The Chemistry of Antimycin A. XII. Dissociation Constants and Iron(III) Chelates of Antimycin A_3 and Some Analogs[1]

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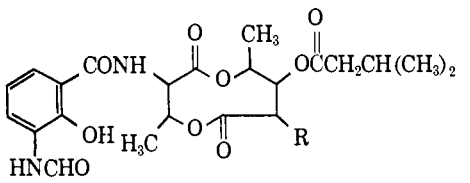
The pK_a values of antimycin A_3, 3-formamidosalicyl-N-(n-butyl)amide (FSBA), 3-formamidosalicylanilide, and o-formamidophenol were determined to be 5.5, 6.5, 6.2, and 8.1, respectively. The coordination of antimycin A_3 and FSBA with Fe(III) was investigated by the method of continuous variation. FSBA coordinated with Fe(III) in a 1:1 ratio at an observed pH of 2.9. Antimycin A_3 was found to form a 2:1 chelate with Fe(III) at pH 2.6. As the pH was lowered, the ratio changed to a 1:1 chelate at pH 1–1.5. The stability constants of the 1:1 Fe(III)–FSBA (log K, 8.5) and Fe(III)–antimycin A_3 (log K, 7.8) chelates were comparable with the previously reported constant for a 1:1 Fe(III)–salicylamide chelate (log K = 9.0). The stability of the 2:1 antimycin A_3–Fe(III) complex (log K = 15) was considerably less than that reported for a 2:1 salicylamide–Fe(III) chelate (log K = 18.3). The Fe–antimycin chelate was postulated to be a bidentate, six-membered ring structure involving the 1-carboxamide and 2-hydroxyl functions of the aromatic moiety of antimycin.

Antimycin A, a very powerful inhibitor of the electron transport system,[2–4] is a substituted 3-formamidosalicylamide.[5]



antimycin A_1, R = *n*-hexyl
antimycin A_3, R = *n*-butyl

Extensive studies with the respiratory enzymes of highly purified submitochondrial particles have localized the antimycin-inhibited site at a point in the electron transport chain after coenzyme Q and cytochrome *b* but before cytochrome c_1.[4,6–8] The antimycin-sensitive reduced coenzyme Q–cytochrome *c* reductase particle contains nonheme iron and cytochromes *b* and c_1 in the ratios 2:2:1.[9] This particle is also powerfully inhibited by 2-alkyl-4-hydroxyquinoline N-oxides and by 3-alkyl-2-hydroxy-1,4-naphthoquinones in those cases where the alkyl groups contain 6–9 carbon atoms.[4,10]

Dickie, *et al.*,[11] found that the N-hexadecyl- and N-octadecylamides of 3-formamidosalicylic acid were quite powerful inhibitors of the reduced coenzyme Q–cytochrome *c* reductase particle and concluded that the inhibitory activity was associated particularly with the aromatic portion of the antimycin molecule. Since all of these inhibitors contain potential ligands for chelation of iron, it has been suggested that the inhibitory action is related to nonheme iron binding capacity.[12]

The properties of the potential ligands available on the aromatic portion of antimycin as well as the nature and stability of the iron(III) complexes of antimycin A_3 and its analogs are reported in the present paper.

Experimental

Materials. Spectral quality reagent grade *p*-dioxane, b.p. 100–101°, was obtained from Matheson Coleman and Bell Co. and maintained in a frozen state under nitrogen between experiments to retard peroxide formation. The entire contents of a 1-l. bottle was used over a time period not exceeding 1 month. Ethanol (95%) was freshly distilled prior to use. Ferric perchlorate (nonyellow) from the G. Frederick Smith Chemical Co. was freed of excess perchloric acid by repeated washings with cold 95% ethanol, dried *in vacuo*, and stored at −18°. Iron-free potassium perchlorate (Parr Instrument Co.) was used without further purification. Perchloric acid solutions were prepared by diluting 70% Mallinckrodt A.R. grade perchloric acid with deionized water and standardizing with 0.11 *N* sodium hydroxide.

The N-(*n*-butyl)amide and -anilide of 3-formamidosalicylic acid were prepared as previously described[11] and had melting points of 100–100.5° and 175–176°, respectively. Antimycin A_3 (blastmycin) was obtained from Dr. H. Yonehara, Institute of Applied Microbiology, Tokyo University, Tokyo, Japan, and was shown[13] to be free of antimycin A_1 and A_2. *o*-Formamidophenol, m.p. 128–129°, was prepared by the method of Bamburger.[14]

Apparatus. pH measurements were made on a Model 7664 Leeds and Northrup pH meter equipped with a Model GK 2021 B Radiometer concentric glass electrode. All visible and ultraviolet spectra were

(1) Supported in part by Grants No. G-22249 and GP-1983 from the National Science Foundation.
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Table I. Acid Dissociation Constants of Antimycin A_3 and Representative Model Compounds

Compd.	Solvent	Concn., m*M*	pK_a Present study	pK_a Lit.
Antimycin A complex	Ethanol (95%)	0.12		5.1 ± 0.1[a]
Antimycin A_3	Dioxane (50%)	0.13	5.5 ± 0.1	
FSBA[b]	Ethanol (10%)	0.13	6.5 ± 0.05	
FSA[c]	Ethanol (10%)	0.07	6.2 ± 0.05	
o-Formamidophenol[d]	Water		8.1 ± 0.1	
Salicylamide[d]	Dioxane (50%)			8.89 ± 0.05[e]
o-Aminophenol[d]	Dioxane (50%)			11.57 ± 0.05[f]

[a] See ref. 5. [b] 3-Formamidosalicyl-N-(*n*-butyl)amide. [c] 3-Formamidosalicyl-N-phenylamide. [d] The pK_a values of these compounds were determined by potentiometric titration; the others were determined by spectrophotometric measurements; see ref. 15. [e] A. Agren, *Acta Chem. Scand.*, **9**, 50 (1955). [f] R. G. Charles, H. Frieser, and W. D. Johnston, *J. Am. Chem. Soc.*, **74**, 1383 (1952).

determined on a Cary Model 11 spectrophotometer in 1-cm. quartz cells at 25°.

Procedure. Spectrophotometric determination of acidic dissociation constants was performed by the method of Bendich.[15] Stock solutions were prepared in either dioxane or 95% ethanol, and suitable small aliquots were diluted in 10-ml. volumetric flasks to volume with appropriate buffers. The buffers were prepared by the method of Clark and Lubs.[16] After thorough mixing the pH of each solution was determined with the glass electrode. Solvent blanks were

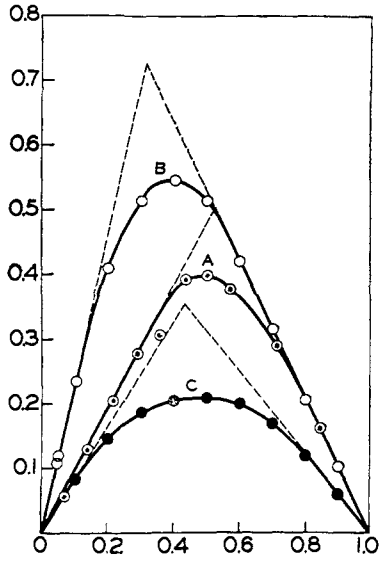

Figure 1. Curve A, Fe–FSBA at 550 mμ; pH 2.9 ± 0.05; [Fe(III)] + [FSBA] = 7.0 μmoles/10 ml. Curve B, Fe-antimycin A_3 at 542 mμ; pH 2.6 ± 0.05; [Fe(III)] + [antimycin A_3] = 10 μmoles/10 ml.; μ = 0.05 *M*. Curve C, Fe-antimycin A_3 at 550 mμ; pH 1.5 ± 0.05; [Fe(III)] + [antimycin A_3] = 10 μmoles/10 ml.; μ = 0.05 *M*.

prepared by diluting 1-ml. portions of ethanol or 5 ml. of dioxane to 10 ml. with the corresponding buffer solutions. Absorbance values at the alkaline λ_{max} were plotted *vs.* pH and the inflection point was determined. The dissociation constant for antimycin was determined in 50% aqueous dioxane at an ionic strength of 0.05 *M* maintained with $KClO_4$. The calculated apparent pK_a values were corrected by subtracting 0.2 in the case of 10% ethanol and 1.2 for the 50% dioxane solutions, because the aqueous buffers in the pH range 4–8 were consistently observed to read 0.2 and 1.2 pH units lower, respectively, than the same buffers prepared in these solvents. This is in agreement with the observations of Van Uitert, *et al.*[17] The pK_a value of *o*-formamidophenol was determined by potentiometric titration. For the iron chelate studies, pH values are quoted as measured with the glass electrode and are not corrected.

Molar ratios and stability constants of the chelates were determined from data obtained by continuous variation experiments as described by Job[18] and by successive approximation using the law of additive absorbancies.[19] The stability constant, $K_n = [FeL_n]/[Fe^{+3}][L^-]^n$, where L^- is the unprotonated ligand and n = 1 or 2, was calculated from the observed constant and the respective pK_a values. In these studies 3-formamidosalicyl-N-(*n*-butyl)amide (FSBA) and antimycin were used. FSBA was dissolved in 4% aqueous ethanol and the solution was adjusted to pH 2.9 ± 0.05 with perchloric acid. Antimycin was prepared in 50% aqueous dioxane and the solution was adjusted to an ionic strength of 0.05 *M* with 0.125 *M* potassium perchlorate and to the desired pH with perchloric acid. At pH 1.5 absorbances were determined between 10 and 15 min. after mixing because of limited stability of the colored complexes formed. The colors at higher pH values were stable for 30–45 min.

Results

Acid Dissociation Constants. The results of the pK_a determinations are listed in Table I together with literature values for structurally related model compounds. The most strongly acidic of the phenolic protons are those of the *ortho*-disubstituted phenols represented by antimycin and its N-(*n*-butyl)- and N-phenylamide analogs. This is probably due to a combined inductive effect of the *ortho* substituents coupled with a greater resonance stabilization of the phenolate ion. The model compounds having single HCONH– or $-CONH_2$ groups *ortho* to the hydroxyl are several hundredfold less acidic than antimycin A.

Coordination of 3-Formamidosalicyl-N-(n-butyl)-amide (FSBA) with Iron(III). FSBA was chosen as the antimycin analog to study because it was readily obtained in a high degree of purity[11] and was fairly water soluble. Continuous variation results (Figure 1A)

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(17) L. G. Van Uitert, C. G. Haas, W. C. Fernelius, and B. E. Douglas, *J. Am. Chem. Soc.*, **75**, 455 (1953).
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(19) H. Diehl and F. Lindstrom, *Anal. Chem.*, **31**, 417 (1959).

Table II. Composition and Stability Constants of Fe(III)–Antimycin A_3, Fe(III)–3-Formamidosalicyl-N-(*n*-butyl)amide, and Fe(III)–Salicylamide Complexes[a]

Ligand	Ligand–Fe(III) ratio	λ_{max}, mμ	log K_1	log K_2	[Ligand]/[H^+]	Molar absorptivity × 10^{-3} M^{-1} cm.$^{-1}$
FSBA	1:1	550	8.5 ± 0.1	...	0.1–0.3[b]	1.48
Antimycin A_3	1:1	550	7.8 ± 0.1	...	0.003–0.03[b]	0.645
Antimycin A_3	2:1	542	...	15 ± 0.1[c]	0.025–0.25[b]	2.30
Salicylamide	1:1	525	9.0[d]	...	1 or less	1.50
Salicylamide	2:1	475	...	18.3[d]	10 or more	2.90

[a] See ref. 20. [b] Based on the range of FSBA or antimycin concentrations in the continuous variation experiments at constant pH. [c] Calculated by successive approximations using the law of additive absorbances. [d] Calculated from Agren's values[20] for the K_a of salicylamide and the chelate stability constant.

were consistent with the formation of a 1:1 FSBA–Fe(III) complex. The stability constant, K_1, as determined from the continuous variation data is given in Table II. The log K value of 8.5 is in good agreement with the potentiometric results of Agren[20] on salicylamide. Calculation of the stability constant for this complex from Agren's data and the pK_a value for salicylamide gave a log K value of 9.0.

Antimycin A_3–Iron(III) Chelates. Attempts to carry out continuous variation studies with antimycin at pH 3.0 or above were unsuccessful because of the formation of unstable ferric–antimycin complexes. This instability was attributed to the formation of strongly solvated ferric hydroxide species in competition with the ferric–antimycin complex at pH 3 and higher pH values. When the experiments were performed at pH 2.6 in 50% dioxane, a 2:1 antimycin–Fe(III) complex was formed (Figure 1B) having a λ_{max} at 542 mμ. The stability constant of this complex (equal to $[Fe(A_3)_2][H^+]/[FeA_3][HA_3]$, where HA_3 is antimycin A_3) was calculated, by use of the law of additive absorbances, from those points in Figure 1, curve B, where antimycin was in excess (*i.e.*, [Fe(III)]/([Fe(III)] + [ligand]) less than 0.5). The necessary molar absorptivity values were also read from Figure 1, that of the 2:1 complex from the extrapolated peak of curve B, and that for the 1:1 complex at that point on extrapolated curve C corresponding to equimolar concentrations of Fe(III) and antimycin. The assumption was made that no Fe^{+3} was present in the experimental mixtures involved in these particular calculations. The error introduced by the two different wave lengths (542 and 550 mμ) is negligible as the spectrum of the 1:1 complex shows a very broad maximum. Calculations utilizing data from those points in Figure 1 corresponding to an excess of Fe(III) over antimycin were also used once the stability constant of the 1:1 complex was determined as described below.

As the pH was lowered at identical antimycin concentrations, the continuous variation plots shifted gradually toward a characteristic 1:1 slope intercept. At pH 1.5 an apparent molar ratio of 1.17:1 antimycin–Fe(III) was observed (Figure 1C). Attempts to work below pH 1.5 were unsuccessful as the initially formed purple complex was rapidly decomposed and a black precipitate formed within 5 min. The stability constant for the 1:1 complex at pH 1.5 was therefore calculated by use of the law of additive absorbances plus the value for the stability constant of the 2:1 complex corrected for the pH change. The resulting value was used to calculate the stability constant of the 2:1 complex at pH 2.6 in the region where the antimycin concentration was not in excess. Table II gives the values of the stability constants, $K_1 = [FeA_3]/[Fe^{+3}][A_3^-]$, and $K_2 = [Fe(A_3)_2]/[Fe^{+3}][A_3^-]^2$, calculated from the observed constants and the pK_a value of antimycin.

(20) A. Agren, *Acta Chem. Scand.*, **9**, 47 (1955).

Discussion

The above results are in agreement with the observations of Agren[20] who reported that the coordination ratio of salicylamide to Fe(III) was dependent on the ratio [salicylamide]/[H^+]. A 2:1 salicylamide–Fe(III) complex was formed when this ratio had a numerical value of 10 or more, while a 1:1 complex predominated when it was 1 or less.

In the antimycin studies, limited solubility coupled with iron hydrolysis at pH values above 3 made it impossible to carry out experiments under conditions where the ratio [antimycin]/[H^+] was more than 0.003. However, the experimental results obtained (Table II) indicate that a 1:1 chelate is formed near this value, as would be expected from the lower pK_a value of antimycin, 5.5, as compared to 8.89 for salicylamide.

Structure of the Antimycin–Iron(III) Chelates. The aromatic portion of the antimycin molecule possesses three potential bonding groups which may form five- or six-membered rings with iron(III), namely, the 1-carboxamide carbonyl, the 2-hydroxyl, and the 3-formamido nitrogen. Ferric iron chelating with the first or second of these pairs would form six- or five-membered ring chelates, respectively. Although *o*-aminophenol does react to form a purple five-membered ring chelate,[21] *o*-formamidophenol failed to give a visible chelate with ferric perchlorate under the same conditions. The fact that addition of pyridine to the reaction mixture in this case had no effect on color formation precludes hydrogen bonding as the sole inhibitor of chelate formation.[22] Steric hindrance by the formamido carbonyl to the formation of a five-membered ring coupled with the instability of a possible seven-membered ring (including the carbonyl oxygen) would explain the difficulty of chelate formation in the case of *o*-formamidophenol.

Second, the 1-carboxamide together with the 2-hydroxyl function should provide a system having properties similar to those of salicylamide in the formation of a six-membered iron chelate ring. Comparison of Agren's studies[20] on the iron(III)–salicylamide

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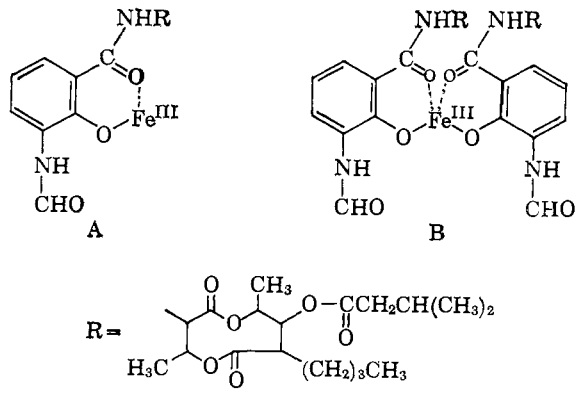

Figure 2. Proposed structure of antimycin A_3–Fe(III) chelates: A, 1:1 chelate; B, 2:1 chelate. The suggested structure, B, is not intended to exclude other possible stereochemical arrangements of the ligands around the Fe atom. The other coordination positions, presumably occupied by water or other solvent molecules, are not included in the structure.

chelates with the results observed with antimycin A_3 confirmed these similarities (Table II). In further support of this type of ring formation Loomans[23] noted that the infrared spectrum of the ferric chloride–antimycin coordination compound was identical with that of antimycin except for a marked decrease in the 1-carbonyl peak at 6.01 μ.[24] Finally, Pfeiffer[25] reported that six-membered ring chelates are favored when one or more double bonds are present in the ring. The aromatic carbons of the benzene ring would contribute this double bond character in the case of antimycin.

The above evidence taken together strongly indicates that antimycin forms a chelate with iron(III) which contains a six-membered ring formed by coordination between the 1-carboxamide carbonyl and the 2-hydroxyl group. The proposed structures of the 1:1 and 2:1 complexes are given in Figure 2. In contrast, Folkers[26] has proposed a π-type 2:1 coenzyme Q–Fe(III) complex for the nonheme iron associated with the flavoproteins of succinic dehydrogenase and DPNH dehydrogenase.

Application to Electron Ttransport in Vivo. Extrapolation of the above results concerning the nature of the ferric–antimycin chelate to the intact reduced coenzyme Q–cytochrome *c* reductase particle as it occurs *in vivo* would be of doubtful validity. Whether or not antimycin will bind nonheme iron in the lipoprotein environment must depend very greatly on the accessibility of the bidentate ligands to the six coordination sites of iron(III). Clarification of this point must await further knowledge of the lipoprotein-bound iron. Furthermore, *in vitro* iron chelation studies carried out in the present work were of necessity performed in partly aqueous media and at low pH. These conditions differ widely from the environment of the reduced coenzyme Q–cytochrome *c* reductase particle where pH has little or no meaning in the absence of water. The stability of the antimycin A–iron chelates described in this study may, however, be compared with the stability of similar chelates of other electron transport inhibitors, such as those described by Tappel.[12]

Estabrook[27] has demonstrated that high concentrations of ferrous iron do not prevent electron transport inhibition or compete for antimycin. It might be possible to test the hypothesis that antimycin A inhibition is due to binding of nonheme ferric iron by adding the purified antimycin–iron(III) chelate to the reaction medium. If the purified chelate failed to show electron transport inhibition, the possibility of an iron(III) binding mechanism by antimycin would be considerably strengthened.

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(24) E. E. van Tamelen, *et al.*, ref. 5, p. 1641.
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Communications to the Editor

The Neighboring Anthryl Group in Solvolysis[1]

Sir:

2-Phenyl-1-ethyl systems[2] (I) are marginal with respect to the competition in solvolysis between anchimerically unassisted ionization (k_s) and anchimerically assisted ionization (k_Δ). The latter leads to the "nonclassical" phenyl-bridged or "ethylenephenonium"[3] cation. The two modes of ionization are associated with characteristically different ΔS^*,[2c] β-D isotope effects[4] and response to solvent nucleophilicity and ionizing power.[2] From kinetic criteria the k_Δ/k_s ratio in solvolysis of I-OTs is judged[2b,c] to be low in EtOH and AcOH and substantial in HCOOH. For 2-*p*-anisyl-1-ethyl *p*-toluenesulfonate (2-An-EtOTs), with a *p*-MeO group accelerating k_Δ but not k_s, the kinetic criteria[2c] indicate k_Δ/k_s is *ca.* 1 in EtOH and high in both AcOH and HCOOH. In typical solvolysis of such simple primary systems leakage from any intermediate III associated with k_s to the bridged ion II or the rearranged open ion IIIa is negligible, so that product composition[2d,5] from suitably labeled starting

(1) (a) Reported in summary at the Japanese-American Seminar in Physical Organic Chemistry, Kyoto, Japan, April 6–10, 1965; (b) research sponsored by the U. S. Army Research Office (Durham).
(2) S. Winstein, *et al.*: (a) *Bull soc. chim. France*, **18**, 55 (1951); (b) *J. Am. Chem. Soc.*, **75**, 147 (1953); (c) *ibid.*, **78**, 4801 (1956); (d) *Helv. Chim. Acta*, **41**, 807 (1958).
(3) (a) D. J. Cram, *J. Am. Chem. Soc.*, 71, 3863 (1949); (b) *ibid.*, **86**, 3767 (1964).
(4) (a) W. H. Saunders, *et al.*, *ibid.*, **80**, 242 (1958); (b) *ibid.*, **82**, 3586 (1960).
(5) C. C. Lee, *et al.*, *Can. J. Chem.*, **35**, 1417 (1957); *Tetrahedron*, 7, 206 (1959).