Articles

Spectroscopic Studies on Iron Complexes of Different Anthracyclines in Aprotic Solvent Systems

Hartmut Drechsel*,†

Mikrobiologie und Biotechnologie, University of Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany

Marina Fiallo

Departement de Chimie, Institut Universitaire Technologique, Universite Paul Sabatier, avenue Georges Pompidou, F-81100 Castres, France

Arlette Garnier-Suillerot

Laboratoire de Chimie Bioinorganique, UFR Medecine, Université Paris Nord, 74 rue Marcel Cachin, F-93012 Bobigny, France

Berthold F. Matzanke*,‡

Isotopenlabor, Medizinische Universität Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany

Volker Schünemann

Institut für Physik, Medizinische Universität Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany

Received March 10, 2000

Iron complexes of daunorubicin, idarubicin, pirarubicin, and doxorubicin in anhydrous DMF were studied by UV/vis, CD, fluorescence, Mössbauer, and EPR spectroscopy. Titration studies of the metal-free anthracyclines showed one (UV-detectable) deprotonation step requiring 2 equiv of base, compared to 1 equiv for quinizarine. Metal complexation was studied at three different metal/ligand ratios, and with increasing amounts of base. The results obtained from optical spectroscopy show the existence of two different complex species and give clear indications for the requirements of metal complexation. Complex species **I**, formed at a low iron-to-ligand ratio, is less dependent on base addition than complex species **II** formed with equimolar ferric ion. EPR and Mössbauer experiments provide further insight into the structures of both complex species. Lack of spin density of the Mössbauer samples in EPR indicates spin coupling between the metal centers. Mössbauer spectra consist of single quadrupole doublets with values typical for high-spin ferric ion in an octahedral arrangement. The Mössbauer spectroscopic features at 7 T exclude the presence of $S = 0$ dimers. Complex **I** represents a monomeric ferric iron complex whereas complex **II** is consistent with a more or less aggregrated oligomeric Fe−anthracycline system.

Introduction

Anthracycline-based antibiotics have gained widespread application in the treatment of neoplasia.[1] Among their different modes of action under investigation are intercalation into DNA with subsequent topoisomerase-induced strand breaks,[2] reductively activated alkylation of DNA,[3] and radical-induced oxidative damage.[4,5] The usefulness of anthracyclines is limited by conventional toxicity as well as cardiotoxic side effects.[6] Thus, both their useful action and most of their side effects are related

† Tel: +49-7071-2976925. E-mail: hartmut.drechsel@uni-tuebingen.de.
‡ Tel: +49-451-5004140. E-mail: matzanke@physik.mu-luebeck.de.

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10.1021/ic0002723 CCC: $20.00
Published on Web 09/11/2001

to radical formation.[7,8] The redox activity of anthracyclines is related both to their structure[9] and to the formation of complexes with ferric ion.[10] Therefore, research has been directed at modulating efficacy and reducing cardiotoxicity by modifying the structures of anthracyclines[11] and applying anthracyclines as metal complexes.[12] A great deal of research has been devoted to the study of complexes of anthracyclines with different metals,[13−15] especially with iron.[16−18] The partly controversial data collected so far has shown the complexity of the subject. The anthracyclines alone as well as their metal complexes are prone to self-association in polar solvents,[19,20] and different spectroscopies (fluorometry, UV−vis absorption, circular dichroism, EPR, NMR, and Mössbauer) need to be performed in very different concentration ranges, limiting comparability of the results. To overcome these difficulties, we decided to study the formation and properties of ferric complexes in anhydrous DMF. The use of DMF serves two purposes: first, avoiding (at metal-to-ligand ratio 1/10) or greatly reducing the problems of self-association between anthracycline molecules.[36] Second, in an anhydrous environment, the formation of hydroxo- and/or oxo bridges can be excluded, simplifying the structural interpretation of the metal complexes formed. Using the solvent system DMF, which is not acting as a ligand for ferric iron, and using the appropriate molar ratios of anthracycline ligand and ferric iron, we were able to show that the observed features are definitely a consequence of the interaction of this particular ligand system with iron. The use of DMF as a solvent for metal complexes of anthracyclines has also been successfully demonstrated earlier on tin(IV) complexes of doxorubicin.[21]

Experimental Section

Chemicals. Daunorubicin, doxorubicin, and idarubicin, each as hydrochlorides, were obtained from Pharmacia-Upjohn, France. Pirarubicin was purchased from Roger Bellon, France. Dimethylformamide (anhydrous), ferric chloride (anhydrous), sodium ethoxide, and quinizarine were purchased from Aldrich, and morpholinopropanesulfonic acid was purchased from Sigma, anhydrous ethanol from Carlo Erba, and deferrioxamine B mesylate from Ciba Geigy. All other reagents were of the highest quality available. $^{57}FeCl_3 \cdot 6H_2O$ was prepared from $^{57}Fe_2O_3$ (Wissel Elektronik, Starnberg, Germany), by dissolving in concentrated HCl, heating, filtering from residual solid particles, and evaporating gently to dryness.

Spectroscopic Measurements. UV/vis absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrometer equipped with an IBM-compatible personal computer for data acquisition, storage, and evaluation. Samples were measured in quartz cuvettes with a 0.2 cm path length, relative to a blank of the pure solvent. For titration series, the spectra were recalculated to identical concentrations.

Circular dichroism (CD) spectra were measured on a Jobin Yvon Mark V dichrograph. The samples were recorded in 0.2 cm quartz cuvettes, the spectra were accumulated from three acquisitions, and the blank of the pure solvent was subtracted from each spectrum. In titration series, the spectra were corrected for the added reagent volumes, as described for the absorption spectra. Processing and evaluation of the spectra was performed with JY CD Soft Ver. 3.0 on an Apple II e microcomputer.

Uncorrected fluorescence spectra were recorded at 20 °C on a Jobin Yvon JY 3 CS spectrofluorometer. For fluorescence spectra, a small aliquot of the anthracycline solutions in DMF was diluted to 1/200 with anhydrous DMF, and the diluted solutions were measured in quartz cuvettes with a 1 cm path length. The excitation wavelength was set at 480 nm. Processing and evaluation of the spectra was performed on an Apple II e microcomputer. 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 γ source was 0.9 GBq ^{57}Co/Rh (Amersham Buchler). 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 (MD 306, Oxford Instruments). A small field of 10 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 absorption area were obtained by least-squares fits of Lorentzian lines to the spectra. For the high-field measurement a cryostat with a superconducting magnet was used (Oxford Instruments). This spectrum was analyzed with the spin Hamiltonian formalism.[25]

EPR measurements were made between 10 and 150 K at X-band (9.6 GHz) using a conventional EPR spectrometer (Bruker ER 200 D SRC) equipped with a helium-flow cryostat (Oxford Instruments ESR 910). The data acquisition system, based on a personal computer, is our own development. Spin quantitations were performed by double integration of the EPR spectra and by comparison with a 0.93 mM ferric low-spin cytochrome $P450_{CAM}$ standard. The cytochrome $P450_{CAM}$ was measured at $T = 20$ K, because at $T = 10$ K the signal is obscured by passage effects. A Curie behavior of the signal intensity was assumed. The temperature was calibrated by measuring the resistance of a silicon diode, which was put into an EPR tube filled with oil, with the help of a four-pole technique. In order to account for differences in g values, the integrated areas were weighted with Aasa correction factors.[22]

For compensation of zero-field splitting of the $S = {}^5/_2$ species at $g = 4.3$ we assumed equal population of the three Kramer's doublets at 10 K and multiplied the integral values of the $g = 4.3$ signal by 3. This procedure has been motivated by the fact that zero-field splittings of related iron compounds like siderophores are generally low ($D \leq 0.5$ cm^{-1}).[18] We checked this assumption by quantitations of the sample Ida(5) which has also been used for the Mössbauer measurements (see Figure 8) at 10, 20, and 30 K. The so obtained values were the same within the experimental error of ± 20%. There is a small signal around $g = 2$ originating from a Cu impurity in the cavity. Subtraction of the baseline removes this impurity.

Sample Preparation. Anthracyclines were used as hydrochloride salts and dissolved in anhydrous DMF to a standard solution. The concentration of this standard was checked by the absorption of an aliquot, diluted to the micromolar concentration range ($\epsilon_{480} = 11500$

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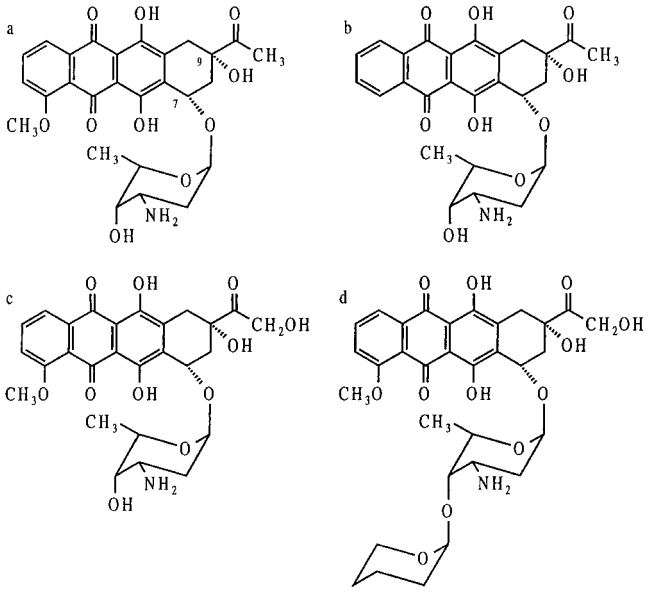

Figure 1. Structures of anthracyclines used in this study: (a) daunorubicin, (b) idarubicin, (c) doxorubicin, and (d) pirarubicin.

M^{-1} cm^{-1} [23]). Since anthracycline solutions are sensitive to light and oxygen, stock solutions were prepared just prior to use. Samples for measurement were prepared in series of aliquots from the same standard, diluted with DMF to the intended concentration, with metal and base added in specified ratios. For the metal-containing solutions, a standard of anhydrous ferric chloride in anhydrous DMF was prepared and standardized with deferrioxamine B. To an aliquot of ferric chloride in anhydrous DMF, an excess of aqueous deferrioxamine B was added, the complex equilibrated, and its absorption spectrum recorded. The iron content was calculated from the molar extinction of 2800 M^{-1} cm^{-1} at 428 nm.[24]

Base was added as a solution of sodium ethoxide in anhydrous DMF, which was prepared fresh for each sample and was added immediately to the anthracycline and ferric anthracycline solutions.

For Mössbauer and EPR spectroscopy, the lactose-containing commercial anthracycline preparations were dissolved in 0.1 M $NaHCO_3$ and extracted three times with 4/1 $CHC1_3/CH_3OH$. The organic solvent was evaporated, yielding pure anthracyclines. The ferric complexes were prepared by adding solutions of $^{57}FeC1_3$ in DMF to the stock solutions of the anthracyclines in DMF. The iron content was kept constant at 1 mM, and the anthracycline content was varied for different iron-to-ligand ratios. After the UV−vis absorption, circular dichroism, and fluorescence spectra of the solutions were recorded and checked, the solutions were immediately frozen in Mössbauer sample holders made of Delrin as well as in EPR tubes in liquid nitrogen and stored at 77 K until measurement.

Results

Deprotonation Studies. Solutions of daunorubicin, idarubicin, pirarubicin, and doxorubicin (Figure 1) in anhydrous DMF at concentrations of 0.5 mM were titrated with freshly prepared sodium ethoxide in anhydrous DMF, in steps of 0.3 equiv of base, relative to anthracycline. The absorption spectra were recorded and the molar extinctions at selected wavelengths plotted against the base content. All anthracyclines showed one deprotonation step, the spectral changes occurring between 1 and 2 equiv of base. The spectra remained stable from 2 equiv and above, until decomposition became apparent at 4−5 equiv of base. A test experiment was carried out with quinizarine, which is lacking the side chain functionalities on the anthracycline ring system. Titration of quinizarine in anhydrous DMF, carried out analogously to the experiments with anthracyclines, showed one deprotonation step. The spectra remained stable above 1 equiv of base, relative to quinizarine. The final base consumption was only 1 equiv. The influence of the solvent has been tested by titrating doxorubicin in DMF, with increasing content of ethanol (Figure 2). At a composition of 30% ethanol,

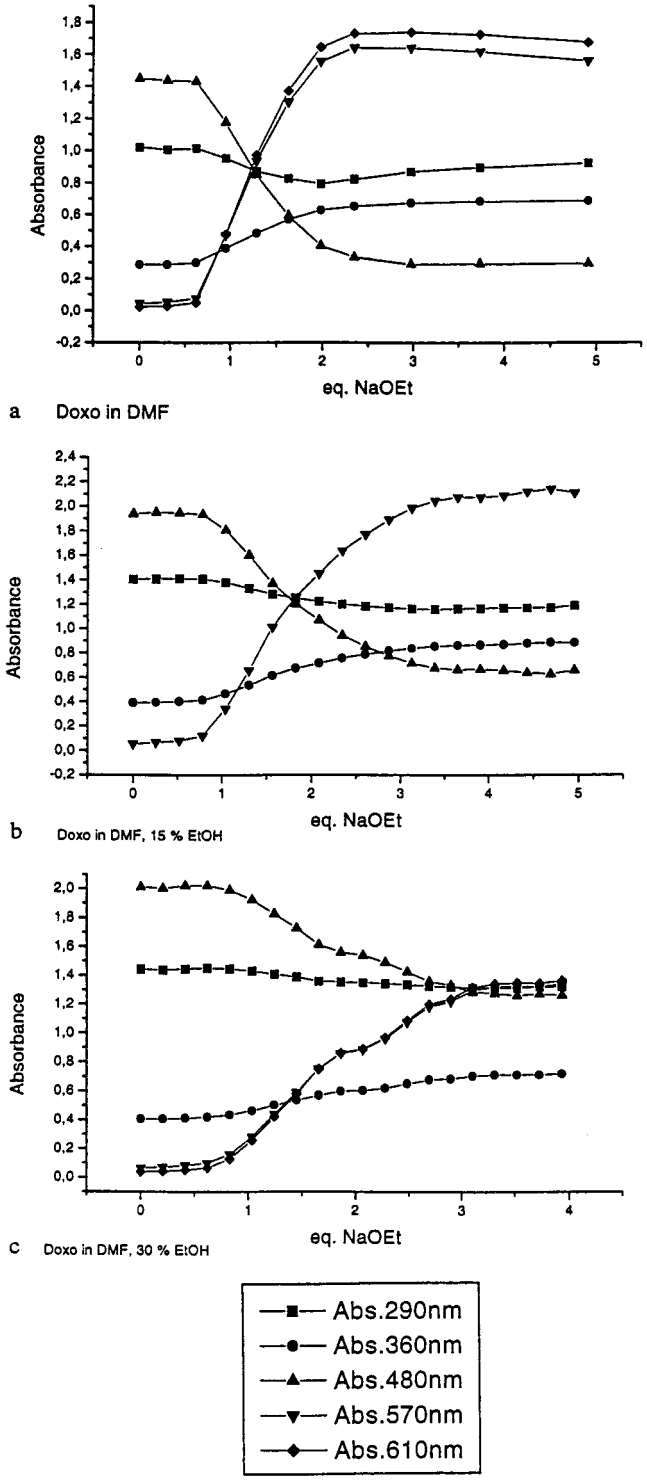

Figure 2. (a) Titration of doxorubicin, $c = 0.67-0.37$ mM in anhydrous DMF, with sodium ethoxide. (b) Titration of doxorubicin, $c = 0.60-0.39$ mM, in anhydrous DMF, 15 vol % ethanol, with sodium ethoxide (c) Titration of doxorubicin, $c = 0.73-0.45$ mM, in anhydrous DMF, 30 vol % ethanol, with sodium ethoxide.

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70% DMF, two deprotonation steps could be observed, corresponding to titrations of anthracyclines in water or ethanol. With 15% ethanol, 85% DMF, an intermediate situation with a broad, "smeared" step was observed.

CD spectra of the deprotonation series of the anthracyclines were recorded primarily for the purpose of comparison with the metal complexation series. Taken alone, the circular dichroism spectra cannot yield conclusive information on the extent of deprotonation; they give, however, information on the accompanying conformational changes induced by changes in net charge of functional groups. Anthracyclines in DMF have a negative band at 295 nm ($\pi \rightarrow \pi^*$ (short axis)), a positive band at 350 nm ($n \rightarrow \pi^*$), and a positive band at 480 nm ($\pi \rightarrow \pi^*$ (long axis)). On deprotonation, the $\pi \rightarrow \pi^*$ transitions show a decrease of intensity. The same holds for the $n \rightarrow \pi^*$ transitions where, in addition, an accompanying shift from 325 to 350 nm was observed. Idarubicin differs from the other anthracyclines in that the $n \rightarrow \pi^*$ transition shifts, but retains its full intensity, as for the protonated species.

Metal Complexation. For an estimation of the degree of complexation, a reference experiment was carried out for the spectral properties of ferric ions in the solvent dimethylformamide. Series of increasing iron concentrations in anhydrous DMF were prepared, the iron concentrations being 0.06, 0.2, and 0.6 mM, analogous to the iron concentrations used in the anthracycline complexation studies. Different series contain 0.0, 0.6, 1.2, and 1.8 mM sodium ethoxide. The characteristic contribution of ferric ions to the UV/vis spectra was an absorption at 360 nm, independent of base content. Corresponding characteristics are thereafter referred to as "free iron", denoting iron not bound to anthracyclines.

In preparing ferric complexes of anthracyclines in DMF, an increasing amount of iron (0.1, 0.33, and 1 equiv, relative to anthracycline) was added to a solution of anthracycline in anhydrous DMF. The experiments were performed in several series with varying base content (no base, 0.1, 0.33, 1 equiv, relative to anthracycline). The base, sodium ethoxide, was added in quantities corresponding to the iron content of the solutions, to compensate for the protons liberated from the ligand by the complexation process. Thus, the base would only assist in complexation, avoiding an excess of base which would produce deprotonated free ligand. The spectral characteristics of the metal complexes are thus not disturbed by the superposition from spectra of the free anionic anthracyclines.

The absorption spectra of the anthracycline/iron complexes provide insight into the conditions necessary for complex formation without differentiating between different types of complexes. The series of absorption spectra of daunorubicin/iron samples prepared without base show, with increasing iron content, a weak gain of absorption at 600 nm, with a slight decrease of the free anthracycline maximum at 480 nm. At high iron content (1/1 against anthracycline), the absorption at 360 nm becomes prominent, indicating free iron. With 1 equiv of base, relative to iron, the absorption band at 600 nm increases more, whereas the "free iron" fingerprint at 360 nm is disappearing completely. For 2 equiv of base, the absorption at 600 nm further increases and remains virtually the same for 3 equiv of base (Figure 3). The changes in the CD spectra of the anthracycline/iron complexes occurring with increasing iron content have to be compared with the base titrations of the anthracyclines without iron (see above). Daunorubicin, after addition of iron without accompanying base, is not affected much; i.e., the spectral characteristics below 530 nm do not change significantly. At 595 nm, a single maximum appears,

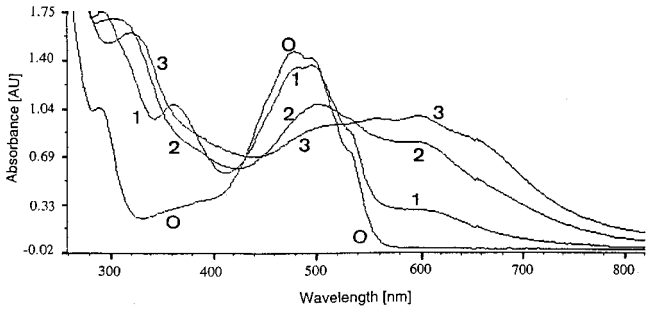

Figure 3. UV−vis absorption spectra of daunorubicin/iron complexes, *c*(daunorubicin) = 0.65 mM in anhydrous DMF, metal/ligand ratio 1/1. Curve 1: no base. Curve 2: 1 equiv of base sodium ethoxide, relative to iron content. Curve 3: 2 equiv of base sodium ethoxide, relative to iron content. Curve 0: iron-free daunorubicin.

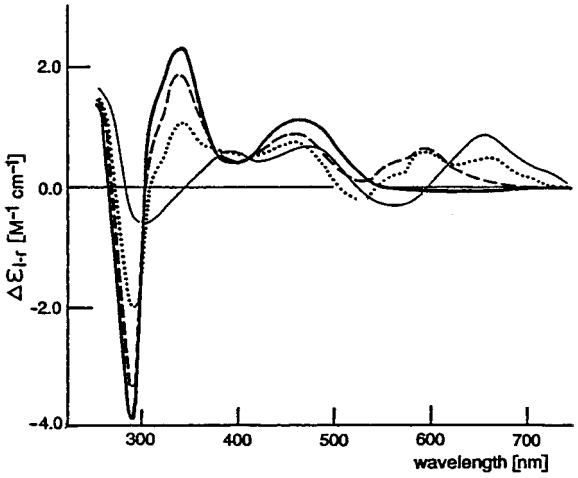

Figure 4. Circular dichroism spectra of daunorubicin/iron complexes in anhydrous DMF, 2 equiv of base sodium ethoxide, relative to iron content. Dashed line: *c*(daunorubicin) = 0.58 mM, metal/ligand ratio 1/10. Dotted line: *c*(daunorubicin) = 0.53 mM, metal/ligand ratio 1/3. Thin line: *c*(daunorubicin) = 0.41 mM, metal/ligand ratio 1/1. Thick line: daunorubicin in DMF, *c*(daunorubicin) = 0.62 mM, no metal, no base.

most prominently for 1/10 iron content, decreasing again at higher iron content. When 1 equiv of base is used, relative to the increasing iron content, the maximum at 595 nm appears the same as for the base-free series, with increased amplitude. For 1/1 iron and 1 equiv of base, a new feature appears, a maximum at 665 nm, combining with the residual amplitude at 595 nm to a broad maximum. This feature becomes even more prominent at higher base contents, i.e., with 2 and 3 equiv relative to the iron content. These spectra show that the maximum at 665 nm combines with a minimum at 575 nm, producing an S-shaped curve, best visible for 1/1 iron and 2 equiv of base (Figure 4). The curves for 1/3 iron have the characteristics of both the 1/10 and 1/1 CD spectra and are presumably superpositions of these curves. This could be confirmed by a plot of $\Delta\epsilon$ at 575, 600, and 665 nm against increasing iron content, at 2 equiv of base each, as shown in Figure 5. An isodichroic point is found at 629 nm. The formation of the complex species at low (1/10) iron content is less dependent on base addition than the complex species formed with an equimolar ratio of iron. The base-induced features of the iron-free anthracyclines, especially the double maximum at 565/595 nm, are not visible in the iron-containing series, even at 3 equiv of base. This validates the assumptions for the base additions outlined above. The time course of daunorubicin/iron complex formation was monitored by CD spectroscopy. The

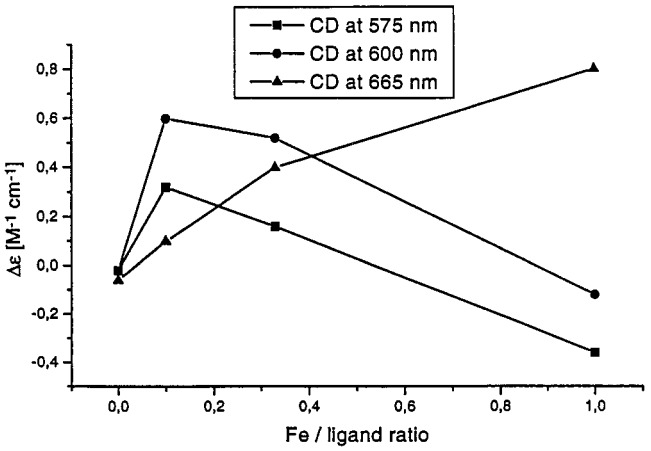

Figure 5. Evolution of CD spectra of daunorubicin with increasing iron content. Sample data same as in caption to Figure 4.

samples with 1/1 iron content were kept at room temperature, shielded from direct light and from contact with air. Without base, the CD spectra of the samples do not change significantly. With 1 equiv of base, the maximum at 665 nm increases within 2 h, indicating slow kinetics of formation of this complex species. After 3 and 5 days, the spectra collapse progressively, presumably due to decomposition of the ligand. Samples with 2 and 3 equiv of base usually precipitate within several hours after preparation.

The absorption spectra of the iron complexes of idarubicin indicate complex formation only with the addition of at least 1 equiv of base. Complete complex formation is reached with 2 equiv of base and does not increase further with 3 equiv. This behavior is analogous to the daunorubicin series.

The CD spectra of idarubicin/iron complexes differ to some extent in several series from the daunorubicin/iron complexes. Without addition of base, the CD spectra are comparable to the situation described for daunorubicin. When base is added, from 1 to 3 equiv, the features at shorter wavelength shift bathochromic. The minimum at 285 nm and the maximum at 350 nm decrease in intensity, accompanied by a shift from 285 nm to 315 nm and from 350 nm to 385 nm. The maximum at 465 nm, most pronounced in iron-free anthracyclines, decreases with the addition of iron, while the positive bands above 530 nm appear at 1/10 iron and increase with increasing iron content. With 1 equiv of base, 1/10 iron produces an unsymmetrical double maximum at 565/595 nm, resembling the CD characteristics of free, deprotonated idarubicin. With 1/3 iron, the double maximum develops into a broad single maximum, which shifts to 665 nm with 1/1 iron. With 2 and 3 equiv, the 1/1 iron complex shows a distinct negative CD contribution, resulting in an S-shaped curve. The 1/10 iron samples still have an unsymmetrical double maximum at 565/595 nm, as in the iron-free preparations. The 1/3 iron samples have contributions from both the high- and low-iron ratio samples and are obviously superpositions of CD spectra from the two different complex species therein (Figure 6). This could be confirmed by plots of the CD effects at different wavelengths against the iron/ligand ratio, as demonstrated for daunorubicin. The formation of the complex species at low (1/10) iron content is less dependent on base addition than the complex species formed with an equimolar ratio of iron.

The absorption spectra of iron complexes of doxorubicin in anhydrous DMF are analogous to those of the other anthracyclines. Upon increasing iron, the absorption of free, protonated anthracyclines at 480 nm decreases, while a new broad absorption band is formed around 600 nm.

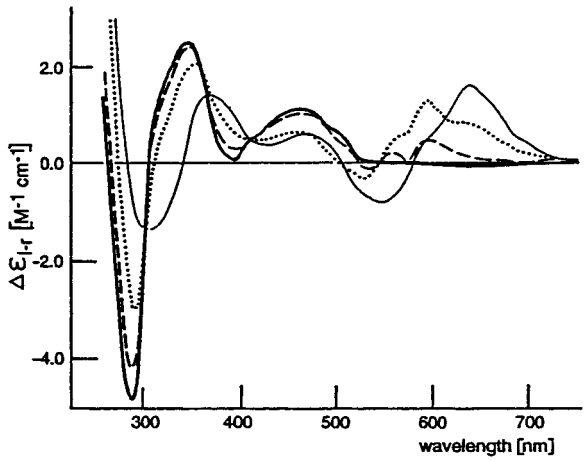

Figure 6. CD spectra of idarubicin/iron complexes in anhydrous DMF, 2 equiv of base sodium ethoxide, relative to iron content. Dashed line: *c*(idarubicin) = 0.57 mM, metal/ligand ratio 1/10. Dotted line: *c*(idarubicin) = 0.49 mM, metal/ligand ratio 1/3. Thin line: *c*(idarubicin) = 0.35 mM, metal/ligand ratio 1/1. Thick line: idarubicin in DMF, *c*(idarubicin) = 0.61 mM, no metal, no base.

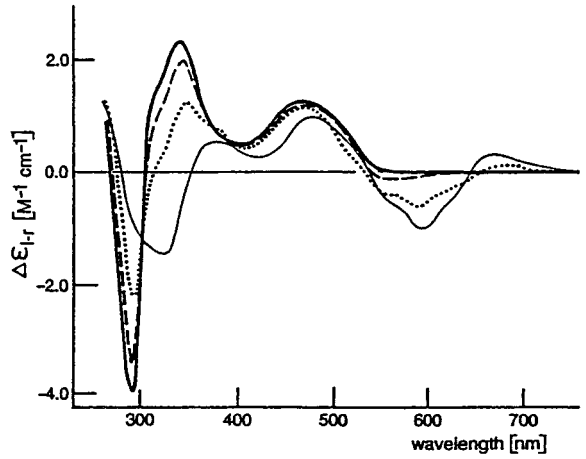

Figure 7. CD spectra of doxorubicin/iron complexes in anhydrous DMF, 3 equiv of base sodium ethoxide, relative to iron content. Dashed line: *c*(doxorubicin) = 0.60 mM, metal/ligand ratio 1/10. Dotted line: *c*(doxorubicin) = 0.49 mM, metal/ligand ratio 1/3. Thin line: *c*(doxorubicin) = 0.32 mM, metal/ligand ratio 1/1. Thick line: doxorubicin in DMF, *c*(doxorubicin) = 0.66 mM, no metal, no base.

The CD spectra of doxorubicin/iron samples without base do not change much upon increasing content of iron, indicating the necessity of base for complex formation. With 1 equiv of base, relative to iron content, increasing iron decreases the minimum at 295 nm, the maximum at 360 nm, and the maximum at 485 nm. The changes above 530 nm are negligible. With 2 and 3 equiv of base, the decrease in intensity of bands is accompanied by a bathochromic shift in the CD spectra of the samples with high iron/ligand ratio (1/1), from 295 to 325 nm, from 360 to 385 nm, and from 465 to 485 nm. In the series with 3 equiv of base, relative to iron content, increasing amounts of iron lead to a negative band at 575 nm accompanied by a smaller maximum at 665 nm. The CD spectra of the 1/1 iron/doxorubicin preparations are distinctly different from the other CD spectra at lower iron content. The underlying complex species is visible at 2 equiv of base and fully formed with 3 equiv of base (Figure 7).

The absorption spectra of iron complexes of pirarubicin in anhydrous DMF are analogous to those of the other anthracyclines. Upon increasing iron, the absorption of free, protonated anthracyclines at 480 nm decreases, while a new broad

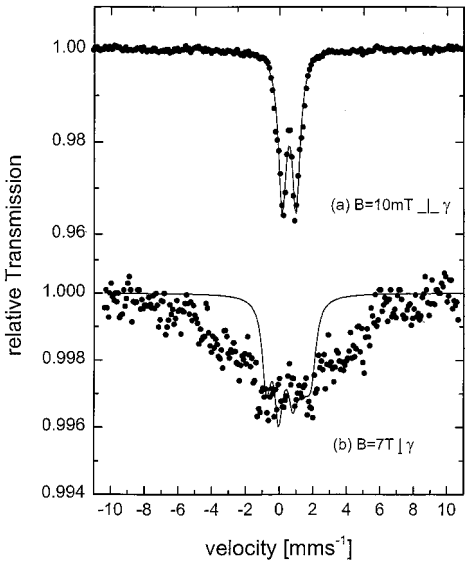

Figure 8. (a) Mössbauer spectrum of Ida(5) idarubicin/iron complex in anhydrous DMF (c(^{57}Fe) = 1.1 mM, c(idarubicin) = 1.1 mM, 1 equiv of base relative to iron content), taken at 4.2 K. The solid line is a Lorentzian fit with the parameters: $\delta = 0.56$ mm s^{-1}, $\Delta E_Q = 0.82$ mm s^{-1}, $\Gamma = 0.56$ mm s^{-1}. (b) Mössbauer spectrum of Ida(5) taken at 4.2 K in a field of 7 T perpendicular to the γ-beam. The solid line is a simulation with the parameters obtained from Figure 8a assuming a diamagnetic ground state and a positive sign of ΔE_Q.

absorption band is formed around 600 nm. The spectral characteristics of "free iron" in DMF are only observed for high iron content without base.

The evolution of CD spectra of pirarubicin/iron complexes upon increasing iron content in series with different base content is similar to the changes observed for doxorubicin/iron complexes. The minimum at 295 nm decreases upon increasing content of iron, with only a slight shift to 305 nm. The maximum at 330 nm decreases and shifts bathochromic to 395 nm in samples with a 1/1 iron/ligand ratio. The maximum at 465 nm decreases without significant shift, and a new minimum at 600 nm is formed, beginning with at least a 1/3 iron/ligand ratio and 2 equiv of base. This complex species is fully pronounced with 1/1 and 1/3 iron/ligand ratio and 3 equiv of base.

The fluorescence of anthracyclines is diminished by addition of base, due to formation of the nonfluorescent anionic anthracycline species. The addition of iron produces a similar quenching effect. The effect is most pronounced from zero iron content through 1/10 to 1/3 iron/ligand ratio, and less for further increasing the iron/ligand ratio to 1/1. This indicates that the assumed iron species formed at low iron content is fully active in suppressing fluorescence of the anthracycline chromophore. Changes in solvent composition, effected by adding increasing amounts of ethanol to the DMF solutions, do not change the fluorescence intensity, either for the iron-free anthracyclines or for their iron complexes in the presence of 1, 2, or 3 equiv of base, relative to iron content.

Mössbauer spectra were recorded for samples of daunorubicin in anhydrous DMF and of idarubicin in acetate buffer and in anhydrous DMF. The samples used for Mössbauer spectroscopy were at a constant iron concentration of 1 mM, with varying anthracycline content. As an example the spectrum of Ida(5) obtained at 4.2 K in a weak applied field of 10 mT perpendicular to the γ beam is shown in Figure 8a. The spectrum consists of a relatively broad quadrupole doublet. Table 1 displays Mössbauer parameters of Ida(5) and of the other frozen solutions of the different samples. With the exception of an aged sample,

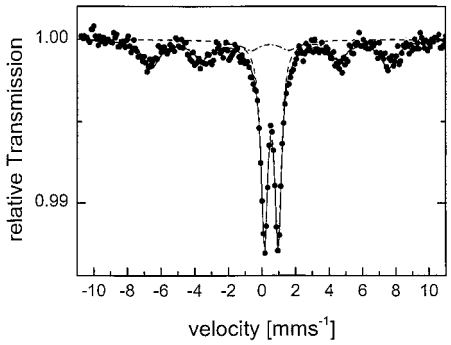

Figure 9. Mössbauer spectrum of daunorubicin/iron complex in anhydrous DMF (c(^{57}Fe) = 1.1 mM, c(daunorubicin) = 1.1 mM, 2 equiv of base relative to iron content), taken at 4.2 K. The sample was kept for 64 h at −20 °C before being frozen in liquid nitrogen. The spectrum has been analyzed with parameters given in Table 1.

the spectra consist merely of quadrupole doublets ($\Gamma = 0.40-0.52$ mm s^{-1}), the Mössbauer parameters of which ($\delta = 0.56-0.58$ mm s^{-1}, $\Delta E_Q = 0.66-0.93$ mm s^{-1}) are typical for high-spin ferric ion bound to oxygen/nitrogen in an octahedral arrangement. The nonzero quadrupole splitting reflects a symmetry lower than cubic at the iron site.

The samples of idarubicin allowed the comparison of the aqueous and the anhydrous solvent systems. The isomer shift was virtually identical for both solvents. Exchange of the solvent (water by anhydrous DMF) yields an increase of the quadrupole splitting: +0.09 mm s^{-1} for Ida(l)/Ida(3) and +0.15 mm s^{-1} for the samples Ida(2)/Ida(5). Likewise, increasing base content also increases ΔE_Q at either metal/ligand ratio (+0.04 mm s^{-1} in the Fe/ligand = 1/3 and +0.05 mm s^{-1} in the iron/ligand = 1/1 preparations). Finally, replacement of idarubicin by daunorubicin again results in a slight increase of ΔE_Q, possibly due to a slightly altered configuration of the anthracycline D-ring.

The line widths Γ (full width at half-maximum) of all samples are fairly large ($\Gamma = 0.40-0.52$ mm s^{-1}). At least for Ida(5) ($\Gamma = 0.58$ mm s^{-1}) and Ida (6) ($\Gamma = 0.52$ mm s^{-1}) we attribute this finding to the presence of two or more species in solution exhibiting very similar Mössbauer parameters.

For 1/1 daunorubicin/iron, with 2 equiv of base, another sample was prepared, equilibrated for 64 h at −20 °C, before freezing in liquid nitrogen. The Mössbauer spectra of this sample (in Table 1: Dau(2), aged 64 h) showed the additional appearance of a magnetic sextet (Figure 9), comprising about 60−70% of total iron. This indicates a process of aging of the initial complex species through time dependent rearrangement reactions. Spectra similar to this aged sample have been obtained earlier in aqueous preparations of ferric daunomycin.[18]

In order to elucidate the magnetic ground state of the samples we have performed high-field Mössbauer experiments at $T = 4.2$ K at a field of 7 T perpendicular to the γ-ray for Ida(l), Ida(5), and Dau(1). As an example the high-field spectrum of Ida(5) is given in Figure 8b. A relatively broad magnetic pattern is observed, which is also present in the high-field measurements of Ida(l) and Dau(l) (data not shown). Such a pattern is consistent neither with the presence of spin-coupled ferric dimers with $S = 0$ ground state nor with the presence of significant amounts of uncoupled ferric ion. The latter would show a well-defined magnetic structure depending on the values of the ligand field parameters D (zero field splitting) and E/D (rhombicity).[25,26]

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Table 1. Mössbauer Parameters of Various Ferric Anthracyclines[a]

compound[b]	solvent	ratio[c]	base[d]	δ (mm s^{-1})	ΔE_Q (mm s^{-1})	$\Gamma_{1/2}$ (mm s^{-1})[e]
Ida(l)	aq	1/3	na	0.58(2)	0.67(3)	0.22(2)
Ida(2)	aq	1/1	na	0.57(5)	0.68(9)	0.22(6)
Ida(3)	DMF	1/3	1	0.57(2)	0.76(3)	0.20(2)
Ida(4)	DMF	1/3	2	0.56(1)	0.81(2)	0.21(2)
Ida(5)	DMF	1/1	1	0.56(2)	0.83(3)	0.28(2)
Ida(6)	DMF	1/1	2	0.54(2)	0.88(4)	0.26(3)
Dau(1)	DMF	1/1	2	0.56(2)	0.94(4)	0.21(3)
Dau(2)	DMF	1/1	2	0.56(4)	0.82(2)	0.23(5)
Dau(2), aged 64 h[f]						
magnetic species, 58% contribution				0.50(6)		0.87(1)
doublet species, 42% contribution				0.56(6)	0.82(2)	0.46(2)

[a] Measurements were performed at 4.2 K in an external magnetic field of 10 mT perpendicular to the γ-beam. For details see the Experimental Section. [b] Ida: idarubicin. Dau: daunorubicin. [c] Ratio: molar ratio of iron/anthracycline, c(Fe)= 1.1 mM. [d] Base: molar equivalents of sodium ethoxide added, with respect to Fe^{3+}. [e] $\Gamma_{1/2}$: half-widths at half-maximum. [f] H_{eff} = 43.9 T.

Ferric dimers with $S = 0$ ground state would exhibit a pattern which is shown as a solid line in Figure 8b. This simulation of a diamagnetic component has been performed with the parameters obtained from the measurement at low fields (Figure 8a). The experimental data clearly differs from the simulation which shows that the majority of the iron is not in a diamagnetic ground state. Thus, on the basis of the high-field experiments, the formation of ferric dimers exhibiting strong Heisenberg interaction which causes antiparallel coupling and a diamagnetic ground state can be excluded. Obviously, the present samples are different from ferric ion dimers which are connected via μ-oxo bridges and which exhibit strong antiferromagnetic exchange like the ferric forms of ribonucleotide reductase[27] and methane mono oxygenase[28] as well as various model complexes.[29]

EPR spectra were recorded on samples of the same concentration as for the optical spectroscopy (iron concentration 0.05−0.5 mM). The series was performed on daunorubicin in anhydrous DMF, with 1/10, 1/3, and 1/1 iron/ligand ratio, at 1, 2, and 3 equiv of base, relative to iron content. All EPR spectra of the preparations show very weak signals near $g_{eff} = 4.3$ and $g_{eff} = 2$. A selection of the spectra is shown in Figure 10 in comparison to a signal from a cytochrome P450 standard. In Table 2 the calculated spin densities of the series of ferric daunomycin complexes are listed. At high Fe(III) contents the so obtained spin concentration of the EPR signal is very low if compared to the iron concentration; the samples with 167 and 500 μM iron concentration are basically EPR-silent. Also at 50 μM a substantial portion of the iron is EPR-silent. Since the magnetic Mössbauer data revealed that the samples are not diamagnetic at 4.2 K but all iron is in the ferric high-spin state, the fact of EPR-silent species can only be explained by spin coupling of the ferric $S = {}^5/_2$ ions. In order to investigate whether large oligomers as in ferritin-type structures are present in the samples, we have performed additional EPR measurements at 150 K. But also at 150 K, the samples remain EPR-silent (data not shown).

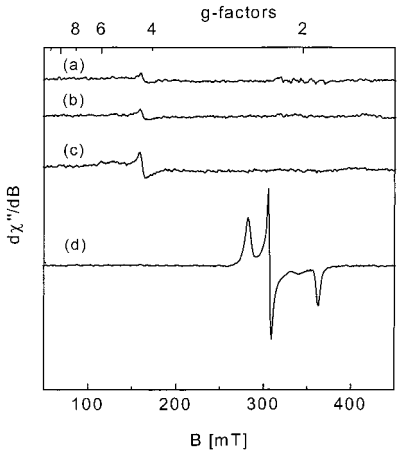

Figure 10. EPR spectra of ferric daunomycin in DMF. Spectrum a corresponds to sample Dau(6), b to Dau(5), and c to Dau(4). As a standard (spectrum d), a ferric low-spin form of cytochrome P450 (0.930 mM) measured at 20 K was used. The daunomycin spectra were recorded at 10 K with 9.6456 GHz microwave frequency at 20 μW microwave power, modulation amplitude 0.5 mT, and modulation frequency 100 kHz. A copper impurity originating from the cavity was eliminated by baseline subtraction. Spectrum a contains a small Mn impurity. For spin quantitation see Table 2.

Discussion

As can be seen from the structure of anthracyclines (Figure 1), there are three sites for deprotonation. Since the deprotonation of the amine function is not visible in UV spectra, only the deprotonation of phenol to phenolate can be followed by UV spectroscopy. The deprotonation of the anthracyclines in anhydrous DMF shows only one deprotonation step. These results differ markedly from the titrations of anthracyclines in water and protic solvents, where two UV−visible deprotonation steps with the final consumption of 3 equiv of base occur.

With the additional results from the titration of quinizarine, we conclude that, in DMF, the deprotonation of anthracyclines and of quinizarine does not proceed beyond the monoanion. The dianionic species, resulting from complete deprotonation in titration experiments of the anthracyclines in water or protic solvents, cannot be obtained in anhydrous DMF, because of the much smaller dielectric constant of DMF (38.25, at 293 K) compared to water (78.54, at 293 K).[30] For anthracyclines, the first equivalent is necessary to deprotonate the ammonium functionality, without accompanying changes in absorption spectra. The visible deprotonation step is due to the deprotonation of the first phenolic group, yielding a monoanion. The dianion is energetically unfavorable in a solvent of low dielectric constant, i.e., the pK_a of the second phenol group is shifted upward, such that ethoxide is insufficient for its deprotonation.

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Table 2. Percentage of Total Spin Density of the $g_{eff} = 4.3$ Signal of EPR Samples of Different Ferric Daunorubicin Complexes[a]

	Dau(1)	Dau(2)	Dau(3)	Dau(4)	Dau(5)	Dau(6)	Dau(7)	Dau(8)	Dau(9)
EPR-visible (%)	35	11	4	38	8	1	45	14	17
Dau(1)	c(Dau) = 500 μM, c(Fe) = 50 μM, c(OEt) = 50 μM								
Dau(2)	c(Dau) = 500 μM, c(Fe) = 167 μM, c(OEt) = 167 μM								
Dau(3)	c(Dau) = 500, μM, c(Fe) = 500 μM, c(OEt) = 500 μM								
Dau(4)	c(Dau) = 500 μM, c(Fe) = 50 μM, c(OEt) = 100 μM								
Dau(5)	c(Dau) = 500 μM, c(Fe) = 167 μM, c(OEt) = 333 μM								
Dau(6)	c(Dau) = 500 μM, c(Fe) = 500 μM, c(OEt) = 1000 μM								
Dau(7)	c(Dau) = 500 μM, c(Fe) = 50 μM, c(OEt) = 150 μM								
Dau(8)	c(Dau) = 500 μM, c(Fe) = 167 μM, c(OEt) = 500 μM								
Dau(9)	c(Dau) = 500 μM, c(Fe) = 500 μM, c(OEt) = 1500 μM								

[a] Areas were obtained by double integration of the EPR derivative signals. As a standard, a ferric low-spin form of cytochrome P450 (0.930 mM) measured at 20 K was used. The estimated error is 20% of the calculated values. For details of this procedure, see the Experimental Section, Spectroscopic Measurements. Experimental parameters: the daunorubicin spectra were recorded at 10 K with 9.6456 GHz microwave frequency at 20 μW microwave power, modulation amplitude 0.5 mT, and modulation frequency 100 KHz

For quinizarine in DMF, 1 equiv of base produces the monoanion, the dianion being likewise inaccessible. Titration experiments of doxorubicin in DMF with increasing content of ethanol support the interpretation based on solvent polarity.

The UV−vis absorption characteristics of anthracycline/iron complexes in anhydrous DMF indicate the necessity of base addition for complex formation. This is clear from the missing buffering capacity in the solvent used. The pK_a for the protonation of DMF is reported to be −1.3 to −1.4.[31] No more than 2 equiv is needed for complete complexation. The CD spectra of anthracyclines show distinct features even without metal complexation due to the presence of chiral centers in the molecule (Figure 1). The aglycon part of the molecule contains two chiral carbons, C(7) and C(9), both of which have the *S* configuration in all anthracyclines used in this study. Another contribution to the overall dissymmetry arises from different conformations of the cycle A, the aliphatic six-membered ring.[32] The amino sugar, daunosamine, contains five chiral carbons. And finally, the orientation of daunosamine relative to the chromophore leads to different conformations of the glycosidic bond.[33]

In the presence of a metal, complexation occurs, the extent being dependent on the addition of base which compensates for the protons liberated in the process of complex formation. Changes in the conformation of the anthracycline as well as the configuration introduced at the metal center profoundly change the CD spectra.

The CD spectra of daunomycin/iron complexes, with the addition of, ideally, 2 equiv of base, relative to iron content, indicate the formation of two different complex species. One complex is formed at low iron content, i.e., at a 1/10 iron/ligand ratio relative to the anthracycline; the other is formed at high iron content, i.e., at a 1/1 ratio. The formation of the latter is strongly base dependent and requires 2 equiv of base for full development of its CD features.

The CD spectra of idarubicin/iron complexes in anhydrous DMF show the formation of two different complex species, one at a low iron/ligand ratio, the formation of which is independent of base, and another species at a high iron/ligand ratio. The base dependency of these two complex species is analogous to that of daunorubicin, formation of the complex at an equimolar ratio being strongly base dependent and requiring 2 equiv of base for full development of its CD features.

The CD spectra of doxorubicin/iron complexes indicate the formation of a complex species at 1/1 iron/ligand ratio, requiring at least 2 equiv of base for its formation. The existence of another, different complex species at low iron content cannot be proven from the spectral data acquired in this study.

Evaluation of the CD spectra of pirarubicin/iron complexes yielded results similar to those obtained for doxorubicin. A distinct complex species is formed with high iron content (1/3 and 1/1 ratio). The formation of this species starts with 1 equiv of base (relative to iron content) and reaches saturation with 3 equiv of base. Another, differerent complex species cannot be conclusively characterized.

All ferric complex species suppress the fluorescence of the anthracycline chromophore. This is analogous to the nonfluorescent behavior of deprotonated anthracyclines produced by titration with base.

In the Mössbauer measurements, three dependencies of ΔE_Q from the experimental conditions were observed. First, an increase of ΔE_Q was found when the aqueous solvent was replaced by DMF. This reflects increased net charge on the donor atoms in a solvent of lower dielectric constant, consistent with the dielectric effect discussed in the context of deprotonation of the metal-free ligands. In water, the solvent obviously shields the negative charges of the ligand oxygen to some extent by dipole−charge interaction. Second, increase of base in the DMF preparations increases ΔE_Q. We attribute this shift to the presence of additional charges in the vicinity of the metal center. Finally, replacing idarubicin by daunorubicin also yields an increase of ΔE_Q. The two anthracyclines differ in their sugar moieties. Although the sugar portion is not directly involved in complex formation, it obviously affects the complex structure—e.g., by steric means (altered configuration of the anthracycline D-ring)—giving rise to the observed shift.

In an earlier EPR study of ferric daunomycin in aqueous solution a signal near $g_{eff} \cong 2$ observed at 10 K was attributed to a doublet species with $\delta = 0.58$ mm s^{-1} and $\Delta E_Q = 0.77$ mm s^{-1}. This species was assigned to polynuclear magnetic aggregates. The majority of the spectrum, however, was EPR-silent.[18] However, the results of the latter and the present investigations are not directly comparable. Here, samples, prepared in DMF, were quickly frozen, whereas in the earlier study pH adjustment of aqueous samples was made slowly within a time period of 1 h.

The EPR data of the daunorubicin samples clearly disclosed that the portion of the iron (at 500 μM almost all iron) is EPR-silent. Since Mössbauer spectroscopy clearly reveals that all iron in the sample is in the ferric high-spin state, the iron sites must be magnetically coupled to an integer total spin. The observed

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broad Mössbauer pattern at high external fields revealed that the major portion of the iron is not diamagnetic. Thus, on the basis of the high-field experiments, the formation of spin-coupled ferric dimers with $S = 0$ can be excluded. Ferric iron centers can interact through relatively strong exchange interactions which are mediated via μ-oxo bridges, but there are also cases where, in the absence of μ-oxo bridges, only weak dipolar spin−spin interactions are effective. Such a situation can also be detected by the application of magnetic fields on the order of 5−7 T.[34]

The large field supresses the spin−spin relaxation rate which reveals a spectrum showing a well-defined sextet. This is not the case in our samples, and therefore we can also exclude the presence of only dipolar coupled iron sites.[23]

It is obvious that none of the above scenarios describes the experimental facts correctly. An explanation could be a very weak magnetic exchange interaction in the range of approximately 2−10 cm^{-1}. At 4.2 K and an external field of 7 T already several excited states are populated. Such a situation cannot be handled in the strong interaction limit anymore. A $S = 0$ Hamiltonian is not sufficient as can be seen from the simulation given in Figure 8b, because one set of ligand field parameters for each single site and a coupling term have to be taken into account. The present data do not allow such an analysis, and even if we had measured over a wider range of temperatures and magnetic fields, at least 15 significant unkowns need to be determined even if all tensors share a common principal axis system as was stated by Münck and co-workers for the case of the biferrous form of the hydroxylase of MMO from *Methylosinus trichosporium* OB3b.[28] Of course, these data do not allow a definitive statement about the degree of oligomerization (dimer, trimer, etc.) (see Conclusions). Also a stacking of the complexes and the formation of small aggregates (<10 nm) might be possible. Nevertheless, the lack of a broad resonance around $g = 2$ in the EPR spectra at higher temperature (150 K) also excludes the presence of large antiferromagnetically coupled clusters as found for ferritin and bacterioferritin.[35]

The amount of the EPR-visible species very likely corresponds to the monomeric complex. At an iron concentration of 500 μM (Fe/ligand ratio 1/1) this species exhibits virtually no contribution to the EPR spectrum. Consequently, this component cannot be observed in the Mössbauer spectra, where the iron concentration is higher (c(Fe) = 1.1 mM).

Finally, we attribute the aging effect of the ferric daunomycin complex, identified by the formation of a magnetic sextet species, to a cluster growth of the originally formed species.

The current study reveals the presence of iron−anthracycline complexes at concentrations as low as 50 μM in a solvent system which avoids self-association between anthracycline molecules and the formation of hydroxo-bridged metal centers. On the basis of the solvent chosen, complex formation is solely possible via the ligand. As shown in earlier studies, summarized in ref 1, there exist two primary ligating sites of anthracyclines for metals, namely, at positions 11 and 12 as well as at positions 5 and 6 of the anthracycline ring system; the amino group on the sugar moiety is rarely involved, as in Pd complexes.[15] The ligating groups (O−C═C−C═O) act as bidentate ligands and not as bis-monodentates. The latter arrangement is neither thermodynamically nor sterically favorable.

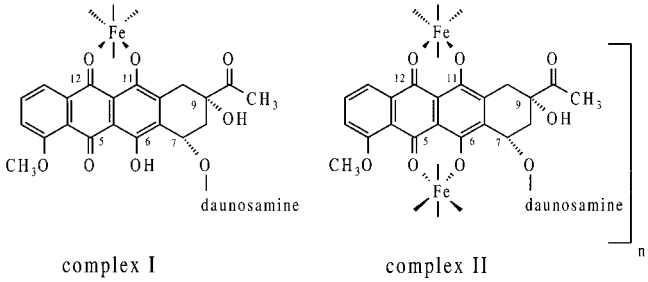

Figure 11. Proposed structures for complexes **I** and **II** on the example of daunorubicin.

We therefore propose two structural models for the ferric anthracycline complexes in DMF. The first species, formed preferentially at low iron-to-ligand ratio, and visible in its spectral characteristics in the samples with a 1/10 iron/metal ratio, binds one ferric ion, at positions 11 and 12, acting as a keto-phenolate bidentate. This is in agreement with complex **I** described in another publication on this topic.[36,37]

The second species, which evolves at a 1/3 iron-to-ligand ratio and reaches full expression in preparations equimolar in iron and ligand, is presumed to be structurally identical to complex **II**,[36] in which two ferric ions are bound to one anthracycline molecule, occupying the coordination sites of C11/C12 and C5/C6 (Figure 11).

The lack of spin density observed in the EPR spectra is seen to be a result of spin coupling between the metal centers. Field dependent Mössbauer spectroscopy indicates that the exchange coupling between the two iron sites is weak, since no diamagnetic ground state could be detected at 4.2 K. An exchange path may be possible via the ring system of the idamycin and daunomycin ligands. However, based on the present data we cannot exclude the formation of oligomers which could be arranged in a ladderlike fashion.

A chainlike oligomer exhibiting long-range couplings can be established through interchain associations, possibly on the basis of $\pi-\pi$ interactions which are operative in more concentrated solutions of metal-free anthracyclines.[19,20] This proposal is in agreement with the observed tendency of flocculation and precipitation of aged samples of 1/1 complexes between ferric ion and anthracyclines. Similar structural conclusions have been reached by studies on colloidal preparations of ferric anthracycline complexes.[16]

At low iron concentration (50 μM) and a metal−ligand ratio of 1/10, optical spectroscopic data show almost exclusively the presence of monomeric complex **I**. The contribution of this species decreases with increasing iron concentration. Qualitatively, the same tendency is observed in the EPR spectra showing a decreasing contribution of the EPR-visible species at $g = 4.3$ (45% spin density) with increasing iron concentration. This, in turn, indicates an increasing contribution of an EPR-silent magnetically coupled system. Complex **II**, which is almost exclusively present (83−99%, Table 2) at high iron concentration (500 μM), is invisible in EPR.

In conclusion, this study shows the existence of two distinct anthracycline/iron complexes, dependent on the stoichiometric

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(37) On the basis of the evaluation of optical data, the stoichiometry of complex **I** of idarubicin in DMF has been proven to be 1/1 at a molar ratio of metal to ligand 1/10 (in solution) and an oligomeric to polymeric complex **II**, in equilibrium with complex **I**, at metal-to-ligand ratios (in solution) 1/3 and above.

ratio of metal and ligand. The stoichiometry of these complexes has been (partly) determined in another publication of the same authors.[36] On the basis of the results therein and on the spectroscopic investigations of this study, structural models have been proposed. The iron centers do interact via weak exchange interactions, the nature of which will be investigated in a future combined susceptibility and EXAFS study.

Acknowledgment. This work was part of the EC project "Human Capital and Mobility", No. CHRX-CT94-0589, and it was supported with grants from Université Paris Nord and CNRS. H.D. was supported by a DFG (Deutsche Forschungsgemeinschaft, Bonn, Germany) research grant for the duration of this project.

IC0002723