Solution Structure of Iron(III)−Anthracycline Complexes

Marina M. L. Fiallo,*,‡ Hartmut Drechsel,‡,▽ Arlette Garnier-Suillerot,‡ Berthold F. Matzanke,† and Henryk Kozlowski§

Chimie Bioinorganique, LPBC, ESA 7033, Université Paris Nord, 74, rue Marcel Cachin, 93017 Bobigny Cedex, France, Isotope Laboratory of the Natural Sciences, Medical University Lübeck, Germany, and Faculty of Chemistry, University of Wroclaw, Poland

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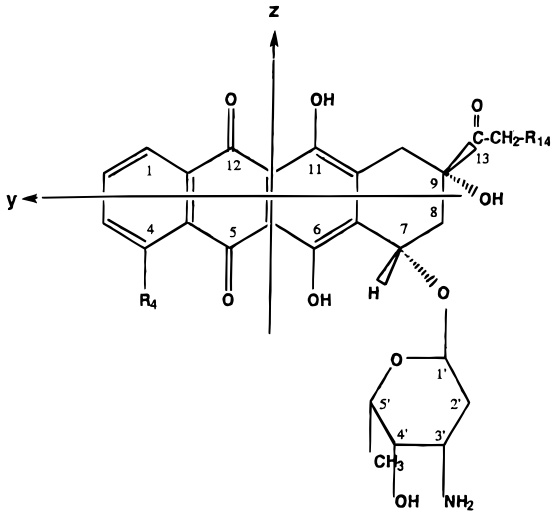
The interaction of Fe^{3+} with the anthracycline anticancer drug idarubicin (Ida) was studied by absorption, CD, Mössbauer, and EPR spectroscopy. The formation of two major Fe^{3+}−Ida complexes, labeled **I** and **II**, was observed. In complex **I**, Fe^{3+} ion was bound to anthracycline at the {C(12)═O; C(11)−O^-} coordination site. In complex **II**, two Fe^{3+} ions were bound at sites {C(5)═O; C(6)−O^-} and {C(12)═O; C(11)−O^-}, respectively. Complex **I** was an equimolar monomeric species with a 1:1 Fe^{3+}:Ida stoichiometry ($\beta_1 = 4.8 \times 10^{11}$ M^{-1}), whereas in complex **II** the anthracycline ligand was bridging two metal ions, alternatively bound to both anthracycline ring chelating sites with the assumption that the ratio of Fe^{3+}:Ida in complex **II** was 2:1 ($\beta_2 = 5.3 \times 10^{24}$ M^{-2}). Alternatively, complex **II** may be oligomeric with Fe^{3+}:Ida = 1:1 and with each Fe^{3+} bridging two Ida molecules. Our findings could be important in understanding the biological effects of the anthracycline−ferric complexes. Thus, providing information about the nature of the Fe^{3+}−Ida system, we suggest that the formal 1:3 Fe^{3+}: anthracycline complexes, reported in the previous literature, could be a mixture of species **I**, **II**, and free ligand.

Introduction

The development of multidrug resistance (MDR)[1] against anthracyclines[2] (Figure 1), as well as against other antitumor compounds, is a major problem in anticancer chemotherapy. Complexation of metal ions to biologically active molecules has been recently proposed as a new strategy to overcome the drug resistance.[3,4] Although this approach, using the anthracyclines, has not been successful,[5,6] the understanding of the interactions between these antitumor compounds and metal ions, especially biometals, such as Fe^{3+}, could be very useful to elucidate the molecular mechanisms in terms of biological activity and cell resistance.[7] In fact, it has been suggested that the adduct formation between anthracyclines and DNA[8−10] or phospholipids[11] could be enhanced by the presence of Fe^{3+}. Furthermore, the interaction between Fe^{3+} and anthracyclines could alter the antitumor properties of the drugs. For instance, the ferric complex of daunorubicin (Dnr) retains its biological properties,[5] whereas the one of doxorubicin (Dox) does not.[12] In the latter case the metal ion coordination leads to considerable ligand degradation,[6,13] which is not the case with the free ligand kept under similar experimental conditions.[14−16]

Within the last two decades, several papers concerning the interaction between Fe^{3+} and anthracycline derivatives have been published. The results of these studies, however, were often conflicting. The first reported Fe^{3+}−anthracycline complex, quelamycin,[17] assumed to be a triferric derivative of doxorubicin, could not exist at physiological pH.[18] Afterward, on the grounds of electronic absorption spectroscopy studies,[19−21] Fe^{3+} was generally accepted to form a major complex in which the metal ion binds three anthracycline molecules. Magnetic susceptibility measurements have shown the coordination of six oxygen atoms[20] to Fe^{3+} and the metal ion binding site has been generally assumed to be located on the {C(12)═O; C(11)−O^-} functions.[22] This



anthracycline		R_4	R_{14}
doxorubicin	Dox	OCH_3	OH
daunorubicin	Dnr	OCH_3	H
idarubicin	Ida	H	H

Figure 1. Structures of anthracyclines.

* Corresponding author. Tel: +33 (0)1.48.38.77.47. Fax: +33 (0)1.48.38.77.77. E-mail: fiallo@smbh.univ-paris13.fr.
‡ Université Paris Nord.
† Medical University Lübeck.
§ University of Wroclaw.
▽ Present address: Mikrobiologie und Biotechnologie, University of Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, France.

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binding preference, according to Myers,[23] was derived from better fitting of the distance between the oxygens of this site and Fe^{3+}. Against the general consensus, in 1984, Bachur suggested the formation of 1:1 Fe^{3+}:Dox complexes.[18] Furthermore, a CD study on these systems revealed the formation of at least two species of Fe^{3+}−anthracycline complexes, depending on the experimental conditions.[20] More recently, the results of ^{57}Fe Mössbauer and EPR spectroscopic studies have suggested that the formation of monomeric Fe^{3+}−Dnr or Fe^{3+}−Dox complexes was possible only in the presence of high ligand excess, whereas at the 1:3 metal:ligand molar ratio, oligomeric and/or polymeric species were formed.[24,25]

As a part of a project aimed at characterization of the interaction of anthracycline with cellular components, such as membranes,[26] DNA, or metal ions, using CD spectroscopy,[27] we have investigated the interaction between Fe^{3+} and idarubicin (Ida). This drug was chosen for the following reasons: (i) Ida is a close derivative of Dnr, which cannot be degraded in the presence of Fe^{3+};[12] (ii) Ida does not easily undergo self-association in aqueous solution;[26] and (iii) this highly lipophilic compound overcomes the MDR by very fast cell uptake.[28]

This paper reports the solution structure of the Fe^{3+}−Ida complexes. To determine the impact of the massive Fe^{3+} hydrolysis in aqueous medium on complex formation, the studies were performed in both DMF and aqueous solutions. Because the metal ion coordination resulted in simultaneous deprotonation of the phenolic function(s) of the aromatic ring, the spectra of the formed metal species resembled those of the anion(s) of the parent molecule, with a bathochromic shift due to the metal ion.[29] Thus, the full assignment of the CD spectrum of the anthracycline, at various deprotonation states of the drug molecule, was essential to interpret the CD data obtained for anthracycline metal complexes. The interactions occurring in the Fe^{3+}−Ida complexes were also studied by Mössbauer and EPR spectra.

Results and Discussion

The structure of Ida and, for comparison, those of Dox and Dnr are shown in Figure 1. The absorption and CD spectra of free Ida in solution, either in water at pH 7 or in DMF (Figure 2a), consisted of two bands centered around 290 and 470 nm, which were assigned to the allowed $\pi \rightarrow \pi^*$ transitions of the chromophore polarized along the short and the long axes of the anthracycline molecule, respectively. Two other bands at around 330 and 350 nm, clearly detected in the CD spectra, were assigned to the $n \rightarrow \pi^*$ transitions of the C(12)=O and C(5)=O chromophores, respectively.[27] Contrary to Dox and Dnr, Ida appeared to be self-associated only marginally in aqueous solution. In fact, its apparent association constant was estimated at 100 M^{-1}, whereas for Dox and Dnr the values are 1.1×10^4 and 8.6×10^3 M^{-1}, respectively.[26]

The Ida solution, either in DMF or in buffer, turned from orange to blue-violet when Fe^{3+} was added, with a broad band centered at 600−640 nm (Figure 3). Furthermore, the 300−400 nm region was also modified by complex formation; the concomitant absorption of Fe^{3+} and its hydrolysis products, however, made it less informative.

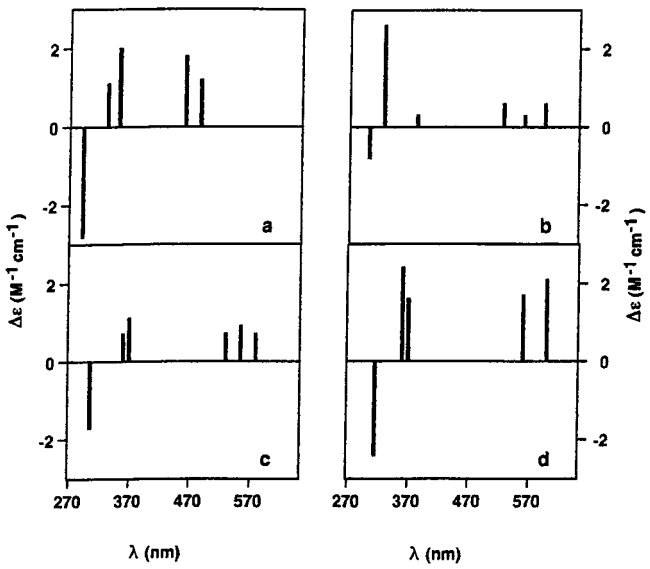


Figure 2. Schematic representation of the CD spectra of Ida under different experimental conditions: (a) DMF or water; (b) DMF and 2 equiv of EtONa; (c) water at pH 11, $[Na_2HPO_4] = 0.05$ M, $[KCl] = 0.1$ M; (d) water at pH 13, $[NaOH] = 0.1$ M, $[Ida] = 10^{-4}$ M.

Fe^{3+}−Ida Systems in DMF. When Fe^{3+} was added to a 5×10^{-4} M solution of Ida in DMF, at metal-to-ligand molar ratios ranging from 0.1 to 1, no appreciable spectral modifications were observed in the CD and absorption spectra. However, the subsequent addition of 1 equiv of EtO^- (per Ida) gave rise to the spectral changes shown in Figure 3a,b. At low metal-to-ligand molar ratio (0.1), the appearance of a band at ∼600 nm and a slight decrease of the intensity of the one at 290 nm were observed, while a new band appeared at 400 nm, in the spectral region associated with the $n \rightarrow \pi^*$ transitions. As the ratio of Fe^{3+} to Ida increased (>0.1), the bands associated with the $\pi \rightarrow \pi^*$ transitions polarized along the short and the long axes shifted to 650 and 315 nm, respectively. It follows that, depending on the amount of Fe^{3+} added, two different types of spectra were observed in DMF solution, suggesting formation of, at least, two distinct chemical species.

Fe^{3+}−Ida Systems in H_2O. In this experiment, Fe^{3+}−Ida complexes were prepared by adding the metal ion to the anthracycline solutions, at either pH 4 or 7.[31] At low metal-to-ligand molar ratio (<0.3), the CD spectra of Fe^{3+}−Ida, in acetate buffer (Figure 3d), were close to the first type of spectra observed in DMF solution (Figure 3b) with a band around 585 nm and a shoulder at 550 nm. The increase of the Fe^{3+}:Ida molar ratio (>0.3) led to a further shift of the band at 585 nm to ∼640 nm (Figure 3d). Two different sets of apparent isodichroic points were observed, at low metal-to-ligand ratio (<0.3), at 359 and 514 nm and, at high metal-to-ligand ratio (>0.3), at 367 and 509 nm.

In HEPES buffer, despite the massive hydrolysis of Fe^{3+} species at pH 7, similar CD spectra were obtained, except for the intensity of the band at ∼640 nm, which was increased 2-fold compared with that observed at pH 4 (Figure 3f).

Fe^{3+}−Ida Complexes. Under all experimental conditions, the recurrent presence of two types of CD spectra, depending on the metal-to-ligand molar ratio,

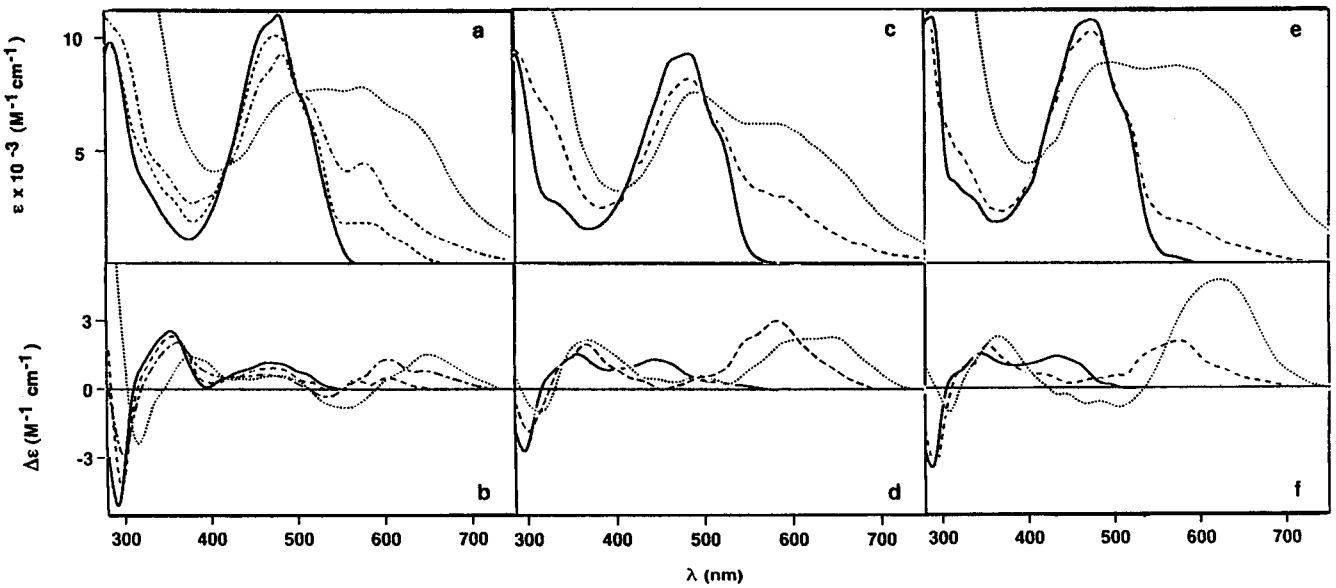


Figure 3. Visible absorption (upper panel) and CD (lower panel) spectra of Ida and Fe^{3+} systems, at different metal-to-ligand molar ratios: 0 (—), 0.1 (– – –), 0.33 (—·—), 1 (···); (a,b) in DMF with 1 equiv of EtONa; (c,d) in water at pH 4, [CH_3COONa] = 0.05 M, [KCl] = 0.1 M; (e,f) in water at pH 7, [HEPES] = 0.05 M, [KCl] = 0.1 M, [Ida] = 5×10^{-4} M.

strongly suggested that two different Fe^{3+}—Ida complexes were formed. For this reason, the dependence of $\Delta\epsilon$ at 600 and 650 nm (640 nm for the experiments done in aqueous solutions) versus the metal-to-ligand molar ratio was reported, under different experimental conditions (DMF, water at pH 4 and 7) (Figure 4). The plot of $\Delta\epsilon$ at 600 nm showed a maximum for a metal-to-ligand molar ratio around 0.3, whereas the maximum intensity of the band at 650 nm was reached for a metal-to-ligand molar ratio of ~1.

We called **I** and **II** the two complexes formed at low and high metal-to-ligand molar ratios, characterized by the CD bands at 600 and 650 nm, respectively. Furthermore, the addition of 1 more equiv of Ida to the complex **II**, at pH 4, favors the formation of complex **I**, whereas at pH 7 the same reaction was not possible anymore. These observations suggested that, in both complexes, Fe^{3+} was also bound to water molecules, which, being deprotonated at pH 7, would change the reactivity of the metal ion.

Stoichiometry of the Complexes I and II. Determination of the stoichiometry of the complexes **I** and **II** with the continuous variation method, or Job's method, was not possible. In fact, at 600 and 640 nm, the two complexes **I** and **II** were present in a wide range of metal-to-ligand molar ratios.

The stoichiometry of complex **I** could be determined, however, in DMF by CD spectroscopy with a high excess of ligand. Under these conditions, the entire available Fe^{3+} reacted with Ida. The intensity of the band at 480 nm was proportional to the amount of free drug (Figure 5). Its reduction, as a function of the amount of Fe^{3+} added, gave a stoichiometry of 1:1 Fe^{3+}:Ida for the complex **I**. This determination was not possible at a metal-to-ligand molar ratio higher than 0.2, when complex **II** started to be formed, because stacking of free and bound drug modified the $\Delta\epsilon$ values at 480 nm.

Thus, an analogous determination of the stoichiometry of complex **II** based on the CD data was not possible. As shown in Figure 4, when species **II** was formed, a certain amount of complex **I** was still present,

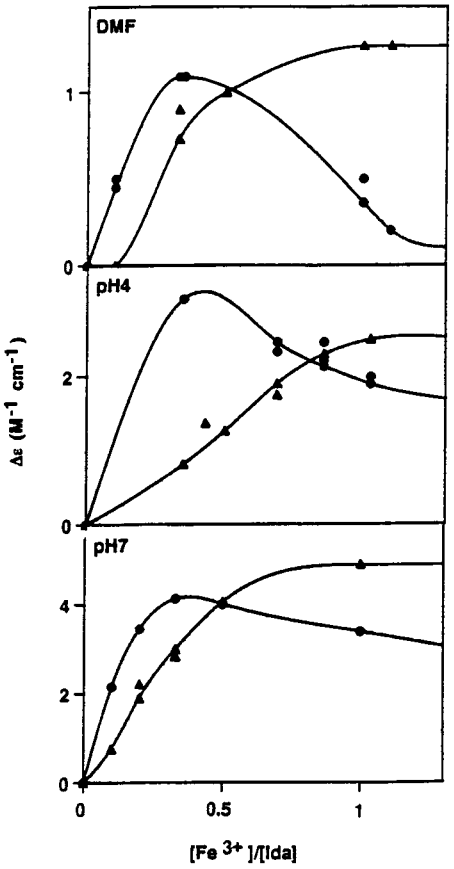


Figure 4. Variation of $\Delta\epsilon$ at 600 nm (●) and 650 nm (▲) as a function of the [Fe^{3+}]:[Ida] molar ratio, in different experimental conditions: (DMF) [EtONa] = 5×10^{-4} M; (pH 4) [CH_3COONa] = 0.05 M, [KCl] = 0.1 M; (pH 7) [HEPES] = 0.05 M, [KCl] = 0.1 M, [Ida] = 5×10^{-4} M.

and we were not able to quantify exactly the amount of coordinated anthracycline. However, clues on its stoichiometry could be derived from the observation that complexes **I** and **II** were in equilibrium only at pH 4, whereas at pH 7 their formation was irreversible and

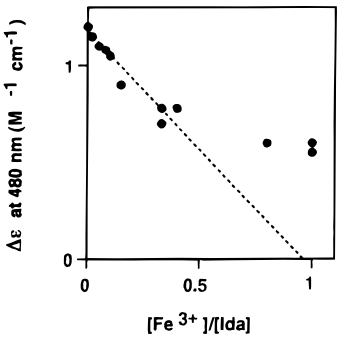


Figure 5. $\Delta\epsilon$ at 480 nm of Fe^{3+}−Ida system in DMF as a function of the metal-to-drug molar ratio: [Ida] = 5×10^{-4} M, [EtONa] = 5×10^{-4} M.

complex **I** was always formed before complex **II**. Two possible models of complex **II** are in agreement with these observations: (i) two Fe^{3+} are bound to the same Ida molecule or (ii) two (or more) molecules of complex **I** dimerized (or oligomerized) forming complex **II**. Depending on the two models, complex **II** would have a 2:1 or 1:1 Fe^{3+}:Ida stoichiometry.

Coordination to Idarubicin. Early, it was generally assumed that Fe^{3+} binds three molecules of anthracyclines. To evaluate the complex stoichiometry, the ϵ values at 600 nm were generally used.[19] Unfortunately, as shown in Figure 5a, because of the superposition of the bands at 600 and 640 nm, assigned to complex **I** and complex **II**, respectively, it was difficult to distinguish between them completely in the vis spectra, especially when besides the formation of two different complexes the metal hydrolysis was a competitive process.

To determine the coordination sites of Ida to Fe^{3+}, we used a phenomenological approach, comparing the CD spectra of Ida at different protonation states[27] with those obtained in the presence of Fe^{3+}. Ida has three potential coordination sites: the {C(5)═O; C(6)−O^-} and {C(12)═O; C(11)−O^-} aromatic functions and the amino group of daunosamine (pK_a = 8.2). The deprotonation of the ammonium group slightly modifies the UV−vis and CD spectra of Ida, whereas the deprotonation of the phenol functions strongly affects the position of all bands in the absorption and CD spectra.[27] In DMF solution, the addition of 1 equiv of EtO^- gave rise to the deprotonation of the ammonium group of the sugar, with no spectral modifications, whereas the deprotonation of a phenolic group occurred when a second equivalent of base was added (Figure 2b). More than 3 equiv of EtO^- (up to 5) did not have a further effect on the spectroscopic features. Thus, the spectral modifications observed in Figure 3a,b could be assigned to the coordination of Fe^{3+} to Ida. In aqueous solution, the deprotonation of the C(11)−OH site (pK_a = 11)[32] is accompanied by the shift of the band at 480 to ∼560 nm and the appearance of a band at 400 nm, corresponding to the $n \rightarrow \pi^*$ C(12)═O transition; by contrast, the position of the band at 290 nm did not change (Figure 2c). Above pH 13, when deprotonation of the C(6)−OH phenolic group occurred (pK_a > 12),[27] the bands, associated with the $\pi \rightarrow \pi^*$ transitions polarized along the long and short axes, further shifted to ∼600 and to ∼313 nm, respectively (Figure 2d).

Table 1. Mössbauer Parameters of 1:1 Fe^{3+}:Ida Systems in Different Experimental Conditions[a]

solution	solvent	base (equiv of EtO^-)	δ (mm s^{-1})	ΔE_Q (mm s^{-1})	$\Gamma_{1/2}$ (mm s^{-1})
a	H_20	(pH 4)	0.573(5)	0.675(9)	0.215(6)
b	DMF	1	0.564(2)	0.827(3)	0.298(3)
c	DMF	2	0.544(2)	0.877(4)	0.260(4)

[a] Measurements were performed at 4.2 K in an external magnetic field of 200 G perpendicular to the γ-beam; [Ida] = 1.1×10^{-3} M.

The spectral modifications associated with the formation of complex **I** (Figure 3), namely the bathochromic shift of the band associated with the $\pi \rightarrow \pi^*$ transition polarized along the long axis, suggested the Fe^{3+} coordination to the {C(12)═O; C(11)−O^-} site, as in the case for most other metals studied with well-defined complexes.[33−36] Unfortunately, in the case of Fe^{3+}, the amount of bound ligand was so low and it was difficult to define precisely the changes in the CD bands in the 280−500 nm region, during the formation of complex **I**. In the case of complex **II**, most of the ligand present in solution was likely involved in metal ion binding, as all the observed CD bands in the 280−700 nm region underwent distinct variations. The band associated with the $\pi \rightarrow \pi^*$ transition polarized along the along axis shifted from 600 to around 650 nm, while the band centered at 295 nm moved to 315 nm with hypochromic effect (Figure 3b). The latter distinct change in transition energies could be explained only assuming that the second potential coordination site at {C(5)═O; C(6)−O^-} was also involved in the metal ion binding. The involvement of both binding sites at the anthracycline ring in complex **II** strongly suggested the formation of the species in which drug molecules were bridged by Fe^{3+} ion, bound to two different anthracycline rings. These coordination modes, identified also for the anthracycline model compound quinizarin,[37,38] were found to be the same in DMF and aqueous solutions.

Mössbauer Spectra. To determine the interactions involving the metal species, Mössbauer and EPR spectra were recorded at 4.2 K. The experiments were done with the Ida solution either in water at pH 4 (solution a) or in DMF, in the presence of 1 equiv of EtO^- (solution b) and 2 equiv of EtO^- (solution c), at 1:1 metal:ligand molar ratio. Table 1 displays Mössbauer parameters of frozen solution of Fe^{3+}−Ida systems. The spectra (Figure 6) consisted merely of single quadrupole doublets with values typical of high-spin Fe^{3+} bound to oxygens in an octahedral arrangement. The quadrupole splitting reflected a symmetry lower than cubic at the Fe^{3+} site. Surprisingly, substitution of the solvent system water with aprotic DMF yielded an increase of 0.152 mm s^{-1} in the quadrupole splitting. The effect derived from water shielding the negative charges of the oxygen ligands by dipole−charge interaction. Also, the addition of base increased ΔE_Q by 0.05 mm s^{-1}. This might be attributed to the presence of additional charges in the vicinity of the metal center. The line widths $\Gamma_{1/2}$ of all samples were fairly large. This can be attributed to the presence of two or more species in solution exhibiting very similar Mössbauer parameters.

EPR Spectra. The EPR spectra of the preparations described above exhibited two extremely weak signals: a rhombic signal near g_{eff} = 4.3 and a signal near g =

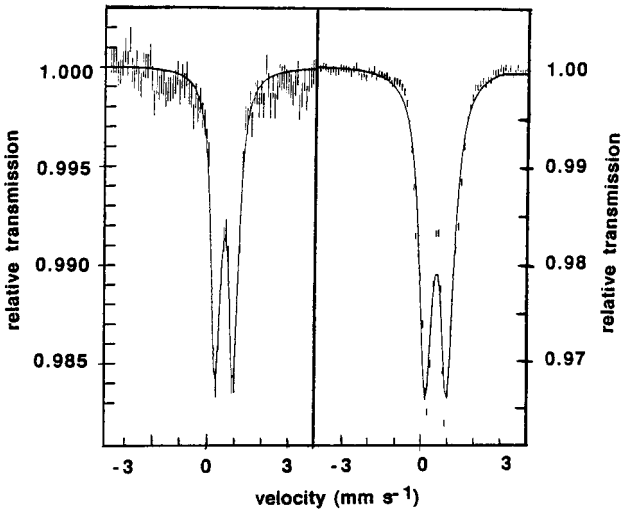


Figure 6. Mössbauer spectra of 1:1 Fe^{3+}:Ida systems: in water at pH 4 (left panel), [CH_3COONa] = 0.05 M, [KCl] = 0.1 M; in DMF with 1 equiv of EtONa (right panel), [Ida] = 1.1×10^{-3} M.

2. The solution b showed a third weak signal near $g = 3$. To quantify the spin density, the areas were obtained by double integration of the EPR derivative signals. The calculated areas were corrected by a factor f yielding the spin density of the detected signals. Assuming equal population of the three Kramer's doublets, the double-integrated $g = 4.3$ signals were multiplied by a factor of 3. The values were corrected for an iron concentration of 10^{-3} M and compared with the area of the reference sample (cytochrome P450 Fe(III) low spin, $S = 1/2$). More than 99% of the spin density of Fe^{3+}−Ida in water at pH 4, more than 93% of Fe^{3+}−Ida in DMF (with 1 equiv of EtO^-), and 100% of Fe^{3+}−Ida in DMF (with 2 equiv of EtO^-) were EPR silent. EPR measurements at 150 K displayed no resonance of antiferromagnetically coupled particles (data not shown).

Spectrophotometric Equilibrium Measurements. To evaluate the equilibrium constants of complexes **I** and **II**, the absorbances in the vis spectrum at 600 and 640 nm were determined for solutions with various concentrations of Fe^{3+} (>[Ida]) (Figure 4). At pH 4 about 20% of the metal ion undergoes hydrolysis. This reduced the rate of the Fe^{3+}−Ida complex formation. The equilibrium measurements in this study referred to the solutions within a few minutes after mixing of the reagents.

In the analysis, formation of the monoiron(III) species (complex **I**) and the diiron(III) species (complex **II**) was assumed. $IdaH_2$ notation means Ida molecule protonated at both the phenol functions. Because the amino sugar is not involved in the Fe^{3+} coordination and remains protonated at pH 4, it was ignored. With the notation Fe^{3+} we have indicated the aquairon(III) complex present in solution.[39]

At low metal-to-ligand molar ratio, complex **I** was formed following eq 1:

$$Fe^{3+} + IdaH_2 \xrightarrow{K_{f1}} [Fe{-}IdaH]^{2+} + H^+ \qquad (1)$$

At high metal-to-ligand molar ratio, complex **II** was formed as:

$$Fe^{3+} + [Fe{-}IdaH]^{2+} \xrightarrow{K_{f2}} [Fe{-}Ida{-}Fe]^{4+} + H^+ \qquad (2)$$

In our approximation, we considered the formation of complex **II** as a diiron(III) species (with a formal 2:1 metal:ligand stoichiometry), simplifying the mathematical analysis for the dimerization (or oligomerization) of **I** to **II**, as in the second model (with a formal 1:1 metal:ligand stoichiometry).

The choice of a model with two steps was already suggested by Jordan on the grounds of kinetic observations in the Fe^{3+}:Dnr ($DnrH_2$) systems.[37] A stopped-flow kinetic study indicated that this reaction was biphasic, and this was attributed to successive formation of $[Fe{-}DnrH]^{2+}$ and $[Fe{-}Dnr{-}Fe]^{4+}$ species. These kinetic results were in agreement with the spectroscopic data discussed above.

The combination of eqs 1 and 2 results in the absorbance relation described by eq 3, where ϵ_1 and ϵ_2 are the molar absorptivity at one specific wavelength of complexes **I** and **II**, respectively, and Fe^{3+} concentration was corrected by eq 4 assuming[40] $K_m = 1.85 \times 10^{-3}$:

$$\frac{A}{[Ida]_{tot}} = \frac{\epsilon_1 K_{f1}[H^+][Fe^{3+}] + \epsilon_2 K_{f1}K_{f2}[Fe^{3+}]^2}{[H^+]^2 + K_{f1}[H^+][Fe^{3+}] + K_{f1}K_{f2}[Fe^{3+}]^2} \qquad (3)$$

The least-squares fitting with the two-species model (eq

$$Fe^{3+} + H_2O \xrightarrow{K_m} [Fe(OH)]^{2+} + H^+ \qquad (4)$$

3) gave a good fit for the values at 600 and 640 nm. Under our experimental conditions ([Fe^{3+}] > [Ida]) complex **II** was preferentially formed, as shown by the CD spectra.

K_{f1} and ϵ_1 were determined for complex **I** and K_{f2} and ϵ_2 for complex **II**, at 600 and 640 nm (Table 2). The values of K_{f1} and K_{f2} were comparable because of similar coordination sites, and this reflected the low degree of association of Ida, compared to other anthracyclines, and less steric hindrance by the daunosamine moiety. The value of K_{f1} can be combined with a pK_a value of the phenol at C(11)−OH to give the conventional formation constant $\beta_1 = [Fe{-}IdaH]/[Fe^{3+}][IdaH] = 4.8 \times 10^{11}$ M^{-1}. The value of K_{f2} was combined also with pK_a of C(6)−OH phenol which was spectroscopically determined at 13 (M. Fiallo, A. Garnier-Suillerot, unpublished results) giving a β_2 value of $[Fe{-}Ida{-}Fe]/[Fe^{3+}]^2[Ida] = 5.3 \times 10^{24}$ M^{-2}.

In our analysis we did not consider the formation of the bis(μ-hydroxo)iron(III) dimer as in eq 5:

$$2Fe^{3+} + 2H_2O \rightarrow [Fe(OH)_2Fe]^{4+} + 2H^+ \qquad (5)$$

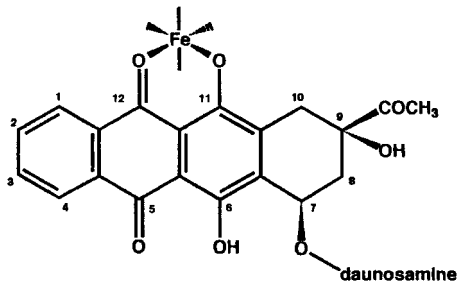
Because of the low degree of association of Ida compared to other anthracyclines,[26] the dimerization constant was not considered in the estimation of β_1 and β_2. The most probable structures for the two complexes for Ida are shown in Figure 7.

Solution Structure of the Complexes I and II. It was previously reported that Fe^{3+} was bound to the anthracycline only at the {C(12)═O; C(11)−O^-} coordination site, because it fitted well with the ionic radius of the metal cation.[23] In fact, Myers[19] showed that 11-deoxydoxorubicin did not bind Fe^{3+}. Furthermore, we have previously reported that another anthracycline

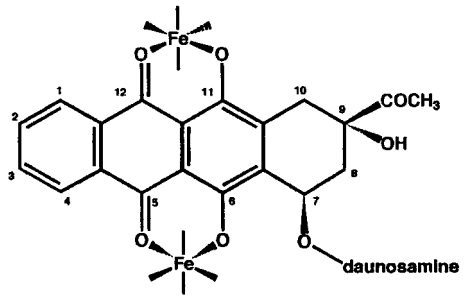
Table 2. Spectrophotometric Results for Fe^{3+}−Ida Complex Formation in Acetate Buffer, pH 4, at 25 °C

	K_f[a]	$\epsilon(600)$ (M^{-1} cm^{-1})	$\epsilon(640)$ (M^{-1} cm^{-1})
Ida		0	0
complex **I**	4.8 ± 0.4	3080 ± 372	3620 ± 330
complex **II**	1.1 ± 0.2	6475 ± 246	5595 ± 45

[a] K_{f1} and ϵ_1 refer to complex **I**, and K_{f2} and ϵ_2 refer to complex **II**.



complex I



complex II

Figure 7. Proposed structures for complexes **I** and **II**.

derivative, aclacinomycin, which lacked the C(11)−OH function, was unable to bind Fe^{3+}, whereas it formed stable complexes with Cu^{2+} and Tb^{3+}.[34,41] In this paper, we have shown that Fe^{3+} can be bound to both {C(12)═O; C(11)−O^-} and {C(5)═O; C(6)−O^-} coordination sites. Our findings are consistent with the hypothesis that the coordination to Fe^{3+} was, first, favored in the {C(12)═O; C(11)−O^-} position, because of the lower acidity of the C(11)−OH phenol, and, then, on the {C(5)═O; C(6)−O^-} position, probably because of the steric hindrance of the daunosamine and the higher acidity of C(6)−OH. Under these circumstances, the lack of coordination of Fe^{3+} with 11-deoxydoxorubicin and aclacinomycin should be due to the competing reaction of Fe^{3+} hydrolysis. The marked difference in the CD spectra of the Fe^{3+}−Ida complexes, with those of Dox and Dnr,[20] could reflect the different degree of self-association of these anthracyclines and the importance of the kinetic parameters in the coordination of Fe^{3+} to these drugs. In fact, because of the higher degree of self-association of these latter anthracyclines, compared to Ida,[26] the two possible coordination sites at {C(12)═O; C(11)−O^-} and {C(5)═O; C(6)−O^-} were not accessible as easily. This observation is in agreement with the K_{f1} and K_{f2} values found by Jordan and colleagues for the Fe^{3+}−Dnr system (23 and 2, respectively).

Finally, it has to be pointed out that, in the absorption spectra of coordinated Ida, the band at 480 nm did not shift to the same extent as in the case of the deprotonated metal-free ligand (Figure 2). This result could have two different explanations. The remaining band at 480 nm could be related (i) to metal-free ligand present in solution in a considerable amount, as in the case of the Al^{3+}−anthracycline complexes,[33] or (ii) to a MLCT transition, as in the case of Fe^{3+}−2-hydroxyacetophenone complex (H. Drechsel, M. Fiallo, A. Garnier-Suillerot, unpublished results). Unfortunately, we were unable to prove any of these hypotheses.

The Mössbauer parameters of the Ida samples were typical of ferric high-spin species. The presence of antiparallel coupled dimers could be excluded.[42] The total lack of spin density of the Mössbauer samples in EPR could, therefore, not be explained by dimer formation (total spin zero). On the other hand, the lack of resonance at higher temperature (150 K) also excluded the presence of ferromagnetic resonance of antiferromagnetically coupled clusters as found for ferritin and bacterioferritin.[43] Our findings, however, can be merely consistent with a small cluster, i.e., oligomeric species, with vanishing total spin.

Biological Implications. Cytotoxic tests of the Fe^{3+}−Ida complexes with K562 erythroleukemic cells showed that the inhibitory effect of the drug was not prevented by the complexation (M. Fiallo, A. Garnier-Suillerot, data not shown) and was comparable with the biological activity of free Ida. The physiological concentration of anthracycline was usually nanomolar (the IC_{50} for K562 erythroleukemic cells is 2×10^{-9} M).[28] However, there was a concentration effect in cells. In particular, in the nucleus the anthracycline concentration could reach a concentration value 1000-fold higher than in the cytosol.[6,45]

The possibility of the formation of Fe^{3+}−anthracycline complexes in vivo was already discussed by Gelvan and Samuni.[21] Taking as a reference the absorption data at 600 nm, they observed that the efficiency of Fe^{3+} binding strongly increased with high anthracycline concentration and a low metal-to-ligand molar ratio. They observed that the maximum of chelation of Dox was not achieved at concentration values below 10^{-4} M. At that value the percentage of anthracyclines coordinated to Fe^{3+} was very low. Their results showed that at physiological pH, at the anthracycline concentrations obtained clinically, very little Fe^{3+} would be bound to the drug.

We obtained for complex **I** an apparent formation constant ($\beta_1 = 4.8 \times 10^{11}$ M^{-1}) which prevented removing iron from transferrin ($K = 4.7 \times 10^{20}$ M^{-1}).[44] However, our previous results showed that, once formed, the Fe^{3+}−Dnr complex was not dissociated in the presence of transferrin probably because the dissociation kinetics was very slow,[20] whereas, in the interactions with the cells, the dissociation was faster.[6] In fact, the Fe^{3+}−anthracycline complexes cannot enter the cells but dissociated at the level of the plasmic membrane, the metal ion interacting with the phospholipid bilayer.[11]

We believe that the results of our work, providing information about the nature of the Fe^{3+}−Ida system, are important in understanding the biological effects of the ferric complexes of anthracyclines. In our findings, the formal [$Fe(Dox)_3$] and [$Fe(Dnr)_3$] complexes were probably a mixture of species **I**, **II**, and free ligand. In turn, the biological effects observed for these systems have to be reconsidered and, may be, attributed either

to the metal complexes or to the free ligand as well as to the degradation products, as in the case of Dox.[6,12] Characterization of the Fe^{3+} complexes of other anthracyclines, in water[46] and in DMF, using CD spectroscopy is currently underway.

Conclusion

CD and UV−vis spectroscopies were used to study the coordination mode of Ida with Fe^{3+}, and Mössbauer and EPR spectroscopies were used to determine the oligomeric structure of the complexes. At low metal-to-ligand molar ratio, Ida could bind Fe^{3+} at the {C(12)═O; C(11)−O^-} coordination site forming complex **I**. At high metal-to-ligand molar ratio, a metal ion was also bound to the {C(5)═O; C(6)−O^-} coordination site, forming complex **II**. This study establishes the solution structure of the Fe^{3+}−Ida complexes, and these results need to be considered in the interpretation of the biological activity of metal−anthracycline systems.

Experimental Section

Ida was a gift from Pharmacia & Upjohn, France. Solution concentration was determined using $\epsilon = 11\ 500\ M^{-1}\ cm^{-1}$ at 480 nm in all solvents. *o*-Phenanthroline (OP) was purchased from Merck and deferoxamine (def) from Aldrich. KCl was obtained from Carlo Erba. $(NH_4)_2Fe(SO_4)_2 \cdot 5H_2O$ (Prolabo) was used to prepare the fresh iron(II) solutions. The concentrations were determined by absorption spectroscopy of the $[Fe(OP)_3]^{2+}$ complex with $\epsilon = 11\ 000\ M^{-1}\ cm^{-1}$ at 510 nm.

Standard solutions of Fe^{3+} were prepared in 0.1 N HCl ($FeCl_3 \cdot 6H_2O$; Prolabo) or in pure DMF (anhydrous $FeCl_3$; Aldrich), and their concentrations were determined by electronic absorption of the $[Fe(def)]^{3+}$ complex with $\epsilon = 2\ 800\ M^{-1}\ cm^{-1}$ at 430 nm.[30] DMF (spectrophotometric grade) and EtONa were purchased from Aldrich. HEPES (Aldrich) and sodium acetate (Carlo Erba) were used as buffer, at pH 7 and 4.3, respectively.

Absorption spectra were recorded on a Varian Cary 219 spectrophotometer and CD spectra on a Jobin Yvon model Mark V dichrograph. The dichrograph was calibrated using a standard solution of epiandrosterone (3.4×10^{-3} M) in a 1-cm cell ($\Delta\epsilon = 3.3\ M^{-1}\ cm^{-1}$ at 304 nm). The CD spectra were recorded using a 0.2-cm cell and the following parameters: $\lambda = 280-800$ nm, step 1 nm, speed 0.3 nm min^{-1}, response 0.3 s, spectral bandwidth 1.4 nm, number of cycles 3. DMF and water blanks were used as references, depending on the experimental conditions.

Results are expressed as ϵ (molar absorption coefficient) and $\Delta\epsilon = \epsilon_L - \epsilon_R$ (differential molar absorption coefficient). The values of ϵ and $\Delta\epsilon$ are expressed as molar concentration of Ida. Potentiometric measurements were obtained with a Metrohm model E603 pH meter at 25 °C using a Metrohm EA147 combined glass electrode.

The Mössbauer spectrometer worked in conventional constant acceleration mode with a source of 0.9 GBq $^{57}Co/Rh$ (Amersham Buchler). The Mössbauer spectra were recorded in the horizontal transmission geometry using a constant acceleration spectrometer operated in conjunction with a 512-channel analyzer in the time-scale mode. The spectrometer was calibrated against a metallic α-iron foil at room temperature yielding a standard line width of 0.24 mm s^{-1}. The Mössbauer cryostat was a helium bath cryostat (MD306, Oxford Instruments). A small field of 20 mT perpendicular to the γ-beam was applied to the tail of the bath cryostat using a permanent magnet. Isomer shift δ, quadrupole splitting ΔE_Q, and percentage of the total absorption area were obtained by least-squares fits of Lorentzian lines to the experimental spectra.

EPR measurements were made between 10 and 150 K at X-band (9 GHz) using a conventional EPR spectrometer (Bruker ER200D) equipped with a helium-flow cryostat (Oxford Instruments ESR910). The data acquisition system, based on a personal computer, is our own development.

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