Comprehensive 2D ^{1}H NMR Studies of Paramagnetic Lanthanide(III) Complexes of Anthracycline Antitumor Antibiotics

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The binding of several lanthanide(III) ions to anthracycline antitumor antibiotics daunomycin and adriamycin in methanol and aqueous solutions has been studied by means of optical and 2D NMR (COSY, TOCSY, and EXSY) techniques. These results indicate that a 1:1 Yb^{3+}−drug complex (**1**) is the predominant complex at a metal-to-ligand ratio <10 with slightly higher proton activities, e.g., ∼pH 4−5 in an aqueous solution. In the presence of a base, a 1:2 (**2**) or 1:3 (**3**) Yb^{3+}−drug complex can be formed. In addition, a 2:1 complex (**4**) is formed when the metal-to-drug ratio is >25. These Yb^{3+}−drug complexes undergo slow chemical exchange with each other relative to the NMR time scale. Therefore, 1D and 2D magnetization transfer experiments can be utilized for the assignment of the isotropically shifted signals arising from the drug nuclei in the various paramagnetic complexes. The spin−lattice (T_1) relaxation times and solution magnetic susceptibilities of these Yb^{3+}−drug complexes confirmed the binding of the metal ion to 11,12-β-ketophenolate in all the complexes (except the second Yb^{3+} in the 2:1 complex which binds to the 5,6-β-ketophenolate). Several other lanthanide(III) ions Pr^{3+}, Eu^{3+}, and Dy^{3+} show similar binding properties to daunomycin based on optical and NMR studies. The binding of Yb^{3+} to daunomycin has a profound effect on the reduction potential of the drug, showing a decrease in the potential by 150 mV upon addition of 1 equiv of Yb^{3+} to the drug solution. This observation indicates that metal ions must play a significant role in the action of these family of drugs in vivo.

Introduction

Daunomycin (Dau) and Adriamycin (Adm) are the prototypical members in the anthracycline antitumor antibiotic family.[1] Despite their severe cardiotoxicity and other side effects, these drugs have been widely used as dose-limited chemotherapeutic agents for the treatment of human cancers such as leukemia since their discovery in the early 1960s.[1] These antibiotics contain a quinone-containing chromophore and an aminoglycoside sugar (Figure 1).[2] The antineoplastic activity of these drugs has been mainly attributed to their strong interactions with DNA in the target cells. There are two major mechanisms for these drugs to deform DNA structure and terminate its biological function:[3] (1) an intercalation of the drugs into the base pairs in the DNA minor grooves, where the major contributions of the intercalative binding arise from hydrogen bonding, electrostatic, van der Waals, and hydrophobic interactions; (2) via a free radical damage of the ribose, where the free radicals are formed during the redox cycle of the anthraquinone. These drugs can be reduced to their semiquinone form by biological reducing agents, such as NADH and NADPH. Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) can be produced via dioxygen receiving electrons from the semiquinone. Then, hydroxyl radical (OH•) can be generated, which can attack cell components, such as membrane and DNA, and stops cell growth.

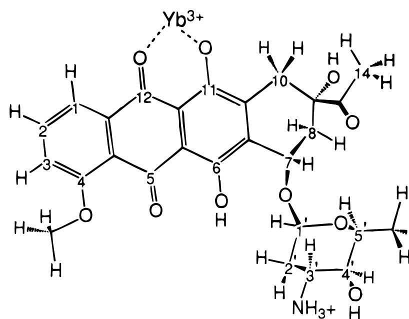

Figure 1. Structure of a daunomycin−Yb^{3+} complex. The structure of daunomycin is drawn on the basis of its crystal structure,[2] and the metal ion is placed at the 10,11-β-ketophenolate site on the basis of the NMR studies described in this report. The 14-methyl is replaced by a $CH_2(OH)$ group in adriamycin.

A number of papers reported that metal ions ($Fe^{2+/3+}$, $Cu^{+/2+}$, and Tb^{3+}) played an important role in altering the biochemical properties of the anthracyclines and indicated a new direction in the pursuit of chemotherapeutic efficacy and lowering toxicity of these antibiotics.[4] The binding of metal ions may cause a significant influence on the redox property of these drugs, thus affecting their activity. The interactions of these metal−drug complexes with DNA and other cell components, and their subsequent damage by the metal complexes, have also been previously studied by the use of various physical and biochemical methods.[5]

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Iron is an important element in that it participates in the action of several drugs (such as bleomycin[6] and streptonigrin[7]) functioning as a redox center that can generate free radicals in the presence of dioxygen under reducing conditions and damage cell components. It has been shown that Fe^{3+} ion can bind three anthracycline molecules in aqueous solution, with the metal chelated by the 11,12-β-ketophenolate group.[4g] A 1:2 Fe^{3+}–adriamycin complex forms a stable complex with calf-thymus DNA in solution, and this tertiary complex is distinct from both the free Fe^{3+}–drug complex and DNA-intercalated drug on the basis of optical and chromatographic studies.[5e] However, Fe^{3+}–anthracyclines have also been observed not being able to intercalate into DNA base pairs until releasing the Fe^{3+} ion, despite the strong binding of Fe^{3+} with the drugs.[4g] Although studies have shown significant interactions between metal–drug complexes and DNA that resulted in DNA damage, the structures of these metal complexes have not been clearly described in all the previous studies.[5]

Since a large variety of metal ions (such as Mg^{2+}, Ca^{2+}, and the transition metal ions) exist in living organisms and are presumably available for binding with these drugs, the study of metal–drug interactions is crucial for a better understanding of the drug action in vivo. A better understanding of metal–drug interactions and the formation of the metal–drug complexes will enable us to gain further insight into their antibiotic mechanisms. In addition to the above-described ions, several other metal ions with preferable spectroscopic and magnetic properties have been utilized as probes for the study of metal–anthracycline interactions. Particularly, the lanthanide(III) (Ln^{3+}) ions have been used as substitutes for the alkaline earth metal ions and have also served as nonredox active metal substitutes for transition metal ions in those studies.[8] However, some questions regarding the metal binding and the structure of the complexes remain unanswered, such as the identity of different species detected in the spectra, the stoichiometry of the metal–drug complexes, and the metal binding mode.

The Ln^{3+} ions are considered useful probes for the study of this drug system because of their unique magnetic and chemical properties:[9,10] (1) The paramagnetic nature ($J = {}^1/_2 - {}^{15}/_2$) of some Ln^{3+} ions allows us to look into the structures of their drug complexes by the use of NMR, where the ^{1}H NMR signals of a coordinated drug are paramagnetically shifted by the Ln^{3+} ions which act as "shift reagents".[11] (2) Like the alkaline earth metal ions, Ln^{3+} ions have a strong affinity to oxygen-rich ligands and can bind to the β-ketophenolate moiety of these antibiotics. This ligand binding property makes Ln^{3+} ions the best substitutes for the alkaline earth metal ions in biomolecules with oxygen-rich metal-binding environments.[10] (3) The radii, charges, Lewis acidity, and preferred oxygen-rich ligand environment of the Ln^{3+} ions are similar to those of Fe^{3+}, despite their different preferred coordination geometries, suggesting that a better study of the physical and chemical properties of Ln^{3+}–drug complexes may afford a better understanding of the iron–drug complexes.

We report here comprehensive studies of several paramagnetic Ln^{3+}–anthracycline complexes by the use of optical, electrochemical, and 1D and 2D ^{1}H NMR techniques. The use of 2D ^{1}H NMR techniques allows us to assign all the proton signals of these Ln^{3+}–drug complexes and to determine their configurations in solution which was not accomplished in previous studies. On the basis of the optical and NMR studies, we conclude that four different Ln^{3+}–drug complexes with metal-to-drug ratios 1:1, 1:2 1:3, and 2:1 (plus an amorphous polymeric form) are formed in solution under different proton and metal ion concentrations. These complexes are under slow chemical exchange with each other in solution. With a complete assignment of the ^{1}H NMR spectrum of the 1:1 Yb^{3+}–drug complex,[12] the spectra of the other species are possible to assign by the use of exchange spectroscopy (EXSY). The configurations of the lanthanide(III) complexes of the drug daunomycin have been determined to be the same as the crystal structure of the free drug. We use these lanthanide(III)–anthracycline complexes as model systems for the understanding of the binding of alkaline earth metal and transition metal ions with these drugs[13] and the effect of metal ion binding on the action of other quinone-containing drugs.[13b]

Experimental Section

Chemicals and Sample Preparation. Daunomycin and adriamycin were purchased from Sigma Chemical Co. and were also supplied as gifts by Farmitalia Carlo Erba, Milan, Italy. All lanthanide(III) chloride salts (99.99%) were obtained from Sigma Chemical Co. All organic

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solvents were HPLC grade. Other reagents were the highest grade available from the commercial sources. Daunomycin and adriamycin solutions were prepared just before the experiments to avoid their degradation. The concentrations of the stock solutions were around 50−200 μM for optical titrations and the continuous variation method (the Job plot method)[14] and were about 1 mM or higher for electrochemistry and NMR studies. The concentrations of the drugs was determined by their absorption at 478 nm (11.5 $mM^{-1}\ cm^{-1}$).[15] The metal concentration was determined by EDTA standard titration with xylenol as an indicator. All the UV−visible absorption spectra and optical titrations were performed on a Hewlett-Packard 8452A diode array spectrophotometer at ambient temperature using a quartz cell of 0.1 or 1 cm path length.

Electrochemistry. The electrochemical studies were performed on an OMNI 90 potentiostat (Cypress Systems, Inc.) with a Linseis recorder. A three-electrode cell with a 0.6-mL sample volume was used. A Pt electrode with 0.1 mm diameter surface was used as the working electrode while another Pt electrode was used as the supporting electrode, with a Ag/AgCl electrode as the reference electrode. The working electrode surface was cleaned with EDTA and 0.1 M HNO_3 following by methanol rinsing before use. Sodium tetraphenylborate or tetrabutylamonium hexafluorophosphate at 50 mM was used as the supporting electrolyte. The solvent was degassed and then saturated with argon. All the experiments were performed under argon.

Nuclear Magnetic Resonance. Proton NMR spectra were acquired on a Bruker AMX360 spectrometer at 360.13 MHz. The ^{1}H chemical shift was referenced to an external TMS (tetramethylsilicane) to avoid the effect of Ln^{3+} paramagnetism on the chemical shift of internal TMS. A 90° pulse (∼7 μs) was used for the acquisition of 1D ^{1}H NMR spectra with 8K data points, while 1024 × 512 data points were used for COSY, TOCSY, and EXSY spectra which were acquired using standard pulse sequences with a presaturation pulse for solvent suppression. A 45−60° shifted sine-squared-bell apadization function was applied to both dimensions prior to Fourier transformation for phase sensitive EXSY and TOCSY spectra. A 0° shifted sine-squared-bell apadization function was applied to COSY spectra and processed in magnitude mode and then followed by symmetrization. For base titration, a certain amount of triethylamine was added to samples with a 1:3 metal-to-drug ratio, and monitored by NMR. These drugs tend to self-aggregate at high concentrations in aqueous solution but not in methanol.[16] Thus, the concentrations of NMR samples in aqueous solution were ∼1 mM to avoid extensive self-association. The proton spin−lattice relaxation times (T_1) for all the metal complexes were determined by the use of the inversion recovery method (180°−τ−90°) with 16 different τ values and a recycle time $>5T_1$. The data were fitted by a three-parameter fitting program on the spectrometer based on a plot of peak intensity versus the τ values.

The magnetic susceptibility values of 1:1, 1:2, and 1:3 Yb^{3+}−drug complexes were determined on the Bruker AMX360 NMR spectrometer by following the Evans method,[17] where a methanol solution of TMS inside a capillary coaxial tube served as the diamagnetic reference substance which was placed inside a 5-mm NMR tube containing the paramagnetic substance (metal concentration at ∼0.5 mM). The formation of different Yb^{3+}−drug complexes was monitored by the ^{1}H NMR spectra of the sample, in which the intensities of the 10-CH_2 proton signals served as the criteria for this purpose. An appropriate amount of the inert reference (TMS), with its intensity comparable to the reference in the capillary tube, was used to monitor the influence by the paramagnetism of the complexes. The difference between the chemical shifts of the TMS inside and outside the capillary tube was measured, and the molar magnetic susceptibility of the metal complexes was calculated according to the Evans method as $\chi_M = \Delta\delta/[1000M(^1/_3 - \alpha)]$, in which $\Delta\delta$ is the difference of chemical shift in ppm (i.e., 10^{-6}), M is concentration in molarity, and $\alpha = 0$ for data obtained in a superconducting magnet.[17]

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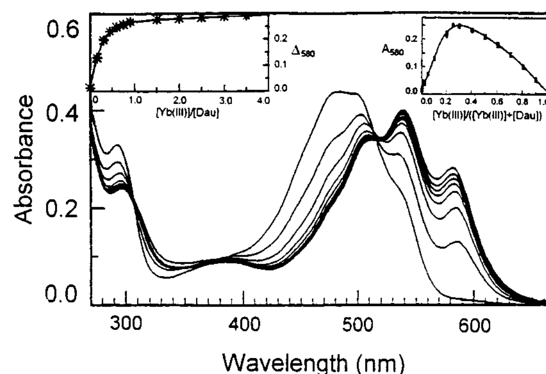

Figure 2. Formation of a Yb^{3+}−Dau complex in HEPES buffer at pH 7.5. A Job plot reveals the formation of a 1:2 or 1:3 Yb^{3+}−Dau complex, similar to the case in methanol in the presence of a base.[12]

Results and Discussion

Optical Studies. The formation of Yb^{3+}−Dau complexes in methanol can be monitored by the use of a spectrophotometer and has been described in our previous communication.[12] In summary, new absorptions appear at 541 and 576 nm while the free drug absorption at 480 nm decreases upon the addition of Yb^{3+} to a Dau•HCl solution in methanol, indicating the formation of a metal−drug complex (cf. Figure 2). Two isosbestic points at 295 and 512 nm are observed, which indicate that only one complex (i.e., a 1:1 complex) is formed during this titration. A plot of $\Delta\lambda_{576}$ against Yb^{3+} concentration gives an affinity constant of $8.21 \times 10^4\ M^{-1}$ for Dau•HCl binding with Yb^{3+}. A Job plot suggests that a complex of 1:1 metal-to-drug ratio is formed without base while species with lower metal-to-drug ratios (1:2 and 1:3) are formed upon the addition of triethylamine in methanol with a concomitant shift of the 576-nm absorption to 580 nm.[12]

The pK_a values for the proton on the phenols C11 and C6 are 10.0 and 13.7, respectively.[18] Therefore, one can expect that the metal binding site in the 1:1 complex is the 11,12-β-ketophenolate. Another β-ketophenolate site at positions C5 and C6 can bind metal ions only when the Yb^{3+}-to-drug ratio is high (>25), to give a 2:1 metal-to-drug complex (cf. NMR studies; vide infra). Upon deprotonation of the C6 phenol at high pH, a precipitate is formed in the presence of Yb^{3+}. This precipitate is not soluble in either polar or nonpolar solvents and does not melt at 420 °C, which is possibly an amorphous polymeric complex formed with Yb^{3+} bound at both β-ketophenolate sites (C5/C6 and C11/C12) between two or more drug molecules. A similar observation was reported previously on the Cu^{2+} complex of this drug under alkaline conditions, in which the formation of a polymeric chain structure was proposed.[4b] These Yb^{3+}−drug complexes with different metal-to-drug ratios are under equilibrium in solution and can be converted from one to the other by changing proton or metal concentration. The same result was obtained when the titration was performed in buffered aqueous solutions, where a 1:1 complex was formed at pH 5.5 as observed previously in methanol[12] and a 1:2 or 1:3 complex was seen at pH >6.5 (Figure 2). The overall spectral features are similar to that of the complex in methanol but with a lower absorption intensity.

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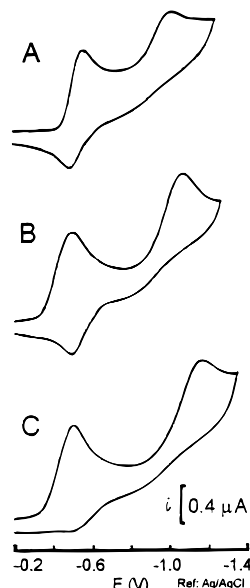

Figure 3. Cyclic voltametric diagrams of (A) ~0.5 mM Dau and Dau with the introduction of (B) 1 and (C) 2 equiv of Yb^{3+} in DMF with a scan rate at 30 mV/s and referenced against Ag/AgCl. A significant shift of the initial reduction potential to more anodic direction by at least 150 mV is observed upon Yb^{3+} binding to Dau.

Other Ln^{3+} ions bind Dau in a similar way except with different binding affinities due to their different charge-to-radius ratios caused by the lanthanide contraction. On the basis of the increase of the absorption at 576 nm upon the addition of Ln^{3+} to Dau·HCl in methanol, the apparent affinity constants for the formation of 1:1 complexes are obtained in the order of $Pr^{3+} < Eu^{3+} < Dy^{3+} < Yb^{3+} < Lu^{3+}$ with the values 13.6, 27.2, 43.1, 82.1, and 97.5 mM^{-1}, respectively, where the largest affinity constant for Lu^{3+} is clearly due to its largest charge-to-radius ratio.

In comparison to these Ln^{3+} complexes, the Ca^{2+}−Dau complex has a dramatically smaller formation constant (21.5 M^{-1}) based on optical titrations. Nevertheless, as anthracycline is taken up in the amount of ~75 mg/m^2 body surface per treatment,[1c] the mM concentrations of divalent alkaline metal ions in the body can bind these drugs and may affect the redox potential (vide infra) and likely the efficacy of these drugs. Because Ln^{3+} ions are good chemical and biochemical mimics for alkaline earth metal ions,[10] the Ln^{3+}−anthracycline complexes can serve as model systems to provide further insight into the physical and molecular properties of Ca^{2+}/Mg^{2+}−anthracycline complexes. Moreover, the effects on the physical and chemical properties of the drugs upon Ln^{3+} binding can be more clearly monitored due to the large formation constants of the Ln^{3+}−drug complexes.

Electrochemistry Study of the Yb^{3+}−Dau Complex. The binding of Yb^{3+} to Dau has been studied by means of cyclic voltometry (Figure 3). The free drug shows an initial reduction potential for the formation of semiquinone at $E_{red} = -0.45$ V in both methanol and DMF, and this electrode reduction is reversible (trace a). A second reduction potential is found at 1.1 V with little reversibility. The first reduction potential of the drug is shifted to more anodic potential with the addition of Yb^{3+} (traces b and c). Our study reveals that the addition of 1 equiv of Yb^{3+} to the drug affects the initial reduction potential of the drug by a significant value of at least 150 mV (trace b), indicating that less work is taken for the reduction of the drug in the presence of metal ions. That is attributable to the formation of a stable Yb^{3+}−semiquinone drug complex upon reduction. It has been previously reported that metal ions form more stable complexes with semiquinones than their corresponding quinone forms due the presence of an extra charge on the semiquinones.[19] The accessibility of the metal−drug complexes to reduction has an important biological consequence, since the reduction of the drugs to their semiquinone forms has been considered the initial step in their antibiotic action.[3]

Nuclear Magnetic Resonance Studies. NMR is one of the most powerful tools for the study of the environment about paramagnetic metal ions, such as Fe^{2+}, Co^{2+}, Ni^{2+}, and some Ln^{3+} ions, in metal complexes and metalloproteins via the assignment of the isotropically shifted ^{1}H NMR signals.[20] Paramagnetic Ln^{3+} complexes have been used as shift reagents for structural determinations of diamagnetic compounds.[11] Furthermore, they have similar radii and ligand binding preferences to that of Ca^{2+} and can easily substitute for Ca^{2+} in proteins.[10] Hence, NMR has been utilized for the structural and mechanistic studies of several Ca^{2+}-binding proteins such as parvalbumin, calmodulin, and α-lactabumin by using paramagnetic Ln^{3+} as probes.[9] The isotropic shift in paramagnetic Ln^{3+} complexes is mainly attributable to the distance and geometry-dependent dipolar shift mechanism (eq 1),[21] where

$$\frac{\Delta\nu^{\text{dip}}}{\nu} = -{}^1/_3 N[\chi_z - {}^1/_2(\chi_x + \chi_y)]\left(\frac{3\cos^2\theta - 1}{r^3}\right) - {}^1/_2 N(\chi_x - \chi_y)\left(\frac{\sin^2\theta\cos 2\Omega}{r^3}\right) \quad (1)$$

χ's are the principal components of the magnetic susceptibility tensor, θ the angle between the **r** vector and the z axis, and Ω the angle between the x axis and the projection of the **r** vector on the xy plane. Of much smaller importance is the contact shift mechanism, since the valence f orbitals are shielded and do not participate in covalent bonding with ligands. Because of this, the environment about the metal can be studied via the isotropically shifted signals and is not restricted to only the first coordination sphere. In this report, we describe the use of Ln^{3+} ions as redox-inactive NMR probes for modeling the binding properties of alkaline earth and transition metal ions[13] with anthracycline drugs.

A. ^{1}H NMR Spectrum of 1:1 Yb^{3+}−Anthracycline Complexes. In our previous communication,[12] we have reported a full assignment of the ^{1}H NMR spectrum of the 1:1 Yb^{3+}−Dau·HCl complex in methanol by the use of chemical exchange (EXSY) and coherence transfer (COSY) NMR techniques. In summary, the proton NMR spectrum of this complex in deuterated methanol exhibits 13 isotropically shifted signals clearly detected in the upfield regions between 0 to −40 ppm. The signals from the free antibiotic are still well resolved in the diamagnetic region, suggesting that the drug is in slow exchange with its metal-bound form on the NMR time scale in solution. We have thus taken the advantage of the fully assigned ^{1}H NMR spectrum of the free drug for the assignment of the isotropically shifted features of the 1:1 Yb^{3+}−Dau complex by the use of the 2D EXSY technique. All the 17 paramagnetically shifted solvent nonexchangeable protons in the metal complex can be correlated to their counterparts in the free drug and

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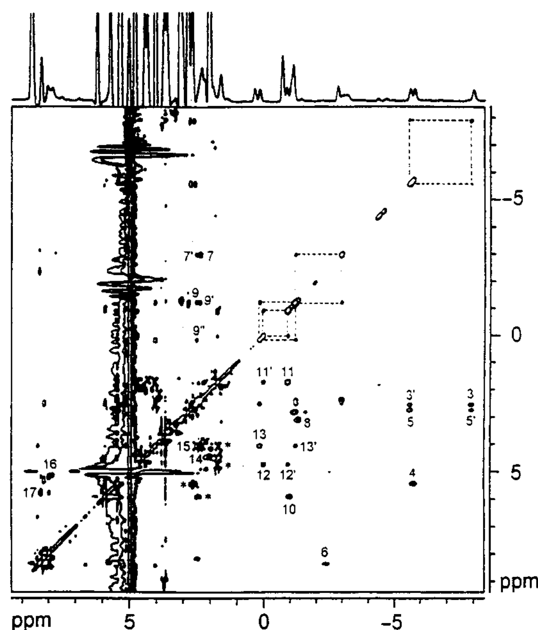


Figure 4. TOCSY spectrum of Yb^{3+}−Dau complex in the presence of excess Dau in methanol-d_4 at 303 K with a spin lock 25 ms. Both coherence transfer (dotted lines), chemical exchange (numbered), and secondary exchange (primed and double-primed numbers) cross-peaks are detected in the rotating frame. In addition, coherence transfer cross signals in free drug are also detected (asterisked). The exchange cross-peaks are associated with the protons 10-CH_2 (1 and 2 at −36.2 and −30.7 ppm, not shown), 8-CH_a (3), 7-CH (4), 8-CH_e (5), 1-CH (6), 2′-CH_e (7), 14-CH_3 (8), 2′-CH_a (9), 1′-CH (10), 5′-CH_3 (11), 5′-CH (12), 3′-CH (13), 4-OCH_3 (14), 4′-CH (15), 3-CH (16), and 2-CH (17). The subscripts a and e indicate the axial and equatorial positions, respectively.

assigned. The metal binding site has been determined to be the 11,12-β-ketophenolate moiety.

One convenient technique that can be utilized for the study of exchange systems is the detection of magnetization transfer in the rotating frame. Particularly, the TOCSY sequence is known to reveal chemical exchange-based saturation transfer and bond-correlated coherence transfer.[21] Chemical exchange-originated cross-peaks can be differentiated from bond-correlated coherence transfer when one of them can be recognized, e.g., by means of EXSY in the former case and COSY in the latter case. The TOCSY spectrum of the 1:1 Yb^{3+}−Dau complex (Figure 4) shows clearly the bond correlation among several protons (dotted lines, which have also been confirmed in a COSY spectrum). The 3′CH proton shows intense cross-peaks with one 2′CH proton, indicating they are anti to each other. Moreover, cross-peaks are also observed for the chemical exchange between the metal−drug complex and free drug (numbered signals). These exchange cross signals (labeled 3−17) are the same as those observed in an EXSY spectrum which, however, does not reveal bond correlations. In addition to the chemical exchange and coherence transfer cross-peaks, several weaker "secondary exchange" cross-peaks, i.e., coherence transfer-mediated exchange cross-peaks (primed and double primed numbers), and coherence transfer cross signals in free drug (asterisked) are also observed in the TOCSY spectrum. A full assignment of the signals (via exchange-based cross-peaks) has been achieved, and an elucidation of the molecular structure (via coherence transfer cross-peaks) becomes possible in the TOCSY spectrum. The TOCSY results corroborate the results acquired from EXSY and COSY studies,[12] suggesting that TOCSY can be a very useful tool for the study of chemical exchange in metal−drug systems.

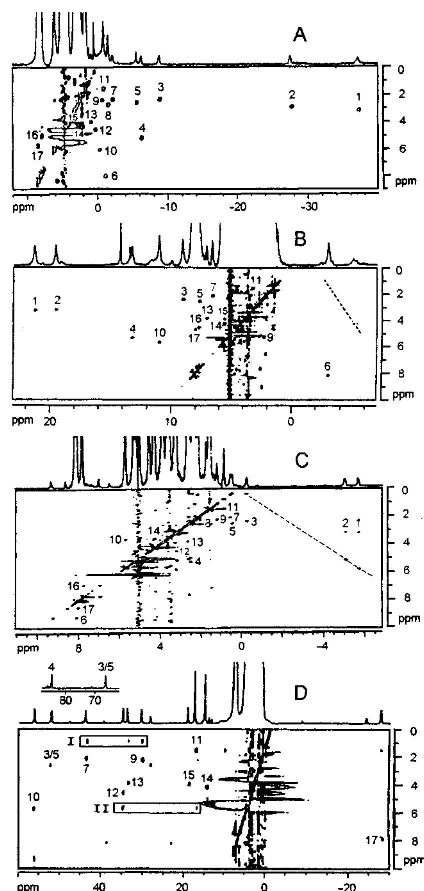


Figure 5. Proton EXSY spectra (30 ms mixing time) of (A) Yb^{3+}−Dau in D_2O at pH 5.8, 298 K, (B) Pr^{3+}−Dau in methanol-d_4 at 298 K, (C) Eu^{3+}−Dau in methanol-d_4 at 303 K, and (D) Dy^{3+}−Dau in methanol-d_4 at 298 K and COSY cross-peaks along 2′-CH_a (inset I) and 5′-CH_3 (inset II) signals. The 10-CH_2 signals in Dy^{3+}−Dau are not detected in the window range ±160 ppm. These protons may shift to >200 ppm on the basis of the chemical shift of the 7-CH proton and eq 1. The numberings in these spectra follow the numbering and assignment in Figure 4. The dashed line in the spectra shows the fold-over diagonal signals due to the use of a small window in the second dimension to enhance the resolution.

Our earlier attempts at studying Ln^{3+} binding with the anthracyclines in aqueous solutions by NMR was not successful owing to drug coagulation and the formation of metal hydroxide precipitates at neutral and higher pHs. This problem was solved by using low drug concentration (~1 mM or less) in slightly acidic solutions (<pH 6). The spectrum of a Yb^{3+}−drug complex in aqueous solution is shown in Figure 5A. The 1:1 Yb^{3+}−Dau complex in aqueous solution exhibits much broader ^1H NMR signals than that in methanol (cf. Figure 6A). The spectra of the Yb^{3+} complex in water and in methanol are very similar, in which 7 signals differ by >1 ppm (Table S1 (Supporting Information)). An average of the difference of all the signals is 1.05 ± 0.86 ppm and is only 0.55 ± 0.24 ppm for the 10 less different signals (in a spectral window ~40 ppm). Despite their broad features and low intensities, the isotropically shifted ^1H NMR signals of the 1:1 Yb^{3+}−Dau complex in aqueous solution have been assigned via their exchanges with the signals of the free drug in an EXSY spectrum (Figure 5A). The two farthest upfield shifted signals at −26.5 and −36 ppm are in chemical exchange with the drug 10-CH_2 protons in the EXSY spectrum and can thus be assigned. Other signals can also be assigned in the EXSY spectrum (cross-peaks 1−17). The spectrum of the complex in aqueous solution is similar to that in methanol, suggesting the structure of the Yb^{3+}−Dau

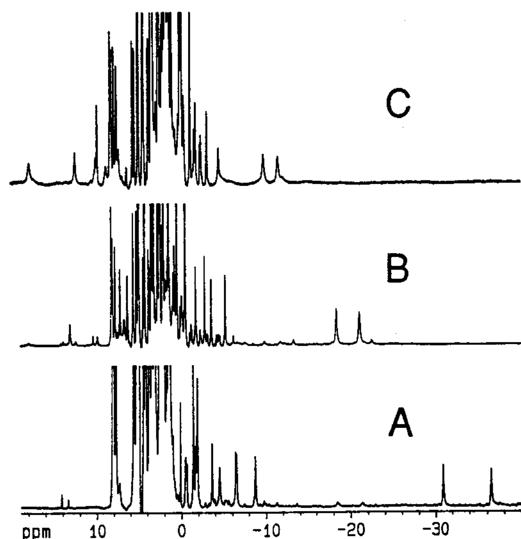


Figure 6. Base titration of Yb^{3+}−Dau complex (in the presence of 3 times excess Dau) in methanol-d_4 at 298 K and monitored by ^1H NMR: (A) Yb^{3+}−Dau complex **1**; (B) complex **1** with ~0.4 equiv of triethylamine added to give **2**, i.e., Yb^{3+}−$(Dau)_2$; (C) complex **1** with ~0.7 equiv of base introduced to give **3**, i.e., Yb^{3+}−$(Dau)_2$. The addition of more base to (C) results in the formation of a precipitate shortly.

complex remains the same in methanol and water. Therefore, the information acquired in methanol is applicable to the understanding of the complex in aqueous solutions.

Daunomycin was the first anthracycline drug used for clinical trial; however, it was later replaced by the more potent and less cardiotoxic adriamycin (Adm) for cancer treatment.[1,3] Despite their similar structure (Figure 1), these two drugs show different clinical properties. However, the ^1H NMR spectra of Yb^{3+}−Dau and Yb^{3+}−Adm show a great similarity to each other, suggesting a similar overall structure. The COSY spectrum of the 1:1 Yb^{3+}−Adm complex indicates that its sugar conformation is similar to that of the 1:1 Yb^{3+}−Dau complex (spectrum not shown).[13b] The ^1H NMR spectrum of 1:1 Yb^{3+}−Adm shows the presence of a minor species that is not seen in the 1:1 Yb^{3+}−Dau spectrum. Slow exchange between the minor species and the 1:1 Yb^{3+}−Adm complex prevents assignment of this species. Nevertheless, this minor species is likely a complex formed by a cooperative Adm binding to the metal to form a complex with higher metal-to-drug ratios, as observed in Yb^{3+}−Dau discussed above. The slightly different metal binding properties between Dau and Adm may reflect their different clinical properties.

B. ^1H NMR of Other 1:1 Ln^{3+}−Dau Complexes. Both Dau and Adm can bind other Ln^{3+} ions to form 1:1 complexes that have virtually the same binding mode as the 1:1 Yb^{3+} complex. Due to their smaller affinities of these Ln^{3+} ions with the drugs, the intensities of the dipolar-shifted ^1H NMR signals in these Ln^{3+} complexes are weaker than those in the Yb^{3+} complexes. The several paramagnetic Ln^{3+} ions have different magnetic anisotropy values ($\Delta\chi$) which result in the shift of signals to different regions; in some cases signals are found in opposite directions in the spectra between different Ln^{3+} ions (such as Yb^{3+} versus Pr^{3+}).[11] The 1:1 Pr^{3+}−Dau complex shows mainly downfield-shifted signals up to 20 ppm in a ~30-ppm spectral range, which can be fully assigned in the EXSY spectrum (Figure 5, spectrum B). The 10-CH_2 protons still exhibit the largest shifted signals at 22 and 19 ppm, indicating that Pr^{3+} binds to the C11/C12 β-ketophenolate site. This is consistent with the results from the study of the 1:1 Yb^{3+}−Dau complex.

As the ground state of Eu^{3+} is $J = 0$, it should be considered diamagnetic. However, its lowest paramagnetic excited state is close to the ground state and is accessible even at room temperature (3.61 μ_B). Therefore, one can expect the ^1H NMR spectrum of Eu^{3+}−Dau complex to exhibit isotropically shifted signals, studied by the use of the EXSY technique (Figure 5, spectrum C). The 10-CH_2 protons at −5 and −5.5 ppm still gain the largest dipolar shifts in this complex. This suggests that Eu^{3+} also binds to the C11/12 β-ketophenolate position, similar to other Ln^{3+}−Dau complexes.

The 1:1 Dy^{3+}−Dau complex shows a ^1H NMR spectrum with sharp paramagnetically shifted signals in a large spectral window due to the fast electronic relaxation rates (~10^{13} s) and the large magnetic moment of Dy^{3+} ($J = {}^{15}/_2$ and 10.5 μ_B). This suggests that NMR is very useful for the study of the interaction of Dy^{3+} with small metal-binding biomolecules, where Curie relaxation is not a concern. Despite the large spectral window, the EXSY spectrum of this complex has been successful acquired. Most of the signals can be assigned in the EXSY spectrum (Figure 5D) and confirmed by the COSY spectrum (insets). The geminal 2′-CH_2 protons at 44.8 and 31.7 ppm show intense cross-peaks in the COSY map, and the latter signal also shows weaker cross-peaks with the 3′-CH proton at 34.9 ppm. This indicates that the 3′-CH proton and the 31.7-ppm 2′-CH proton are at axial positions trans to each other, corroborating with the configuration found in the free drug and in the 1:1 Yb^{3+}−Dau complex.

C. ^1H NMR and Base Titration of Yb^{3+}−Dau. We have utilized Yb^{3+} ion in the study of Ln^{3+}−anthracycline interactions. This is because Yb^{3+} has a large binding affinity with the drugs (82.1 mM^{-1}) and possesses a large magnetic moment (4.4 μ_B) and fast electronic relaxation rates (~10^{13} s^{-1}) that are feasible to NMR studies. Job plots show that the stoichiometry of Yb^{3+}−drug complex varies under different conditions, where a change from the 1:1 Yb^{3+}−drug complex to a 1:3 complex is observed in alkaline solutions. The farthest shifted signals at −30 and −36 ppm in the 1:1 complex in methanol have been assigned to the 10-CH_2 protons by using COSY, TOCSY, and EXSY techniques (Figures 4 and 5) and are used for monitoring the effect on the complex by base in solution. Upon the addition of triethylamine to the 1:1 complex (**1**), different Yb^{3+}−Dau complexes can be formed depending upon the amount of the base added. A second complex (**2**) can be completely formed upon the addition of ~0.4 equiv of base, which exhibits the farthest shifted signals at −20 and −25 ppm that are presumably due to the 10-CH_2 protons (Figure 6B). With addition of the base to ~0.7 equiv, a third complex (**3**) is formed whose 10-CH_2 protons are presumably detected at −10 and −15 ppm (Figure 6C). Upon further addition of the base, a precipitate occurs within a few hours. A gradual shift of λ_{max} from 576 to 580 nm during this base titration can be observed using a 1-mm optical cell, suggesting the formation of 1:2 and 1:3 Yb^{3+}−Dau complexes as determined by the Job plots. These different complexes are under fast equilibria in solution and can be studied in details by using the magnetization transfer EXSY technique (vide infra).

D. Spin−Lattice Relaxation Times of Yb^{3+}−Dau during Base Titration. The spin−lattice (T_1) relaxation times of the protons 1-CH, 7-CH, 8-CH_2, 10-CH_2, 1′-CH, 5′-CH, 5′-CH_3, and 14-CH_3 in the complex **1** are 12.1, 217, 150/180, 16/20.8, 232, 263, 357, and 183 ms, respectively. Relative distances to the metal for the above protons can be estimated with Yb^{3+}−1-CH set to be 4.2 Å as the reference[2] and calculated as $[(T_1/$

Table 1. T_1 and Magnetic Susceptibility Values of the 10-CH_2 Protons and the Magnetic Susceptibility of the Three Yb^{3+}−Dau Complexes in Methanol

	complex					
	Yb^{3+}−Dau, **1**		Yb^{3+}−$(Dau)_2$, **2**		Yb^{3+}−$(Dau)_3$, **3**	
chem shift of 10-CH_2	−30.0	−36.1	−16.4	−18.3	−8.8	−10.1
T_1 (ms)	19.9	17.8	16.6	14.1	20.4	16.7
relative Yb^{3+}−H dist (%)	100	100	97.0	96.2	100.4	98.9
molar magn susceptibility[a] χ_M (10^{-7} m^3 mol^{-1})	1.33		1.37		1.31	

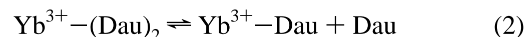
[a] A 1:1 mixture of **1** and **2** gives a value 1.35, a 1:1 mixture of **3** and the complex formed with large excess base gives a value of 1.17, and the latter gives a value 0.85 prior to its precipitation.

$T_{1(1-CH)})^{1/6} \times 4.2]$ Å according to the Solomon equation[22] as following (with distances obtained based on the structure in Figure 1 shown in parentheses): 4.2 (4.2), 6.8 (7.4), 6.4/6.6 (6.7/8.2), 4.4/4.5 (4.4/4.7), 6.9 (8.8), 7.0 (9.7), 7.4 (11.5), and 6.6 (7.4), respectively. Large discrepancies of the values are observed for sugar protons with apparently shorter distances. A free rotation of the sugar along the C7−O−C1′ bonds can afford transient configurations with shorter sugar−metal distances. However, this cannot explain the much shorter estimated distance for the 1′-CH proton. A possible cause might be due to transient interactions of the metal ion with the hydroxyl groups on the sugar, which have significant affinities with Ln^{3+} ions.[11]

The spin−lattice (T_1) relaxation times of the 10-CH_2 protons of the three different Yb^{3+}−Dau complexes **1**−**3** formed during base titration are listed in Table 1. The T_1 values of the two 10-CH_2 protons in these complexes are very similar (averaging 19.0 and 16.2 ms) and shorter than those of the 7-CH and 8-CH_2 protons. This reflects the following: (1) The metal binding site is the β-ketophenolate moiety at positions C11 and C12 rather than the positions C5 and C6. (2) The distances between 10-CH_2 protons and Yb^{3+} do not change much in these three complexes, as the relaxation time (T_1) is proportional to the sixth power of the distance between the proton and the paramagnetic metal (r_{M-H}^6). (3) The significantly different chemical shifts of the same protons in the three complexes are not due to a change of the metal−proton distance in the dipolar shift term (r^{-3} in eq 1). (4) The electronic relaxation rates (τ_e^{-1}) of the paramagnetic Yb^{3+} center in the different complexes are similar, since T_1 is proportional to τ_e^{-1} in Yb^{3+} complexes with large τ_e^{-1} values ($\tau_e < 10^{-12}$ s).[21]

E. Magnetic Susceptibilities of Yb^{3+}−Dau during Base Titration. The solution magnetic susceptibility of the Yb^{3+}−Dau complex is not significantly changed during base titration (Table 1, complexes **1**−**3**). This indicates that the very different chemical shifts (as shown by the 10-CH_2 protons) in these Yb^{3+}−Dau complexes **1**−**3** are not due to a change in solution magnetic susceptibility. The ratios of the chemical shifts of all the signals between two complexes are constants in axially symmetric magnetic systems upon changing of $\Delta\chi$ (e.g., $\delta(\mathbf{1})/\delta(\mathbf{2}) = \Delta\chi(\mathbf{1})/\Delta\chi(\mathbf{2}) =$ constant). A ~20% deviation in the rations of the chemical shifts for the signals 10-CH_2, 7-CH, and 8-CH_2 between the complexes **1** and **3** may indicate that either there is a significant geometric change that affects the terms in eq 1 (which is ruled out in the relaxation study discussed above) or there is significant magnetic rhombicity in the complexes and the three complexes have different $\Delta\chi$ values. Alternatively, the different shifts can be simply accounted by a change in ligand coordination sphere in axially symmetric magnetic systems that result in a change of the relative position of protons (the θ and Ω terms in eq 1) with respect to the magnetic susceptibility tensor. Since these complexes are in chemical exchange with each other, the magnetic susceptibility values can only reflect the average of the different complexes. No matter what the mechanism is, the conclusion made in the above section is still valid. Considering that the magnetic susceptibility of the Yb^{3+} is not significantly altered during base titration, the decrease in the magnetic susceptibility of the complex formed with a larger amount of base suggests a possible presence of an antiferromagnetic coupling in the amorphous polymeric form before its precipitation.

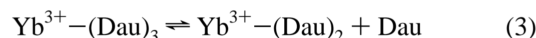
F. ^{1}H EXSY Experiments of Yb^{3+}−Dau Complexes. Since the ^{1}H NMR spectrum of the 1:1 Yb^{3+}−Dau complex **1** has been assigned, it can be used for the assignment of complexes **2** and **3** by the use of the EXSY technique owing to the presence of chemical exchange between the different complexes. The proton signals of complex **2** can be assigned in a one-to-one mixture of **1** and **2** using the EXSY technique. The two signals at −20 and −25 ppm of complex **2** can be clearly assigned in the EXSY spectrum to the 10-CH_2 geminal proton pairs (Figure 7A). Several other signals in complex **2** can also be assigned in the spectrum due to chemical exchange with their counterparts in complex **1** (spectral region not shown). The observation of exchange cross-peaks (marked with arrows) between complex **2** (but not the 1:1 complex **1**) and the free drug indicates that the observed chemical exchange has the equilibrium shown in (2), where only the 1:2 complex Yb^{3+}−



$$Yb^{3+}{-}(Dau)_2 \rightleftharpoons Yb^{3+}{-}Dau + Dau \qquad (2)$$

$(Dau)_2$ shows primary chemical exchange with the free drug. Combining with the results from optical studies, this EXSY study indicates that complex **2** is the 1:2 Yb^{3+}−$(Dau)_2$ complex.

Upon further addition of base, the equilibrium is driven to the formation of complex **3**. The 10-CH_2 signals are detected at −10 and −15 ppm in complex **3**, which are in chemical exchange with the corresponding signals at −20 and −25 ppm in complex **2** as determined in the EXSY spectrum of a one-to-one mixture of **2** and **3** (Figure 7B). The signals in complex **3** show exchange cross-peaks with both complex **2** and the free drug, indicating that this chemical exchange follows the equilibrium shown in (3), in which only the 1:3 Yb^{3+}−$(Dau)_3$



$$Yb^{3+}{-}(Dau)_3 \rightleftharpoons Yb^{3+}{-}(Dau)_2 + Dau \qquad (3)$$

complex shows primary chemical exchange with the free drug, and can be assigned to complex **3**. This corroborates the optical studies.

On the basis of the optical titration of the drug with Yb^{3+}, a forth complex (**4**) is formed when the metal is in large excess (>25 equiv). This complex is partially formed by adding ~20 equiv of Yb^{3+} to the drug and shows a totally different ^{1}H NMR features (asterisked signals in Figure 7C) from the complexes **1**, **2**, and **3**. Complex **4** shows exchange cross-peaks with

(22) Solomon, I. *Phys. Rev.* **1955,** *99*, 559−565.

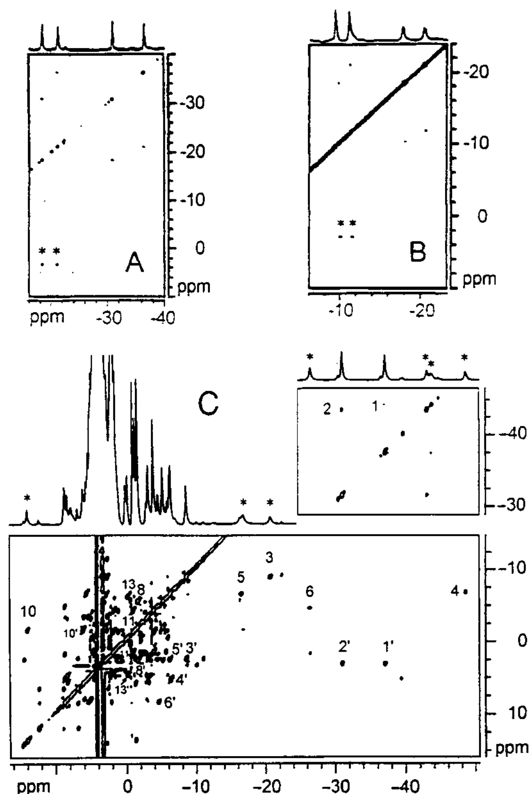
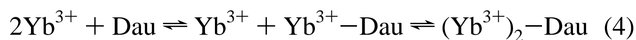


Figure 7. Proton EXSY spectra (at 298 K in methanol-d_4, only 10-CH_2 signals shown) of one-to-one mixtures of (A) Yb^{3+}−Dau (**1**) and Yb^{3+}−$(Dau)_2$ (**2**) in which the detection of the exchange cross-peaks between **1** and **2** and between **2** and free drug (asterisked) indicates the presence of an equilibrium (eq 2) between the two complexes and (B) **2** and Yb^{3+}−$(Dau)_3$ (**3**) in which the exchange cross-peaks between **2** and **3** and between **3** and free drug (asterisked) reflect that the two complexes undergo a fast equilibrium as shown in eq 3. (C) Proton EXSY spectrum (at 298 K in methanol-d_4) of **1** in the presence of 20 equiv of Yb^{3+} to form partially the complex $(Yb^{3+})_2$−Dau (**4**) in which **1** is in exchanges with both free drug (primed numbers) and **4** (numbered) as in eq 4. The asterisked signals in the 1D spectrum are due to complex **4**. The numbering follows the assignment in Figure 4.

complex **1** (numbered signals in Figure 7C) but not with the free drug in the EXSY spectrum. This indicates that the equilibrium is established as shown in (4), in which only Yb^{3+}−



$$2Yb^{3+} + Dau \rightleftharpoons Yb^{3+} + Yb^{3+}\text{−}Dau \rightleftharpoons (Yb^{3+})_2\text{−}Dau \quad (4)$$

Dau shows primary exchanges with both the free drug and $(Yb^{3+})_2$−Dau (primed numbers and numbered signals, respectively, in Figure 7C). The complex **4** can thus be assigned as $(Yb^{3+})_2$−Dau. The signal of the 7-CH proton in this 2:1 complex is found to be further upfield-shifted from −5 ppm in the 1:1 complex to −45 ppm and become the farthest shifted signal. Moreover, the 8-CH_2 protons also shift further upfield, and the sugar 1′ proton gains a significant downfield shift. These results suggest that Yb^{3+} is bound to the 5,6-β-ketophenolate site at higher metal concentrations in addition to the 10,11-β-ketophenolate site. The formation of the 2:1 Yb^{3+}−Dau complex is reminiscent of the result from the study of Fe^{3+} binding with this drug family.[4e] This further suggests that the information acquired from the study of the Ln^{3+} complexes is applicable to the understanding of other metal−anthracycline systems.

Concluding Remarks and Perspectives

The anticancer antibiotics Dau and Adm contain a pair of β-ketophenolate groups and an amino sugar, which are good candidates for the binding of transition metal and lanthanide ions. We present in this report comprehensive NMR studies of Yb^{3+} binding with Dau. The optical and NMR results discussed in this report show that Yb^{3+} is bound primarily to the 11,12-β-ketophenolate site with a configuration similar to the free drug as shown in Figure 1. Depending upon the proton activities, several different metal−drug complexes **1**−**4** with 1:1, 1:2, 1:3, and 2:1 metal-to-drug ratios, respectively, are formed in both aqueous and methanol solutions. The similar Yb^{3+} binding properties of Dau in aqueous and methanol solutions suggest that the information acquired in methanol solution can be used for better understanding of the metal−drug systems in aqueous solutions under physiological conditions. Moreover, with their binding properties similar to those of other metal−anthracyclines,[13] these Ln^{3+}−daunomycin complexes can serve as model systems for the understanding of other metal−anthracycline systems. The use of paramagnetic Ln^{3+} ions as NMR probes for the study of metal−drug interactions has been demonstrated in this report. The methodology present here is also applicable to the study of other metal−antibiotic systems and investigation of the role metal ions play in the interactions of antibiotics with macrobiomolecules.[13b]

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Supporting Information Available: Table S1, listing proton NMR chemical shifts of Yb^{3+}−daunomycin in methanol-d_4 and D_2O, and Pr^{3+}−, Dy^{3+}−, Eu^{3+}−, and Lu^{3+}−daunomycin in methanol-d_4 (1 page). Ordering information is given on any current masthead page.

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