Interaction of Uranyl Ions with Daunorubicin and Adriamycin

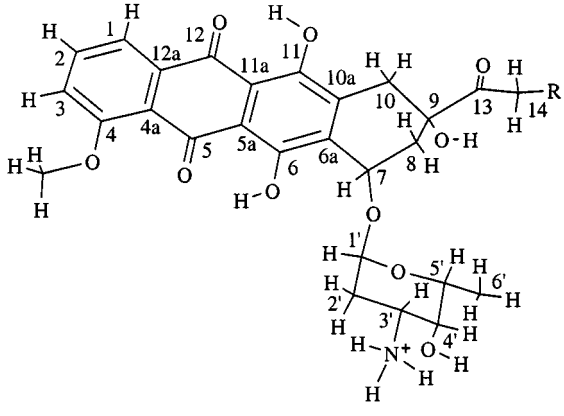
Athanasios Papakyriakou,[a] Angeliki Anagnostopoulou,[a] Arlette Garnier-Suillerot,[b] and Nikos Katsaros*[a]

Keywords: Antibiotics / Antitumor agents / Anthracyclines / O ligands / Uranium

The interaction of the uranyl(VI) ion (UO_2^{2+}) with two anthracyclines, adriamycin (Adr) and daunorubicin (Dnr), has been studied by absorption, circular dichroism, fluorescence and NMR spectroscopy. We have shown that both drugs can form two complexes with UO_2^{2+}, a process strongly dependent on the dielectric constant of the solvent, the metal-to-ligand ratio (R), and the pH. The first complex (**I**) involves coordination of the metal ion to the [C(11)–O$^-$, C(12)=O] chelating site, while the second complex (**II**) involves coordination to the [C(6)–O$^-$, C(5)=O] site. In aqueous solutions at pH 6.5, the formation of complex **I** takes place with two drug molecules per UO_2^{2+} ion. At higher pH or R values, complex **II** forms, and probably has a polymeric structure with one uranyl ion bridging two drug chromophores through both the keto-phenolate sites. In methanolic solutions the simultaneous formation of both complexes **I** and **II** is demonstrated by both CD and NMR spectroscopy. Dissociation of the complexes occurs upon interaction of the drug complexes with native DNA, with the free anthracyclines intercalating between the base pairs of the nucleotide.

Introduction

Adriamycin (Adr, or doxorubicin) and daunorubicin (Dnr, or daunomycin) are two representative members of the anthracycline antibiotic family. These antibiotics contain an anthraquinone chromophore and an amino glycoside sugar (Scheme 1).[1] Despite their severe cardiotoxicity and other side effects, they are both used clinically as chemotherapeutic agents, especially Adr, which exhibits a rather broad spectrum of activity.[2,3] Their antineoplastic activity has been attributed mainly to their strong interaction with DNA, either via an intercalation process of their chromophore into the base pairs of the minor groove, or via free-radical damage of the ribose through the redox cycle of the anthraquinone moiety.[4]



Scheme 1. Structures of daunorubicin (R = H) and adriamycin (R = OH) based on the crystal structure of Dnr[1]

Complexation of anthracyclines by metal ions has been suggested to provide less cardiotoxic compounds,[5,6] while their toxicity has also been related to the formation of reactive oxygen species generated by redox cycling of anthracycline metal complexes.[7,8] The uranyl ion has been used as a staining agent in electron microscopy,[9,10] mainly due to its high affinity for the phosphates of the DNA backbone.[11,12] In an effort to gain more information on the biological mechanisms of the action of anthracyclines we examined the interaction of UO_2^{2+} ion with both Adr and Dnr.

Results

The behavior of both daunorubicin (Dnr) and adriamycin (Adr) in the presence of uranyl (UO_2^{2+}) ions is almost identical. Therefore, we depict only the most characteristic results for either Dnr or Adr, indicating the potential differences for each case. We have also used the same symbols when referring to the corresponding complexes formed with the two drugs.

Formation of the Complexes in Aqueous Solution

Addition of uranyl nitrate to buffered solutions of the drugs at pH 6.5 resulted in a change of color from orange

[a] NCSR "Demokritos", Institute of Physical Chemistry, 15310 Agia Paraskevi Attikis, Athens, Greece
Fax: (internat.) + 30-1/651-1766
E-mail: katsaros@mail.demokritos.gr
[b] Chimie Bioinorganique LPBC CNRS ESA 7033, Univeristè Paris Nord, 74 rue Marcel Cachin, 93017 Bobigny Cedex, France
E-mail: garnier@lpbc.jussieu.fr

  1434–1948/02/0505–1146 $ 20.00+.50/0 *Eur. J. Inorg. Chem.* **2002**, 1146–1154

to purple. Further addition of the metal ion gradually changed the color of the solutions to deep violet and then to blue, while a precipitate was formed. In order to characterize the species formed at this pH, titration of the drugs with increasing quantities of the metal was monitored by both absorption and CD spectroscopy. In the visible region of the absorption spectra, two new bands appeared at around 560 and 605 nm, while the main band of the free drug at 480 nm decreased in intensity. Two clear isosbestic points were observed at 408 and 529 nm (Figure 1) for solutions with a metal-to-ligand molar ratio (R) of up to 0.5. Upon further addition of metal, the two new bands were further shifted to 575 and 625 nm, and the isosbestic points were replaced by two other points at 426 and 572 nm, which were present up to R = 1.5 (Figure 2).

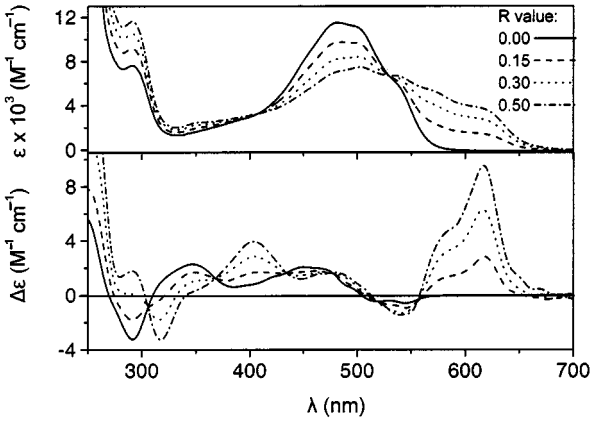


Figure 1. UV/Vis absorption (upper) and CD (lower) spectra of Adr and UO_2^{2+} in aqueous solutions at R < 0.5; [Adr] = 0.1 mM, Hepes buffer 0.01 M, pH = 6.5, NaCl = 0.01 M; the spectra were recorded 10 min after the addition of the metal ion at room temperature

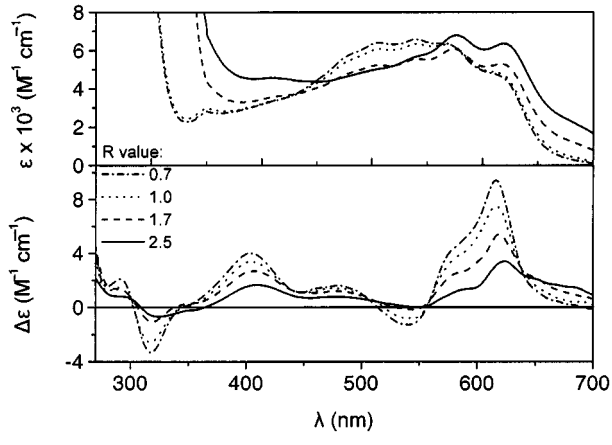


Figure 2. UV/Vis absorption (upper) and CD (lower) spectra of Adr and UO_2^{2+} in aqueous solutions at R > 0.5; [Adr] = 0.1 mM, Hepes buffer 0.01 M, pH = 6.5, NaCl = 0.01 M; the spectra were recorded 10 min after the addition of the metal ion at room temperature

The CD spectra of both drugs exhibited very characteristic changes upon addition of UO_2^{2+} at pH 6.5. The negative band at 290 nm decreased in intensity and moved to 317 nm. The positive band at 330 nm exhibited a red shift to 405 nm, and increased in intensity, while the 350 nm band also decreased in intensity and appeared as a shoulder at the same wavelength. In the visible region the band centered at 480 nm shifted by about 10 nm to higher wavelength, while the negative band at 540 nm increased in intensity (in the case of Adr, Δε increased in intensity almost threefold). Additionally, a band around 625 nm appeared with quite a high hyperchromic effect (Δε = 9.1 for Dnr at 626 nm and 9.5 for Adr at 618 nm) and a shoulder at about 585 nm. These changes occur with isodichroic points at 305, 368, 434, and 477 nm up to R = 0.5 (Figure 1). Further addition of UO_2^{2+} resulted in the hypochromicity of all the above bands, with isodichroic points at 343, 559, and 637 nm up to R = 1.5. At R > 2 a new band became visible at around 685 nm, while the bands at 350 and 317 nm gradually shifted by about 10 nm to higher wavelengths. At R = 3.0 the negative band at 535 nm had almost become positive (Figure 2).

In the solutions with R higher than two, a precipitate began to form after a couple of hours. This blue-violet precipitate was insoluble in either polar or nonpolar solvents. It readily dissolved in dilute HCl, accompanied by a color change to orange (probably due to dissociation to the free drug). Similar observations with such precipitates have been made previously with the Cu^{2+} [13] and Yb^{3+} [14] anthracycline complexes. In these cases, the authors suggested the formation of polymeric chain structure complexes, with the metal ion bound at both the keto-phenolate sites (C5/C6 and C11/C12) of two (or more) drug molecules.

The plots of ε and Δε versus R (molar ratio method, Figure 3) indicate the formation a 1:2 (metal:drug) complex at R ≈ 0.5 (**I**). This conclusion is supported mainly by the plot of ε at 575 nm, which is parallel to the *x*-axis up to R = 1.5. The plot at 625 nm exhibits a break point at R = 0.5 and continues to increase up to R = 1.5. The dependence of the CD bands upon the R value is also shown clearly in Figure 3b: the characteristics of the 1:2 complex fade out upon increasing R to above 0.5 (see Discussion). The 1:2 stoichiometry for complex **I** is also supported by the continuous variation method[15] (Job's method) applied to solutions with a total concentration of 50 μM. As shown in Figure 4a there is a clear maximum at a molar fraction of Dnr equal to 0.65 that corresponds to this stoichiometry.

The CD band at 625 nm has been used to monitor the pH dependence of the 1:2 species formed. As shown in Figure 4b, the complexation begins at pH > 4.5 and is completed at pH 6.5, a point above which the intensity of the band decreases. Furthermore, in solutions at pH higher than 7.5, a colored precipitate was formed. From this plot we can estimate that the pK_a for the complex formation is about 5.6, a value that is the same for both drugs.

The kinetics of the formation of the 1:2 species (complex **I**) is quite fast, whereas the intensity of the absorption band takes around 10 minutes to reach the maximum value. Both complexes are relatively stable, especially that of Dnr, which only dissociated by about 5% after four days at room temperature in the dark. Under the same conditions, the complex with Adr dissociated significantly more (about 35% as

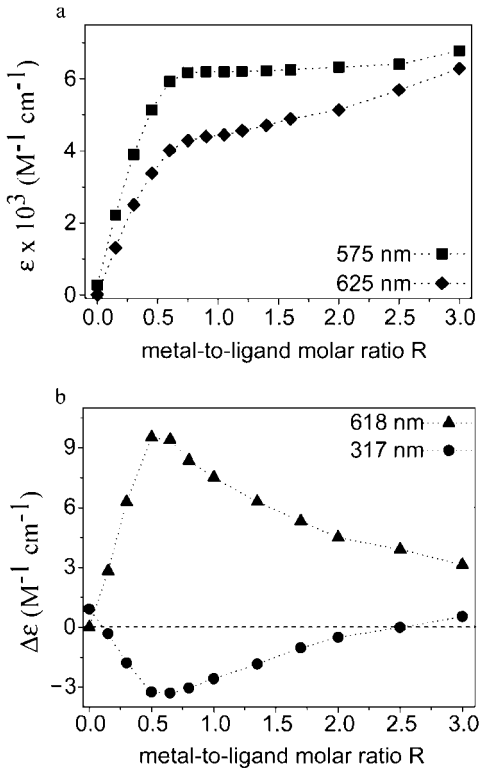


Figure 3. (a) Plots of ε at 625 and 575 nm and (b) Δε at 618 and 317 nm versus R (molar ratio method), of Adr titrated with UO_2^{2+}; experimental conditions as in Figure 1 and 2

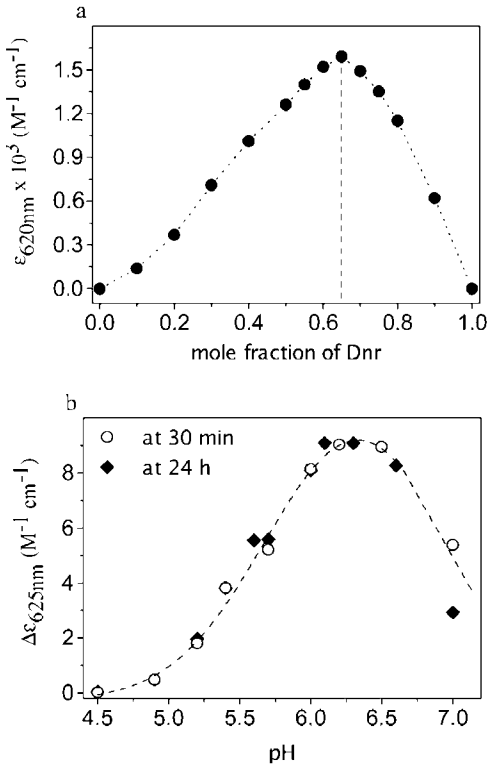


Figure 4. (a) Job's plot for Dnr 50 μM in Hepes buffer 0.01 M, NaCl 0.01 M, pH = 6.5 and (b) the pH dependence of the 1:2 UO_2^{2+}:Dnr complex monitored by the Δε at 625 nm; the pH of the solutions was measured just prior to the spectra acquisition, 30 min and 24 h after the addition of aliquots of NaOH (0.025 M) to solutions of Dnr 0.1 mM, with UO_2^{2+} 0.05 mM and NaCl 0.01 M; the dashed line is an empirical fit of the former data set

measured by the decrease in the intensities of the visible absorption bands).

The complexation with uranyl ions quenched the fluorescence of Dnr and Adr (Figure 5), as has been reported for most of the metal ions studied[16] (except the complex of Al^{3+} and Adr[17]).

From the decrease in the fluorescence intensity at λ_{max} = 555 nm (excitation at 480 nm) we have estimated the association constants K_s for the formation of the 1:1 species. Using Equation (1),[18] where F_o is the fluorescence intensity before the addition of the quencher, and F is the fluorescence intensity at concentration [Q] of uranyl ions, we obtained the linear plots shown in Figure 5. Their slope corresponds to the K_s values, calculated to be 25 mM^{-1} for Dnr and 14 mM^{-1} for Adr. At this anthracycline concentration (5μM) and up to an added [UO_2^{2+}] with R = 10, the linearity of these plots (> 0.997) indicates the formation of a species with a stoichiometry equal to 1:1.

$$F_o/F = 1 + K_s[Q] \quad (1)$$

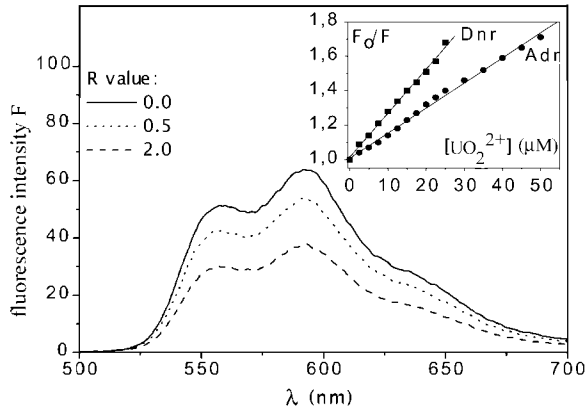


Figure 5. Uncorrected fluorescence spectra of Dnr and UO_2^{2+} at different R values recorded after 24 h (excitation wavelength at 480 nm); [Dnr] = 5 μM in Hepes buffer 0.01 M, pH = 6.5, NaCl 0.01 M; insert: plots of Fo/F at 555 nm versus the uranyl concentration added to solutions of the drugs made as above; the measurements were taken 1 min after the addition of the metal from a solution of [UO_2^{2+}] = 0.5 mM

Formation of the Complexes in 50% Methanolic Solutions

The complexation of UO_2^{2+} with Dnr and Adr was also studied in 50% (v/v) methanol/water solutions in order to examine the dependence of this process upon the aggregation state of the drugs. As has already been reported,[19] under such experimental conditions these compounds exist fully in the monomeric form, a fact that is reflected by the disappearance of the doublet centered at 480 nm in the CD spectra of the free anthracyclines, exhibiting only a positive band. The absorption spectral changes in the visible region of both drugs upon addition of uranyl (Figure 6) are very similar to those in pure water, with the main band at 480 nm decreasing in intensity and giving rise to the appearance of two new bands at 565 and 610 nm. These two

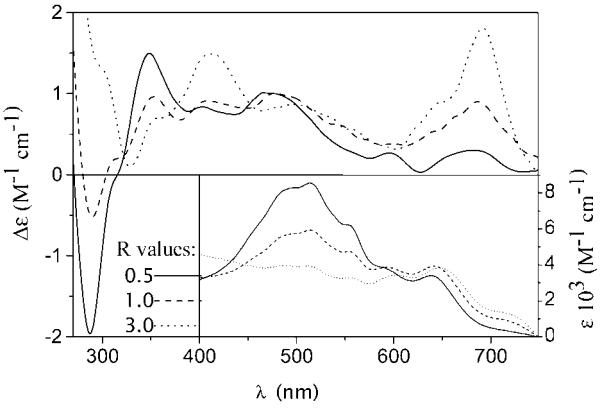


Figure 6. CD (upper) and visible absorption (lower) spectra of Adr and UO_2^{2+} in 50% MeOH solutions at different R values; [Adr] = 0.1 mM, Hepes buffer 0.01 M, pH = 6.5, NaCl 0.01 M

bands gradually shift by about 10 nm to lower energy when more metal is added (R > 2).

In contrast, the CD spectra exhibit much more informative changes following the addition of the metal ion. The negative peak at 290 nm readily decreased in intensity, and above R = 2 shifted to 330 nm. The positive band around 320 nm moved to 410 nm from an early stage of the titration (R ≈ 0.5) increasing in intensity, whereas the band at 350 nm gradually decreased in intensity and energy, shifting to 360 nm for R > 2. An intense red shift of the main band at 480 nm was also observed, yielding a broad band ranging from 600 to 700 nm. At low R values this band consists of a sharper band at 600 nm and a broader band centered at 660 nm, while at R > 2 the low energy band is formed at 675 nm and the high at 625 nm. At these high R values, a precipitate also formed with the same characteristics as those of the respective precipitates in pure aqueous media. There is no evidence for stacking of the drug molecules throughout the titration, at least as far as the spectra are concerned, which do not exhibit any negative band in the 530 nm region.

These changes indicate the formation of the same species as those in aqueous solutions, but with no clear step-points. Complex **I** (through the C11/C12 site) formation probably occurs along with complex **II** (through both the C11/C12 and C5/C6 sites), a possibility that is attested to by the appearance of a broad band at 600 to 700 nm from an early stage of the titration with the metal ion. Thus, the stoichiometry of both species could not be determined, since neither the molar ratio method nor the Jobs plot exhibited any clear break point. The intensity and the position of these CD bands can only support the conclusion that at low R values, complex **I** seems to be present in a higher concentration, while at higher R values (> 1) complex **II** begins to prevail, forming oligomers and polymers like those in pure water.

NMR Experiments in Methanol

Addition of UO_2^{2+} to aqueous solutions of Dnr and Adr in D_2O at pD > 5 resulted in an extensive line broadening of the acquired NMR spectra, therefore making the assignment of the peaks impossible. In order to examine the complexation process we performed NMR experiments in [D_4]methanol. Up to R = 10 one major complex was being formed (complex **I**), while increasing the R value above 20 resulted in the formation of another complex (complex **II**), which appears at lower fields. These two species are both present in the solution along with the free anthracycline, even at a hundredfold excess of UO_2^{2+} (Figure 7). The signals from the free anthracycline are still well resolved, suggesting that the drug is in slow exchange with its metal-bound form on the NMR time scale. The assignment has been successfully accomplished from the 800 MHz ^{1}H NMR spectra of the drugs at 10 mM concentration with UO_2^{2+} in excess (1 M). We have also performed 2D ^{1}H-^{13}C HMQC and HMBC experiments in order to assign the carbons of the anthracyclines and their complexes with UO_2^{2+}.

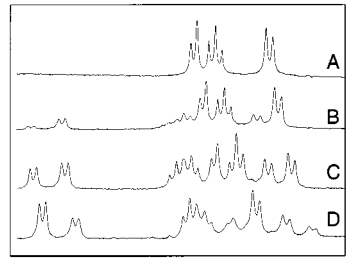


Figure 7. Expanded aromatic region of the 250 MHz ^{1}H NMR spectra of Dnr 10 mM titrated with UO_2^{2+} at R = 0 (A), 20 (B), 50 (C), and 100 (D)

Table 1 contains the ^{1}H NMR chemical shifts of free Dnr along with the chemical shifts of the two complexes formed and the uncomplexed Dnr present in the solution. From the peak intensities at R = 100 we can calculate the presence of the three species in the molar ratios of 2:3:5 (Dnr/complex **I**/complex **II**). The uncomplexed drug's protons present in these solutions exhibit a general upfield shift (0.1−0.2 ppm) upon addition of UO_2^{2+}. Complex **I** exhibits characteristic downfield shifts for H-1 (Δδ = 0.77 ppm) and for both H-10 protons (0.67 and 0.47 ppm for the equatorial and the axial proton, respectively). All other protons are also shifted downfield by 0.1−0.2 ppm. These observations indicate the complexation of Dnr to UO_2^{2+} through the [C(11)−O$^-$, C(12)=O] chelating site, given that the closest protons to C(11)−O$^-$ and C(12)=O are H-10 and H-1, respectively. Proton signals from complex **II** protons are shifted further downfield than those of complex **I** by 0.1−0.2 ppm, except H-1′, H-7, and 4-OCH$_3$, which exhibit rather large downfield shifts (0.84, 0.94, and 0.37 ppm, respectively, compared to free Dnr). These changes indicate the presence of a second UO_2^{2+} ion bound at the [C(6)−O$^-$, C(5)=O] donor site, since H-7, which shows the greatest downfield shift in complex **II** is the proton closest to C(6)−OH, while the 4-OCH$_3$ protons are less influenced by C(5)=O. As far as H-1′ is concerned, its rather large downfield shift in complex **II** reflects the change in the chemical environment around the sugar moiety upon

Table 1. ^{1}H chemical shift values (ppm) of Dnr and UO_2^{2+} in [D_4]methanol measured at 800 MHz and 25 °C; [Dnr] = 10 mM, [UO_2^{2+}] = 1 M; values in parenthesis are the calculated differences between the chemical shift values of the complexes and the respective uncomplexed drug present in the solutions; due to their signal overlapping, H-8 and H-2′ chemical shift values are given with lower accuracy

Protons	Free Dnr	Uncomplexed	Complex I	Complex II
H1	7.92	7.74	8.51 **(+0.77)**	8.67 **(+0.93)**
H2	7.83	7.65	7.88 (+0.24)	7.96 (+0.31)
H3	7.56	7.35	7.48 (+0.13)	7.63 (+0.28)
H1'	5.45	5.37	5.45 (+0.08)	6.21 **(+0.84)**
H7	5.06	4.96	5.10 (+0.14)	5.90 **(+0.94)**
H5′	4.31	4.21	4.26 (+0.05)	4.41 (+0.20)
4OCH$_3$	4.03	3.88	3.95 (+0.07)	4.25 **(+0.37)**
H4′	3.67	3.69	3.71 (+0.02)	3.74 (+0.05)
H3′	3.59	3.60	3.64 (+0.04)	3.68 (+0.08)
H10$_{eq}$	3.01	2.93	3.60 **(+0.67)**	3.66 **(+0.73)**
H10$_{ax}$	2.93	2.80	3.27 **(+0.47)**	3.47 **(+0.67)**
H14	2.36	2.26	2.38 (+0.12)	2.46 (+0.20)
H8$_{eq}$	2.32	~2.1	~2.3 (+0.2)	~2.5 (+0.4)
H8$_{ax}$	2.16			
H2′$_{ax}$	2.04	~1.8	~1.9 (+0.1)	~2.0 (+0.2)
H2′$_{eq}$	1.88			
5′CH$_3$	1.29	1.21	1.23 (+0.02)	1.33 (+0.12)

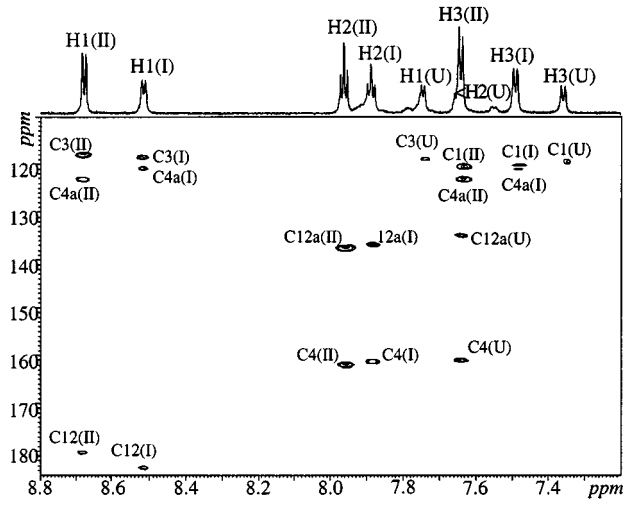


Figure 8. 800 MHz 2D HMBC ^{13}C-^{1}H NMR spectra of Dnr in the presence of a hundredfold excess of UO_2^{2+}; [Dnr] = 10 mM and [UO_2^{2+}] = 1 M; (U): uncomplexed, (I): complex **I**, (II): complex **II**

Table 2. ^{13}C chemical shift values (ppm) of Dnr and UO_2^{2+} in [D_4]methanol measured at 800 MHz and 25 °C; [Dnr] = 10 mM, [UO_2^{2+}] = 1 M; (–): signal not detected

Carbon	Free Dnr	Uncomplexed	Complex I	Complex II
C13	212.4	–	–	–
C5	187.3	187.0	184.7	181.7
C12	186.9	186.6	183.6	180.3
C4	161.5	161.3	161.5	162.2
C6	156.5	156.3	141.2	141.0
C11	155.2	155.0	143.9	143.8
C9	152.5	–	–	–
C2	136.3	–	–	–
C12a	135.4	135.2	137.1	137.9
C4a	134.9	134.6	121.2	123.5
C6a	134.8	134.5	–	–
C1	119.5	119.6	120.9	120.6
C3	119.3	119.3	118.9	118.5
C5a	111.4	–	–	–
C11a	111.2	–	–	–
C1′	100.1	–	–	–
C10a	76.2	–	–	–
C7	70.8	70.4	71.0	71.3
C5′	66.9	66.9	–	–
C4′	66.8	66.7	–	–
4OCH$_3$	56.1	56.0	56.3	56.6
C3′	48.0	47.7	–	–
C8	36.0	35.5	35.7	36.2
C10	32.1	32.1	33.6	34.0
C2′	28.5	28.1	28.2	28.3
C14	23.4	23.7	23.8	23.9
5′CH$_3$	15.9	15.6	15.9	16.1

binding the second UO_2^{2+} ion. We have no indications for participation of the amino group in the complexation process, since no other proton of daunosamine exhibits any significant chemical shift upon addition of the metal ion.

From the 2D ^{13}C-^{1}H NMR experiments (Figure 8) we have managed to assign all the carbons of the free anthracyclines and most of the carbons of the complexes formed (Table 2). Both complexes exhibit remarkable changes in the ^{13}C chemical shifts of the keto-phenolate sites (3−15 ppm upfield shifts), especially those of C(6) and C(11) due to delocalization of the negative charge of the phenolic oxygen atoms upon complexation. This effect was expected to be more striking for C(5a) and C(11a), but this was not the case. None of the other ^{13}C chemical shifts exhibit significant changes (< 2 ppm). Unlike the ^{1}H chemical shifts, the carbons of both keto-phenolate sites in complexes **I** and **II** are similarly influenced by the presence of the metal ion, probably due to ring-current effects.

In the UV/Vis and CD spectra of this system (R = 100) two new bands appear in the absorption spectra at 575 and 620 nm, while the CD spectra is characterized by a red shift of the main band from 480 nm to 610 and 650 nm. It is quite evident from the bands exhibited that the species formed in methanol must be the same as in semi-aqueous MeOH solutions, despite the fact that in the first case the formation constants of the complexes are quite low and a higher excess of UO_2^{2+} is required for the respective changes to take place.

Interaction of the Complexes with DNA

Two different sets of experiments were carried out with native DNA in aqueous solutions. In the first set, a uranyl ion solution was added to a mixture of anthracyclines-DNA in solution at different r values (r = drug-to-nucleotide molar ratio). At r < 0.1, where total binding of the drugs

has been reported,[20] the addition of uranyl to solutions containing the DNA-bound drugs did not cause any changes in the CD spectra, even when a tenfold excess of UO_2^{2+} was added. On the other hand, addition of uranyl to the DNA-bound Dnr and Adr at $r > 0.3$ resulted in almost the same CD spectral features as in the case of complex **I**, probably because of the complexation of uranyl with the free anthracycline molecules. This observation is in agreement with the data reported for the Cu^{II}-Dnr system at $r > 0.1$.[21]

In the second set of experiments a mixture of drug-UO_2^{2+} solution was added to DNA solutions at several r values. In this case the 1:2 complex **I** was formed with both Dnr and Adr. In the presence of DNA, the complexes dissociated in a time-dependent procedure as free Dnr and Adr intercalated between the nucleotide base pairs. This is evident from the CD spectra of a typical experiment at $r = 0.1$ (Figure 9) where the band at 625 nm, associated with the complex decreases in intensity with time, while the negative band at 320 nm red shifts by approximately 10 nm. The characteristic spectra of the intercalated anthracyclines were obtained after 24 h, with bands at around 310, 380, and 500 nm.[22] The half-life of this process was estimated from the plot of $\Delta\varepsilon$ at 626 nm versus time to be 15 min, while for the Fe^{III}-Dnr system it was reported to be 18 h.[23] In the case of Adr, when concentrations above 0.1 mM were used, precipitation occurred, with the precipitate probably containing DNA and the metal-Adr complex, as it indicated by the color of the precipitate, which is the same as the deep-violet solution's color when R is greater than 2 or the pH is greater than 7. This observation is in accordance with the results already reported for Adr complexes with other metal ions.[24]

We have also measured the melting temperature (*T*m) of calf thymus DNA in the presence of both anthracyclines and their 1:2 UO_2^{2+} complexes. The values measured were dependent on r, and were in agreement with those already reported for the Dnr-DNA system,[20] taking into account the variations of the ionic strength. The Tm values in the presence of the complexes were only about 1 °C lower than the respective values for the free drug. Additionally, when the absorbance at 620 nm was monitored while increasing the temperature from 25 to 95 °C for solutions of the complexes in the absence of the DNA, we observed that at 55 °C the complexes had dissociated by about 50%. It is known that UO_2^{2+} solutions are only stable at low pH and that polynuclear complexes and uranyl hydroxide precipitates are formed at higher pH.[25] Thus, under our experimental conditions the released metal ions were bound to hydrolyse. It is not impossible, however, that some may bind to the phosphates, since it has been shown that their affinity for nucleotides is high and it has been proposed that UO_2^{2+} may bind by bridging phosphate groups on opposite strands of the minor groove of DNA.[12]

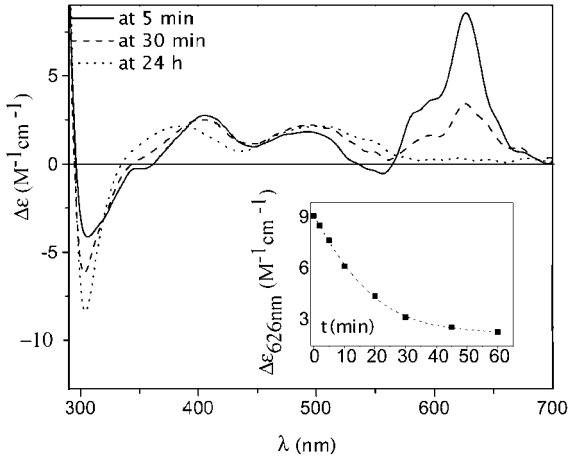


Figure 9. CD spectra of (UO_2^{2+}-Dnr_2)-DNA system as a function of time; [UO_2^{2+}-Dnr] = 50 μM, [DNA] = 0.5 mM, Hepes buffer 0.01 M, pH = 6.5, NaCl 0.01 M; insert: plot of $\Delta\varepsilon$ at 626 nm versus time elapsed after the addition of the nucleotide to the complex solution

Discussion

Our experience during the course of these studies with metal ion complexes of anthracyclines has shown the complexity of these systems.[26] Many conflicting reports have been published over the past 30 years, concerning either the stoichiometry of the complexes formed in solutions or their physicochemical characteristics. The formation of more than one species is evident for most of the metal ions examined, the predominance of which depends on the metal-to-ligand molar ratio, the pH, and the dielectric constant of the solvent. However, spectroscopic analysis, especially the CD spectra, can provide many detailed characteristics of the complexes formed,[27] while NMR spectroscopy has also proved to be a powerful tool for the study of these systems.[14,28]

In pure water and under our experimental conditions most anthracyclines exist in the fully protonated and dimeric state.[19,29] The formation of a major species with a stoichiometry of 1:2 (metal-to-drug) up to R = 0.5 is confirmed by both the clear isosbestic points in the absorption and CD spectra (Figure 1), as well as by the molar ratio and Job's method (Figure 3 and 4). The bathochromic shifts of the visible absorption and CD spectra bands show that complexation takes place to the drug's chromophore after deprotonation of either the C(11) or C(6) phenolic groups, in order of acidity.[19] The position of the absorption bands at 560−600 nm can account for the deprotonation of the first phenolic group[27] and coordination to UO_2^{2+} through the oxygens of the [C(11)−O^-, C(12)=O] donor site, forming a six-member chelate ring. In contrast, the red shift of the UV band from 290 to 320 nm in the CD spectra is characteristic of the deprotonation of the second phenolic group at C(6).[27]

A more detailed examination of these spectra reveal that this is not the case, however, since neither the red shift for the band at 350 nm nor the bathochromic shift of the main visible band from 450 to about 620 nm can account for this

deprotonation. Additionally, the intensity of the band at 317 nm is exactly the same as the original band at 290 nm, while C(6)−OH deprotonation is followed by a decrease in the intensity of this band.[27] Such a decrease in the energy of the $\pi\rightarrow\pi^*$ transitions along the sort axis of the chromophore has previously been observed in the case of Sn^{IV} with Adr.[30] The authors saw no evidence for participation of the phenolic groups, despite the fact that a shift of the band at 290 nm to higher wavelengths accompanied the complexation of Adr to Sn^{IV}. For this reason, it has been suggested that this occurs due to the aggregation of the drug molecules as in the case of Adr gels, for which the proposed structure is a supramolecular aggregate with Adr molecules stacked helically.[31,32] Additionally, when anthracyclines intercalate between the base pairs of DNA this band shifts to 305 nm. This observation can be attributed to a modification of the angle between the sugar moiety and the dihydroanthraquinone part, along with the influence of the hydrogen bonds between the C=O groups and their neighbors. This was confirmed by the crystal structure of Dnr complexed to d(CGTACG), in which the torsion angle around the O(7)−C(1′) bond was found to be 20° different from that of the free drug.[34] In our case the formation of the 1:2 species upon binding of UO_2^{2+} to Dnr and Adr at the [C(11)−O^-, C(12)=O] site probably promotes such a modification.

Upon increasing the metal-to-ligand ratio, the characteristic bands mentioned above decrease in intensity (Figure 2) and at $R > 2$ the appearance of a rather low-energy band at around 685 nm (shoulder), along with the continuous red shift of the band at 350 nm towards 360 nm, indicate further deprotonation of Dnr and Adr at C(6)−OH. As soon as UO_2^{2+} ions bind additionally at the [C(6)−O^-, C(5)=O] site, a precipitate was gradually formed from the blue solutions of the drugs. After 24 h Dnr and Adr have probably formed a polymeric complex, with UO_2^{2+} ions chelating two drug molecules through both the keto-phenolate sites of their chromophore. This has already been reported in the case of Cu^{2+} ions binding to Dnr and Adr,[13] and later in the case of Yb^{3+} binding to Adr,[14] both at high pH. Such a precipitation can occur with UO_2^{2+} at lower R values (< 2) when increasing the pH above 7, indicating that this process is strongly dependent on both the metal-to-ligand ratio and the proton concentration.

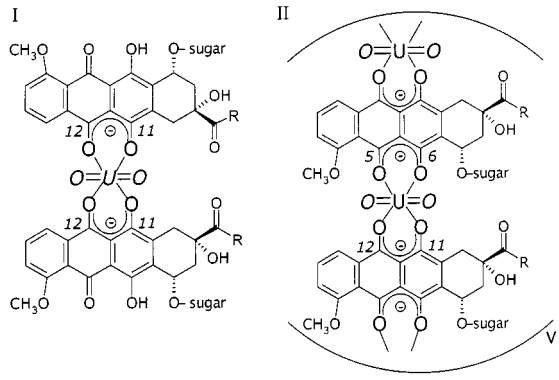
Decreasing the dielectric constant of water by adding 50% MeOH, at $R = 0.5$ at the same pH and ionic strength, resulted in significantly different CD features. First, no stacking interactions occur, as indicated by the absence of the negative band at 530 nm, while the UV band at 290 nm did not shift to higher wavelengths, but rather decreased in intensity. Although the absorption spectra exhibit the same characteristic bands at 565 and 610 nm as in the case of pure water, the CD spectra reveal the appearance of a band centered at 660 nm, which at $R = 3.0$ increased in intensity with a maximum at 675 nm (Figure 5). At this point, the band at 290 nm has red shifted to about 330 nm and the bands at 320 nm and 350 nm have shifted bathochromically to 410 nm and 360 nm, respectively. Therefore, the deprotonation of both the phenolic groups at C(11) and C(6) is demonstrated, a process that probably does not occur step-by-step, as is indicated by the appearance of a low energy band at around 660 nm even at low R values.

The parallel formation of more than one species is also supported by the NMR experiments in pure methanol. Up to $R = 10$ the formation of complex **I** took place with UO_2^{2+} binding to Dnr and Adr through the [C(11)−O^-, C(12)=O] chelating site, while at higher R ratios, complex **II** was also formed with participation of the [C(6)−O^-, C(5)=O] coordination site. The presence of both species is evident even under a very large excess of the metal ion, although complex **II** predominates at $R > 50$ (Figure 6). The participation of the amino group, as in the case of the Adr complexes of Pd^{II}, Pt^{II},[15,28] and Sn^{IV},[30] is excluded by the ^1H NMR chemical shifts of the daunosamine protons, which do not exhibit any significant change upon complexation to UO_2^{2+}, except the equatorial proton H-1′ in complex **II** (Table 1). This should also be true for the semi-aqueous MeOH solutions at $pH = 6.5$, where a gradual change of the CD spectra from the fully protonated to the fully deprotonated Dnr and Adr takes place as more UO_2^{2+} is added. No clear step-points were observed for this process, and as soon as both complexes (**I** and **II**) are present simultaneously in the solutions, the determination of their stoichiometry is very inaccurate.

Upon interaction of UO_2^{2+} with Dnr and Adr 1:2 complexes with calf thymus DNA ($r < 0.1$) at $pH = 6.5$, the complexes appeared to dissociate, with the free anthracyclines intercalating between the base pairs. This process is confirmed by the time-dependent change of their CD spectra (Figure 8) to the characteristic pattern of the intercalated drug. Additionally, no remarkable changes have been observed at the Tm values of the calf thymus DNA bound to the anthracyclines in the presence and absence of uranyl ions. This is probably due to the higher affinity of the drugs for DNA and that of uranyl ion for the phosphate groups of the DNA backbone.[12]

Conclusions

Uranyl ions form two complexes with Dnr and Adr, depending on the metal-to-ligand ratio, the pH, and the solvent. Complex **I** involves binding of UO_2^{2+} to the oxygen atoms of the drugs [C(11)−O^-, C(12)=O] donor site, while in complex **II** coordination also occurs through the [C(6)−O^-, C(5)=O] site. In aqueous solutions up to $R = 0.5$ and $pH = 6.5$ complex **I** is formed with the stoichiometry of 1:2 (metal-to-ligand), while at R above 0.5 or $pH > 7$ complex **II** formation takes place with drug molecules bridging two metal ions and forming oligomeric or polymeric structures (Scheme 2). In methanol these two complexes are formed at higher R values (> 10) and are both present in the solution as was shown by the NMR experiments.



Scheme 2. The proposed structures of the complexes formed upon interaction of UO_2^{2+} ions with Dnr [R = $CH(OH)CH_3$] and Adr [R = $CH(OH)CH_2OH$]; for both drugs, *sugar* = daunosamine

Experimental Section

Daunorubicin and adriamycin hydrochloride 99% were a gift from Pharmacia & Upjohn, Greece. Uranyl nitrate hexahydrate [$UO_2(NO_3)_2{\cdot}6H_2O$] (99%) was purchased from Merck, Hepes buffer 99.5% from Aldrich, while [D_4]methanol (99.9%) and calf thymus DNA were purchased from Sigma and used without further purification. Deionized and doubly-distilled water was used throughout the experiments.

Anthracycline solutions were prepared just prior to use and their concentrations determined by their absorption at 480 nm using ε = 11500 $M^{-1}cm^{-1}$.[25] Uranyl stock solutions were prepared in slightly acidified water to avoid hydrolysis. DNA stock solutions were prepared as described previously[34] and kept at 4 °C for no longer than a week. After dilution to the buffer, the nucleotide concentrations were determined by their absorption at 260 nm using ε = 6600 $M^{-1}cm^{-1}$ (expressed as phosphate).

Absorption spectra were recorded on a JASCO V-560 spectrophotometer. Circular dichroism (CD) measurements were made on a JASCO V-715 dichrograph. All spectra were recorded using a 1.0 cm cell and the following acquisition parameters: λ = 250−750 nm, step 0.5 nm, speed 200 nm min^{-1}, response 1.0 s, bandwidth 1.0 nm, accumulations 4−10. Methanol and aqueous buffer blanks were used as references, depending on the experimental conditions, while the temperature was kept constant at 25 °C. Results are expressed as ε (molar absorption coefficient) and $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ (differential molar absorption coefficient). Both ε and $\Delta\varepsilon$ values are expressed as molar concentration of anthracyclines.

All 1D ^1H NMR spectra were recorded either on a Bruker AC 250 MHz spectrometer or on a Bruker Avance 800 MHz spectrometer at 25 °C. The NMR spectroscopic data were transferred to a SGI O2 workstation (IRIX OS) and were processed using the XWIN NMR software (version 2.6, Bruker). A typical NMR sample contained 10 mM anthracycline in [D_4]methanol. ^1H and ^{13}C chemical shifts were referenced to the solvent peak at δ = 3.31 (25 °C).

The ^1H-1D experiments were recorded with presaturation of the H_2O/HDO signal during the recycling delay. Typical parameters used were: 64 K data domain size, 0.5 s recycling delay, 10 KHz spectral width and 64 transients. The 1D FIDs were multiplied by an exponential window function (0.3 Hz line broadening added) and zero filing was performed prior to Fourier transformation.

^1H-Detected heteronuclear multiple-quantum coherence HMQC [35] and ^1H-detected heteronuclear multiple bond correlation HMBC [36] (optimized for long range couplings with a low-pass J-filter to suppress one bond correlations) experiments were acquired at 800 MHz and at 25 °C. Data sets with 2048 × 512 complex points were acquired with a 9 KHz sweep width in the proton dimension and 40 KHz in the carbon dimension. $J_{C\text{-}H}$ was set to 140 Hz and the solvent signal was selectively irradiated for 1.0 s during the relaxation delay period. A total of 32 scans were collected per t_1 increment and the spectra were processed with an exponential weighting function.

Acknowledgments

The authors gratefully acknowledge Prof. Ivano Bertini and the EU Large Scale Facility at the Magnetic Resonance Center (CERM) of the University of Florence for the 800 MHz NMR experiments performed by Massimo Lucci and Dr. Georgios Spyroulias. We would also like to thank Pharmacia & Upjohn, Greece for the generous supply of the anthracyclines and Dr. Georgios Pistolis for the fluorescence measurements.

[1] C. Courseille, B. Busetta, S. Geoffre, M. Hospital, *Acta Crystallogr., Sect. B* **1979**, *35*, 764−767.
[2] F. Arcamone, *Doxorubicin Anticancer Antibiotics*, Academic: New York, **1981.**
[3] G. Mazué, M. Iatropoulos, A. Imondi, S. Castellino, M. Brughera, A. Podestà, P. Della Torre, D. Moneta, *Int. J. Oncol.* **1995**, *7*, 713.
[4] N. R. Bachur, S. L. Gordon, M. W. Gee, *Mol. Pharmacol.* **1977**, *13*, 901−910.
[5] M. Gosalvez, M. F. Blanco, C. Vivero, F. Valles, *Eur. J. Cancer* **1978**, *14*, 1185−1190.
[6] T. J. Lampidis, A. Garnier-Suillerot, H. Tapiero, 78th Annual Meeting of the American Association of Cancer Research, **1987**.
[7] H. Doroshow, *Cancer Res.* **1983**, *43*, 460−472.
[8] L. Gianni, J. L. Zweier, A. Levy, C. E. Myers, *J. Biol. Chem.* **1985**, *260*, 6820−6826.
[9] H. Huxley, G. Zubay, *J. Mol. Biol.* **1960**, *2*, 10.
[10] P. Testillano, M. Sanchez-Pina, A. Olmedilla, M. Ollacarizqueta, C. Tandler, M. Risueno, *J. Histochem. Cytochem.* **1991**, *39*, 1427−1438.
[11] C. Zobel, M. Beer, *J. Biophys. Biochem. Cyt.* **1961**, *10*, 335−346.
[12] P. E. Nielsen, C. Hiort, S. H. Sönnichsen, O. Buchardt, O. Dahl, B. Nordèn, *J. Am. Chem. Soc.* **1992**, *114*, 4967−4975.
[13] F. T. Greenaway, J. C. Dabrowiak, *J. Inorg. Biochem.* **1982**, *16*, 91−107.
[14] X. Wei, L. J. Ming, *Inorg. Chem.* **1998**, *37*, 2255−2262.
[15] P. Job, *Ann. Chim.* **1936**, *6*, 97.
[16] M. M. L. Fiallo, A. Garnier-Suillerot, *Biochemistry* **1986**, *25*, 924−930.
[17] E. Pereira, M. M. L. Fiallo, A. Garnier-Suillerot, T. Kiss, H. Kozlowski, *J. Chem. Soc., Dalton Trans.* **1993**, 455−459.
[18] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Plenum Press, NY **1983**, IX.3, 264−5.
[19] R. Kiraly, R. B. Martin, *Inorg. Chim. Acta* **1982**, *67*, 13−18.
[20] J. B. Chaires, N. Dattagupta, D. M. Crothers, *Biochemistry* **1982**, *21*, 3933−3940.
[21] M. Spinelli, J. C. Dabrowiak, *Biochemistry* **1982**, *21*, 5862−5870.

[22] E. J. Gabbay, D. Grier, R. E. Fingerle, R. Reimer, R. Levy, S. W. Pearce, W. D. Wilson, *Biochemistry* **1976**, *15*, 2062−2070.
[23] H. Beraldo, A. Garnier-Suillerot, L. Tosi, F. Lavelle, *Biochemistry* **1985**, *24*, 284−289.
[24] A. Garnier-Suillerot, *Bioactive Molecules*, Elsevier: Amsterdam **1988**, Vol. 6, Ch. IV, p. 145.
[25] N. N. Greenwood, A. Earnshaw, *Chemistry of the Elements*, Pergamon Press: Oxford **1986**, p. 1478.
[26] M. M. L. Fiallo, A. Garnier-Suillerot, B. Matzanke, H. Kozlowski, *J. Inorg. Biochem.* **1999**, *75*, 105−115.
[27] M. M. L. Fiallo, H. Tayeb, A. Suarato, A. Garnier-Suillerot, *J. Pharm. Sci.* **1998**, *87*, 967−975.
[28] T. Allman, R. E. Lenkinski, *J. Inorg. Biochem.* **1987**, *30*, 35−45.
[29] I. J. McLennan, R. E. Lenkinski, Y. A. Yanuka, *Can. J. Chem.* **1985**, *63*, 1233−1238.
[30] E. Balestrieri, L. Bellugi, A. Boicelli, M. Giomini, A. M. Giuliani, M. Giustini, L. Marciani, P. J. Sadler, *J. Chem. Soc., Dalton Trans.* **1997**, 4099−4105.
[31] M. Giomini, A. M. Giuliani, M. Giustini, E. Trotta, *Biophys. Chem.* **1991**, *39*, 119−128.
[32] M. Giomini, A. M. Giuliani, M. Giustini, E. Trotta, *Biophys. Chem.* **1992**, *45*, 31−40.
[33] A. H. Wang, G. Ughetto, G. J. Quigley, A. Rich, *Biochemistry* **1987**, *26*, 1152−1163.
[34] E. Tselepi-Kalouli, N. Katsaros, *J. Inorg. Biochem.* **1989**, *37*, 271−282.
[35] A. Bax, R. H. Griffey, B. L. Hawkins, *J. Magn. Reson.* **1983**, *55*, 301.
[36] A. Bax, M. F. Summers, *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.

Received August 8, 2001
[I01314]