calculated orientations of the C_1--C_4 line in both axial conformations are parallel to the symmetry axis to within 1–2°. We must therefore conclude that our calculations failed to recognize a force that acts on the side chains to tilt the benzamide groups and bring the N–H closer to the ring ester. At present, we can only offer tentative suggestions for the origin of such a force. Since the phenyl rings are in close contact with each other and a cavity is formed between the phenyl rings and the lactone ring, some solvent interaction may contribute to such a tilt. Or perhaps the interactions between the π-electron cloud of a phenyl ring and the C–H bonds of a neighboring ring are not sufficiently well represented in our force field. Only further extensive research can perhaps give the answers.

Uncomplexed Enterobactin (1). Six C_3-symmetric low-energy conformations of (neutral) enterobactin were derived from the three obtained for TBA 7 by attaching two hydroxyl groups at the 2,3 positions of the phenyl ring, on either one or the other side of the 1 position. In conformation "a", the carbon at position 2 is trans to the carbonyl oxygen, and its hydroxyl oxygen points toward the amide hydrogen. Conformation "b" is obtained from "a" by a 180° rotation of the phenyl group. Conformation Δa is, like conformation Δ of TBA 7, the most stable. However, the energies of the "a" and "b" conformations are very similar. The hydroxyls are involved in extensive hydrogen bonding to each other and/or to the amide carbonyl oxygen. Perhaps the most significant hydrogen bonding is that from the amide hydrogen to the nearest oxygen of the catechol ring in the "a" conformation, which becomes even stronger in the Fe^{3+} complex. In all other conformational characteristics, the free enterobactin resembles TBA 7. Therefore, the use of TBA 7 as a structural model for enterobactin is also strongly supported by the theoretical conformational analysis.

In particular, the calculated H^{N}--O distances in TBA 7 (2.56 Å) and axial Δ enterobactin (2.52 Å) are very close to each other. The arguments presented above, that the side chains in TBA 7 must be tilted so that the amide–ester hydrogen bond distance is shortened, may therefore hold also for enterobactin. Whether this tentative prediction is borne out by experiment remains to

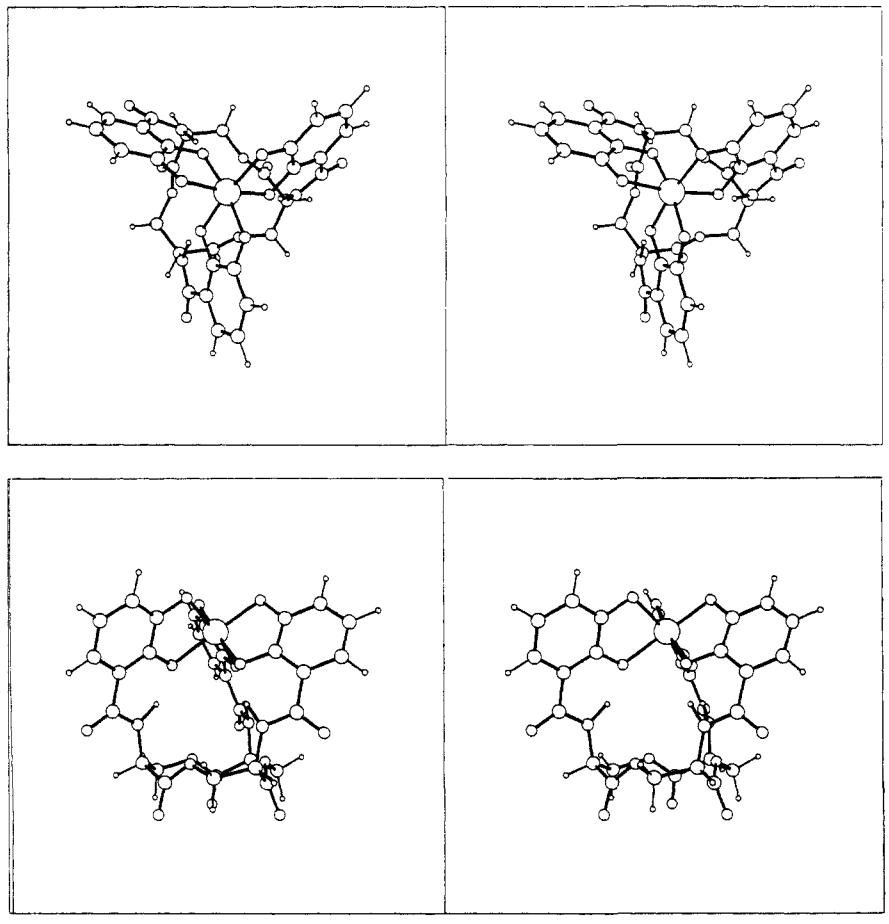


Figure 8. Stereoview of $(Fe{\cdot}enterobactin)^{3-}$ in the calculated Δ orientation.

be seen.

Enterobactin^{6-}·Fe^{3+} Complex (1a). In this section we address ourselves to two questions, the chirality of the complex and its binding strength. Two distinct conformations of the Fe^{3+} complex were calculated. Their stereoviews are shown in Figures 8 and 9. In both, the side chains are rotated so that the catechol oxygens point into the center of the molecular cavity. The ring torsional angles shift relatively little upon binding the ion (see Table IV).

The calculated Δ orientation of $(Fe{\cdot}ent)^{3-}$ is more stable than the Λ by 0.5 kcal/mol. This is approximately consistent with the Δ-cis configuration of $(Fe{\cdot}ent)^{3-}$ observed in its CD spectrum,[10] since even a free energy of about 1 kcal/mol would suffice to exhibit a chiral predominance. The flexibility of the lactone ring is probably the reason why the energy difference between the Δ and Λ orientations of the complex is small. Consequently, the possibility that certain changes of solvent and temperature may modify the complex's Δ-cis preference cannot be excluded. On the other hand, the fact that both experiment and theory show predominance of the Δ conformation invokes the possibility that a preorientation of the Δ conformation in free enterobactin dictates the path of complexation toward the Δ-cis conformation of the complex.

In considering the Fe^{3+} binding strength of enterobactin we have to relate it to both the free catechols and other tricatechol compounds. Tricatechol compounds have the advantage over monocatechols, particularly at low concentrations, in that the order of the binding reaction is reduced from fourth to second order. Therefore the entropy of the uncomplexed state is lower, and the equilibrium is shifted toward complexation. On the other hand, an unfavorable geometry of a tricatecholate may impose upon the complex a distorted and strained conformation, and consequently weaken the ion–ligand interaction energy. Such a distortion may be assessed by evaluating the relative strain of the complex and the accompanying strain energy. We define the relative strain as the conformational change imposed on the molecule by complexation, and the relative strain energy as the accompanying energy change. Its calculation requires the following "Gedanken-Experiment". First, the equilibrium conformation of the $(Fe{\cdot}ent)^{3-}$ complex is calculated in its Δ conformation (the reference state, see Table IV). Next, the $Fe^{3+}{\cdot}(catecholate\ amide^{2-})_3$ complex is separated from the trilactone ring by cutting the C^{α}–N bonds of the three side chains connecting this ring to the catecholamides. The conformations of the two resulting pieces are kept the same as that of the equilibrium $(Fe{\cdot}ent)^{3-}$ complex. We now use the conformations of these "frozen pieces" as the initial states for energy minimizations, making appropriate corrections for the energy of interaction across the severed bonds. The difference between the energies of the pieces in their frozen initial states and their final equilibrium states is their strain energy. The sum of the relative strain energies of the two cut pieces constitutes a reasonable representation of the strain energy imposed on the original ligand upon complexation. This procedure has the great advantage that it avoids the calculation of the energy of the ionization of the catechols by the Fe^{3+} ion, which is common to all Fe^{3+} complexes and therefore cancels out in all comparisons.

The relative strain energy of the $(Fe{\cdot}ent)^{3-}$ complex was calculated to be 11 kcal/mol, namely 3.7 kcal/mol per asymmetric unit. This is small not only relative to the binding energy but also relative to the entropy advantage gained against monocatecholates. On the other hand, it indicates the possibility of seeking new tricatechol ligands with less strain energy which may bind ions even better than enterobactin. Of the 11 kcal/mol relative strain energy, 7.1 kcal reside in the $Fe^{3+}{\cdot}(catecholate\ amide^{2-})_3$ complex and 3.9 kcal in the trilactone ring and its connections to the three amides, thus indicating that the ring energy is less affected by the complexation than the side chains.

The low relative strain of the $(Fe{\cdot}ent)^{3-}$ is also evident from the nature of the conformational change imposed on the enterobactin upon complexation, as can be seen from Table IV by comparing the torsional angles of the free and complexed molecule, both in the Δ and Λ conformations. The only significantly strained torsional angle is ω_N, which is shifted by 27° from its unstrained

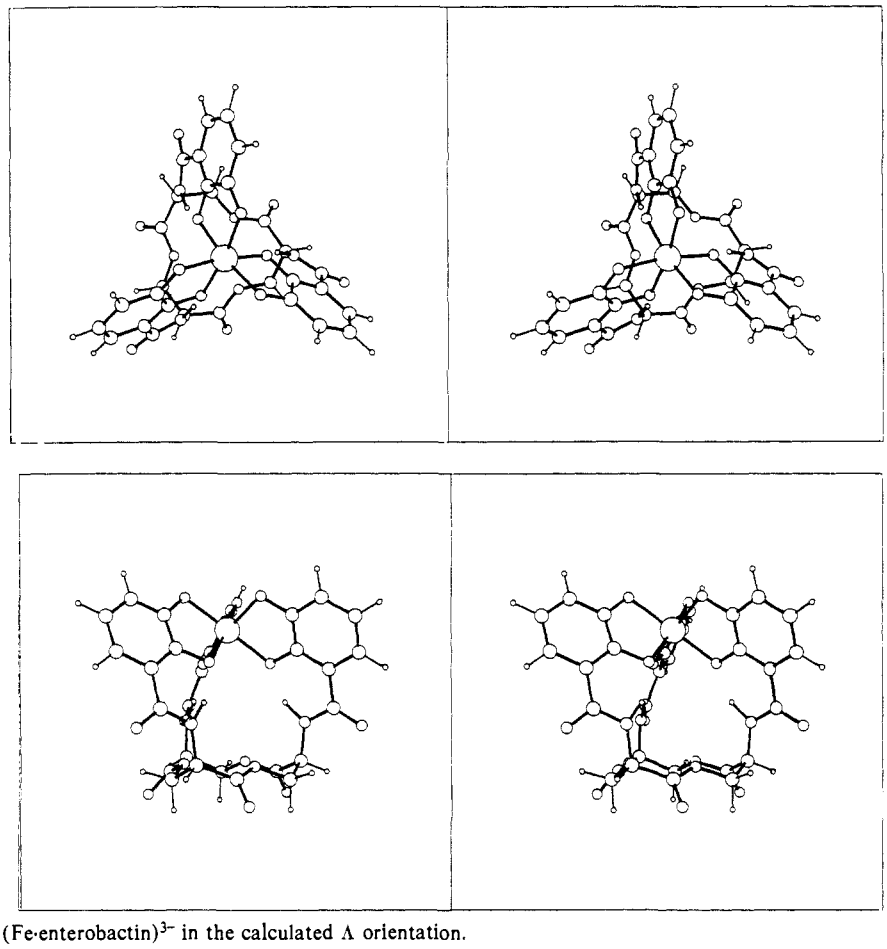


Figure 9. Stereoview of (Fe·enterobactin)$^{3-}$ in the calculated Λ orientation.

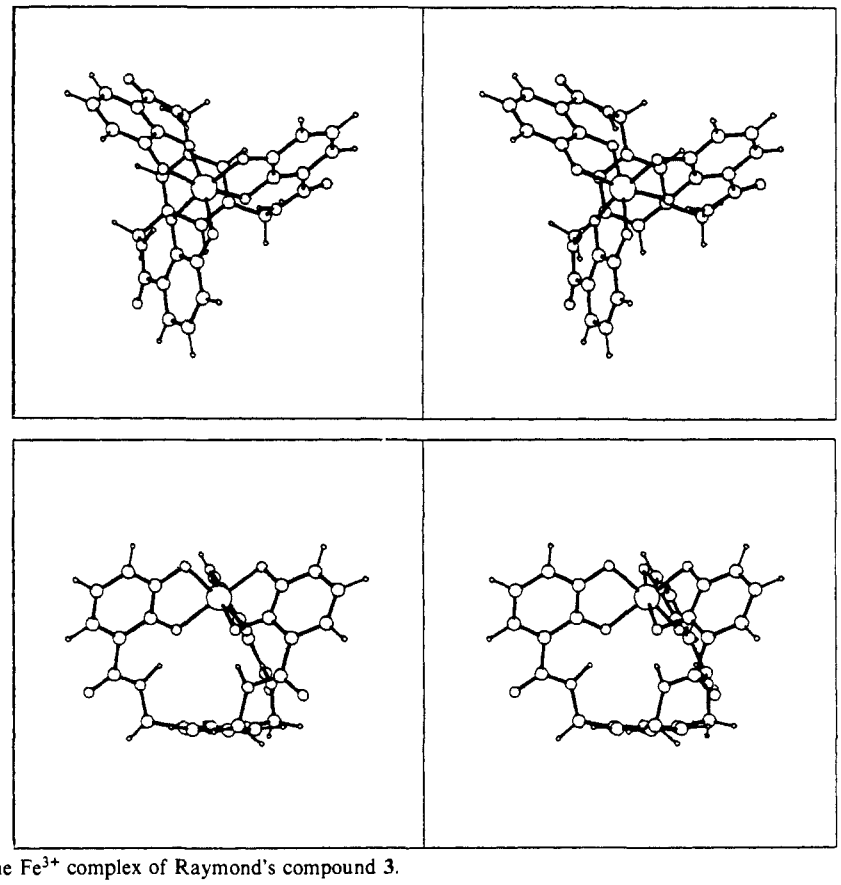


Figure 10. Stereoview of the Fe^{3+} complex of Raymond's compound 3.

value. For the other angles χ_1, χ_2, ϕ, and ψ that are shifted by about 10° to 30°, the rotational barriers are very low (and assumed to be zero in our force field). Indeed, the torsional changes are too small to account for the calculated relative strain energy, which

is distributed over all conformational degrees of freedom, including bond angles, interatomic nonbonded interactions, etc.

The best hitherto known synthetic tricatechol ligand is the mesitylene derivative **3**. It was synthesized by Raymond et al.,[7] who also discussed the relative stabilities of enterobactin and their compound. Our calculations shed some more light on the factors that make enterobactin a better Fe^{3+} binder than the synthetic analogue **3**. We have calculated the equilibrium conformation of this compound (see Figure 10). Following the "Gedanken-Experiment" performed on enterobactin, we also separated this compound **3** into two parts, cutting the Fe^{3+}·(catecholate amide^{2-})$_3$ complex from the mesitylene ring $C_6H_3(CH_2-)_3$ at the C–N bond. Its relative strain energy appears to be 13.0 kcal/mol, which is 2.0 kcal/mol higher than that of enterobactin. The measured binding constant is six orders of magnitude lower, equivalent to an about 8 kcal/mol difference in the binding free energy.[7] The gap may be closed by the difference in the conformational entropy of the two molecules. As we have noted above, enterobactin has only a few low-energy conformations. Its axial Δ conformation is the most stable according to our calculations, and even more stable according to our IR experiments, which indicated stronger hydrogen bonds than those estimated by the force field. The mesitylene derivative **3** has a larger conformational freedom than enterobactin, since its side chains contain an extra CH_2 group, and since it has no hydrogen bonds to stabilize a specific conformation.

The calculated distances of the Fe^{3+} ion to the catechol oxygens $O(C_2)$ and $O(C_3)$ in the mesitylene derivatives **3** are 2.07 and 2.05 Å, respectively. The corresponding values for enterobactin are given in Table IV. The Fe^{3+}--$O(C_2)$ distance in **3** is seen to be 0.05 Å larger. Another difference between enterobactin and the mesitylene derivative **3** may be discerned by comparing the components of their relative strain energies. Of the 13 kcal/mol strain energy of the latter, only 5.3 kcal reside in the iron complex, while 7.7 kcal belong to the mesitylene ring and its connections to the three amides. As was pointed out before, the opposite order holds for enterobactin. This may be qualitatively related to the differences in the conformations of the two molecules. The catechols of the mesitylene derivative are far apart and have to be pulled in to form the complex, while those of enterobactin are packed together in the axial conformation and have merely to be tilted upon binding iron.

Conclusions

When all the experimental data and the calculated results are taken together, the following picture emerges: Uncomplexed enterobactin **1**, like TBA **7**, exists in nonpolar solvents predominantly in a C_3-symmetric conformation with the side chains in an axial right-handed orientation. This predominance is weakened progressively with increasing polarity of the solvent. The axial orientation of the side chains is stabilized by intramolecular hydrogen bonds between the amide protons of the side chains and the lactone oxygens of the ring. The bonding of the amide hydrogen to the lactone oxygen of the same asymmetric unit is preferred over the bonding to the lactone oxygen of the adjacent unit, and this preference is expressed by the predominance of the Δ conformation of the free enterobactin, as well as of TBA **7**, over the conformational diastereoisomer Λ. Upon binding Fe^{3+} the side chains rotate to face directly inward, and the hydrogen bonds of the amides to the ring are broken, while those of the catechol oxygens are shortened. The enterobactin molecule is only mildly strained upon formation of the (Fe·enterobactin)$^{3-}$ complex. The best synthetic catecholate ligand hitherto available, the mesitylene derivative **3**,[7] is somewhat more strained by complexation than enterobactin, and it also looses more conformational freedom, hence its weaker binding. These findings may help the design and synthesis of artificial ionophores with improved binding properties.

Experimental Section

^1H NMR spectra were recorded on a Bruker WH-270 and ^{13}C NMR spectra on a Bruker WH-90 instrument. Chemical shifts were determined in ppm relative to internal Me_4Si. UV spectra were recorded on a Cary 118 and CD spectra on a Cary 60 spectrophotometer. IR spectra were measured on a MX-1-Nicolet FT-IR spectrometer.

Preparation of Trilactone 6. The β-lactone of serine (1.25 g; 4 mmol) and stannoxane (120 mg; 0.2 mmol) were dissolved in 5.0 mL of dry carbon tetrachloride and heated under reflux for 1 h. Then the mixture was concentrated in vacuo and the residue chromatographed on silica gel (woelm 60) with toluene–ethylacetate (98/2) as eluent. Pure trilactone **6** (250 mg) was obtained and recrystallized from chloroform. It decomposes at >260 °C. Anal. Calcd. for $C_{66}H_{57}N_3O_6$: C, 80.2; H, 5.7. Found: C, 79.95; H, 6.00.

The procedure given here for the trimerization of the β-lactone is superior to that reported earlier[11] in providing the cyclic trimer **6** isomerically pure and crystalline. The compound isolated earlier contained linear oligomers as byproducts, which presence had inadvertently been overlooked.

Preparation of Tribenzamide 7. Trilactone **6** (495 mg; 0.5 mmol) was heated for 2 min with 2.0 mL of 0.9 N dry HCl in ethanol. Then the mixture was concentrated in vacuo and washed with four portions of dry ether. The crude ammonium salt was then suspended in 15 mL of dry THF and treated at 0 °C for 10 min simultaneously with 0.35 mL of benzoyl chloride (3 mmol) and 0.63 mL of triethylamine (4.5 mmol). Stirring was continued for 15 min at 0 °C and for 30 min at room temperature. The resulting mixture was concentrated in vacuo and chromatographed on silica gel (Woelm 60). Elution with chloroform–methanol (98-2) provided 71 mg of pure tribenzamide **7**: mp 203–205 °C (chloroform/toluene); molecular weight 573 (determined by CI mass spectrometry in tris(hydroxyethyl)amine).

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Registry No. 1, 28384-96-5; **1a**, 61481-53-6; **6**, 88109-07-3; **7**, 104465-30-7; **8**, 80109-29-1; **9**, 1499-53-2; **12**, 20989-42-8; 3(*S*)-[(triphenylmethyl)amino]-2-oxetanone, 88109-06-2; (3*S*,7*S*,11*S*)-3,7,11-triamino-1,5,9-trioxacyclodecane-2,6,10-trione trihydrochloride, 104530-71-4.