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# Structure-Dependent Complexation of $Fe^{3+}$ by Anthracyclines. 2. The Roles of Methoxy and Daunosamine Functionalities

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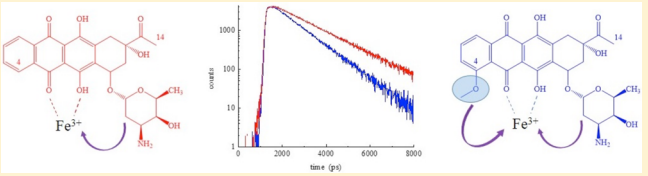
**ABSTRACT:** We have investigated the effects of the methoxy and daunosamine sugar moieties on the stability of anthracycline complexes with $Fe^{3+}$ in aqueous solution. Idarubicin and daunorubicin are structurally very similar, differing only by the presence of the methoxy substituent at the 4-position. We demonstrate that the methoxy group interacts sterically with the proximal quinone oxygen atom and this interaction affects the stability of the anthracycline−iron(III) complex. A similar steric effect is seen for the pendent daunosamine moiety. Free daunosamine in solution does not show any significant interactions with iron(III), whereas the pendent daunosamine functionality destabilizes anthracycline−iron(III) complex formation. This body of information illustrates the important role of methoxy and daunosamine moieties in anthracycline−iron(III) complex formation and stability.



## INTRODUCTION

Daunorubicin and idarubicin are two structurally similar anthracyclines that find use in the treatment of cancer. These two compounds differ only at the 4-position of the "D" ring, where daunorubicin has a methoxy substituent and idarubicin does not (Figure 1).[1] The absence of the methoxy group makes idarubicin more lipophilic, enabling it to penetrate deeper into the plasma membrane bilayer than daunorubicin.[2] The presence of the methoxy functionality influences the bioactivity of the anthracyclines as well. Daunorubicin and idarubicin are used to treat different types of cancers,[3] and these two compounds exhibit different rates of cellular uptake.[1] The spectral properties of these compounds are well-known and are very similar.[4]

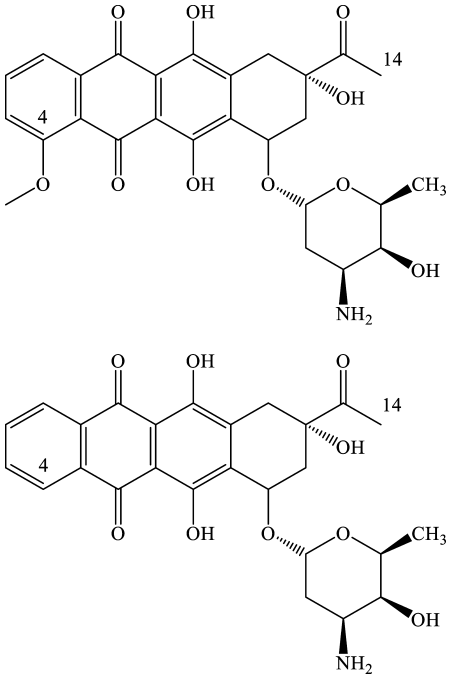


**Figure 1.** Structures of daunorubicin (top) and idarubicin (bottom).

All anthracyclines have a daunosamine sugar moiety attached to the "A" ring (Figure 1). This pendent group plays an important role in making anthracyclines water-soluble and in increasing their affinity toward DNA.[5,6] The major drawback to the use of anthracyclines is their characteristic propensity to induce chronic cardiomyopathy and congestive heart failure.[1] The cardiotoxicity of this family of compounds has been linked to their affinity for complexation of $Fe^{3+}$. The mechanism of the cardiotoxic action remains to be understood fully, but it is known to be related to the disturbance of iron homeostasis.[7]

The nature of anthracycline−iron binding has been examined extensively,[8−10] and we have reported on the kinetics of formation and longer-term stability of the daunorubicin−iron complex in the preceding paper.[11] The goal of this study is to investigate the effects of the methoxy group and daunosamine sugar moiety on anthracycline−iron complex formation. Gianni et al. suggest that the amino group of daunosamine together with the −OH group at the 14-position of doxorubicin might act as a bidentate ligand for iron complexation.[12] In the work presented here we have focused on daunorubicin and idarubicin. Both compounds lack a hydroxyl group at the 14-position, allowing us to study the effect of daunosamine on iron complex formation absent the potentially complicating role of that hydroxyl group. The effect of the hydroxyl group at the 14-

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position on the stability of the iron complex was investigated by us recently.[11] In this work we focus on the role(s) of the methoxy and daunosamine moieties in anthracycline−iron complexation. Our data point to the steric mediation of iron−anthracycline complexation, suggesting the ability to control this potentially adverse chemical process in chemotherapeutic applications.

## MATERIALS AND METHODS

**Chemicals.** Daunorubicin hydrochloride (>99% purity) and idarubicin hydrochloride (>99% purity) were obtained from Selleck Chemicals and used without further purification. Ferrous sulfate heptahydrate was ordered from J. T. Baker Chemicals. Methyl β-L-daunosaminide hydrochloride, HEPES buffer (>99.5% purity), and sodium hydroxide were obtained from Sigma-Aldrich. Sodium acetate (>99% purity) was purchased from Columbus Chemical Industries, Inc. Hydrogen peroxide (15% aqueous solution) was purchased from Rocky Mountain Reagents. All solutions were prepared using Milli-Q water. The concentrations of daunorubicin and idarubicin were determined spectrophotometrically using $\varepsilon_{480} = 1.15 \times 10^4\ M^{-1}\ cm^{-1}$ and $\varepsilon_{475} = 1.20 \times 10^4\ M^{-1}\ cm^{-1}$, respectively.[4] The concentration of ferrous ions in solution was determined indirectly after oxidation with hydrogen peroxide. The absorbance of $Fe^{3+}$ was measured at 340 nm.[13] A 100 μM solution of daunorubicin was aged for 7 days in an amber glass bottle to allow for hydrolytic cleavage of the daunosamine moiety from the anthracycline ring structure. That solution was used without further treatment.

**Time-Correlated Single-Photon-Counting (TCSPC) Instrument.** Fluorescence lifetime and anisotropy data were acquired using a time-correlated single-photon-counting (TCPSC) instrument that has been described in detail previously,[14] and we provided a brief overview of the salient properties of this system in the preceding paper.[11] The light intensity was controlled by optical neutral density filter(s) to avoid anthracycline photodegradation.[15]

**Spectroscopic Kinetics Measurements.** Absorbance measurements were performed using a Cary model 300 double beam UV−visible absorption spectrometer with a spectral resolution of 1 nm. The solutions studied for complexation were made with 1:1 iron to anthracycline stoichiometry. For kinetics measurements the entire spectrum (350−800 nm) was collected every 55 s. Initial concentrations of anthracyclines in a cuvette were 80 μM. The concentration of HEPES buffer (pH 7.0) and $FeSO_4$ were 0.1 M and 80 μM, respectively. The $Fe^{2+}$ solution was introduced to the buffered anthracycline solution and stirred for ∼15 s before kinetics measurements were started. The effect of free solution phase daunosamine on iron binding was determined by adding methyl β-L-daunosaminide hydrochloride solution to the iron−anthracycline solution. The concentration of methyl β-L-daunosaminide hydrochloride in the final solution was 10 times higher than the concentration of daunorubicin. The kinetics of the sample with methyl β-L-daunosaminide was compared with the sample without methyl β-L-daunosaminide and with the sample with aged daunorubicin. The concentrations of all other components were constant.

**Molecular Dynamics Calculations.** The dynamics of the methoxy substituent of daunorubicin were calculated using the Desmond Molecular Dynamics System (version 2.4., D. E. Shaw Research, New York, NY, 2010) and Maestro-Desmond Interoperability Tools (version 2.4, Schrödinger, New York, NY, 2010).[16] The daunorubicin molecule was placed in a cubic box of total volume ∼245 000 Å$^3$ and soaked in water using the TIP3P[17] explicit water model and the OPLS2005 all atom force field.[18] The system was allowed to relax according to the default procedure. Isothermal−isobaric (NPT) ensemble simulation was carried out for 10 ns with both recording interval and trajectory interval set to 1.2 ps.

**Rotational Energy Barrier Calculations.** The rotational energy barrier calculations were performed at the semiempirical level with PM3 parametrization using Hyperchem, version 8.0, software (Hypercube).

## RESULTS AND DISCUSSION

It is known that the toxicity of the anthracyclines varies according to subtle differences in their chemical structures. While the structure−toxicity relationship is not understood from a molecular perspective for this family of compounds, it is thought that the interactions of the anthracyclines with other species, such as $Fe^{3+}$, play a role in the phenomenological observations of toxicity. For this reason it is important to evaluate the structural consequences of the interactions of subtly different anthracyclines with species such as $Fe^{3+}$. In this work we consider the roles of a pendent methoxy group on the anthracycline ring structure and on the presence of a daunosamine group in mediating the interactions of anthracyclines with $Fe^{3+}$. We consider the effect of the methoxy group first (Figure 1).

**Effect of the Methoxy Group.** We have shown that the presence of a hydroxyl group at the 14-position does not affect the excited state lifetime of the anthracycline chromophore.[11] In this work we examine the effect of a methoxy group at the 4-position on the anthracycline ring structure (Figure 1). The fluorescence lifetime for daunorubicin, which contains the pendent methoxy group is 1033 ± 3 ps, and the lifetime for idarubicin, which does not possess the same methoxy functionality, is 1554 ± 4 ps (Figure 2). The only structural

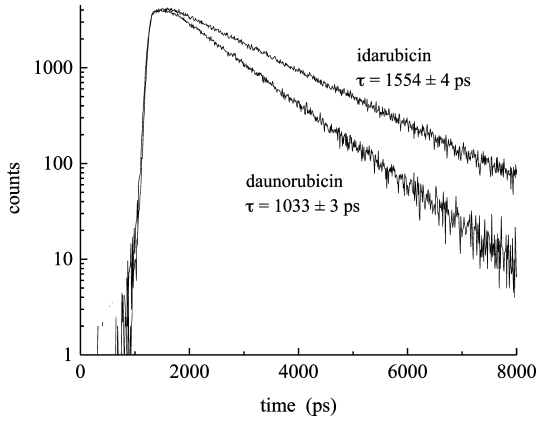


**Figure 2.** Fluorescence lifetime plot for idarubicin and daunorubicin in aqueous buffer solution, with the best-fit lifetimes for each compound as indicated.

difference between these two compounds is the presence of the methoxy group at the 4-position. The absence of the methoxy group gives rise to an increase in fluorescence lifetime, indicating that it is coupled to the electronic structure of the anthracycline chromophore. This is not a surprising result, and it does confirm that the presence of the methoxy group mediates the nonradiative decay kinetics for this system. While


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the details of this coupling are not apparent from the experimental data, it is likely that rotational motion of the methoxy group serves to mediate $S_1$ coupling to nonradiative decay channels in the anthracycline ring system. The mediation of $S_1$ access to the nonradiative decay channel(s) is most likely through oxygen lone pair interactions, either with the aromatic chromophore system or with the solvent medium, or both. To better understand the origin of the observed effect, we have performed molecular dynamics simulations of daunorubicin in water. The results of the methoxy group torsional angle distribution along a 10 ns dynamics trajectory are shown in Figure 3a. The "zero" angle is taken to be where the carbon–oxygen bond of the methoxy group is coplanar with anthracycline ring system with the methyl group pointing away from the carbonyl moiety on the adjacent ring, as drawn in Figure 1. The radial plot shows that the methoxy group does not have an opportunity for free rotation in the anthracycline molecule. Mostly it resides between ±45° of planar, and thermal energy is not sufficient to allow rotation beyond approximately ±130°. The basis for this energetic barrier is steric in nature. The methoxy group cannot move freely because the quinoid oxygen on the adjacent ring blocks its rotation. The methoxy group is thus constrained in its motion. This steric issue can, in principle, have a significant influence on the ability of daunorubicin to form complexes with iron if the quinone–hydroquinone structure of the anthracycline ring is involved in the metal ion coordination.[8] In order to evaluate the rotational freedom of the methoxy group more fully, we have performed semiempirical calculations to assess the methoxy group rotational energy barrier (Figure 3b). We calculate the energy of this barrier to be 122 kcal/mol at 180° torsional angle, but the barrier is sufficiently high (~20 kcal/mol) by ~130° to prevent rotation beyond that point at room temperature. The similarity of the isomerization barriers for the $S_0$ (black) and $S_1$ (red) states demonstrates the dominance of steric contributions relative to electronic contributions in determining the barrier. As expected, this calculated finding is reflected in the experimental iron binding data. We show in Figure 4 a kinetics comparison between the daunorubicin–iron 1:1 complex and the idarubicin–iron 1:1 complex. The aqueous solution of anthracycline in HEPES buffer (pH 7.0) is mixed with ferrous ions in a 1:1 stoichiometric ratio. At pH 7, ferrous ions oxidize rapidly to form ferric ions.[19] This means of adding $Fe^{3+}$ to the solution allows for the facile formation of the anthracycline–iron(III) complex. Such is not the case if ferric ions are introduced directly to the anthracycline solution.[10,11] The broad absorbance band we observe, centered around 600 nm, indicates formation of the anthracycline–iron(III) complex.[8,20] Daunorubicin and idarubicin differ only by the presence and absence, respectively, of the methoxy substituent at the 4-position. The data contained in Figure 4 show clearly that the kinetics profiles of iron binding for the two anthracyclines are different. After the addition of ferrous ions, the solution was stirred for 15 s before measurements were initiated. For daunorubicin, the first stage in the kinetics plot is complex formation, as the newly formed ferric ions bind to the anthracycline (Figure 4a). For idarubicin this process occurs much more rapidly (<15 s) and is not resolved in the data shown in the plot (Figure 4b). The absorbance at 600 nm is higher for the idarubicin complex than for the daunorubicin complex. The time-domain changes in the absorbance spectra suggest that for idarubicin, iron can coordinate to both of the quinone–hydroquinone moieties. In contrast, daunorubicin, with the methoxy group at the 4-position, allows less access to the quinone oxygen adjacent to it, thereby mediating the efficiency of iron binding to this quinone–hydroquinone structure. Even if iron binding is not precluded, its efficiency is diminished for steric reasons. Consistent with this effect, the stability of $Fe^{3+}$ complexes with the two anthracyclines differ. For both anthracyclines, their complex with iron(III) exists in thermodynamic equilibrium with free $Fe^{3+}$ ions in the solution. At pH 7.0, where our experiment was carried out, the free ferric ions form insoluble ferric hydroxides. This phenomenon lowers the concentration of free ferric ions in the solution. As $Fe^{3+}$ ions are removed from solution, the concentration of anthracycline–iron(III) complex will decrease as well, according to Le Chatelier's principle. This effect can be seen clearly in the kinetics plots (Figures 4), and the extent to which it operates depends on the relative stability of the $Fe^{3+}$:anthracycline and $Fe(OH)_3$ species. The daunorubicin complex exhibits faster hydrolysis kinetics,[11] implying that idarubicin forms stronger complexes than does daunorubicin with iron(III).

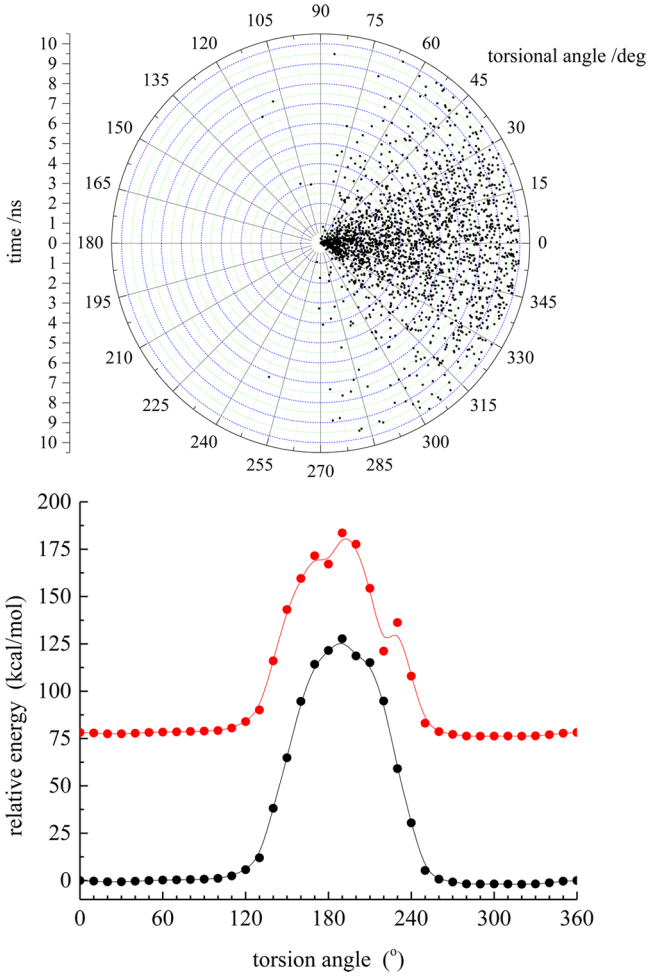


**Figure 3.** (a, top) Variation of methoxy group torsional angle during a 10 ns molecular dynamics calculation. (b, bottom) Calculated isomerization barrier for daunorubicin as a function of the methoxy group orientation relative to the planar ring structure. The similarity of the isomerization barriers for the $S_0$ (black) and $S_1$ (red) states demonstrates the dominance of steric effects relative to electronic effects in determining the barrier.

**Effect of the Daunosamine Group.** In order to gauge the effect of free daunosamine on iron binding with anthracyclines,


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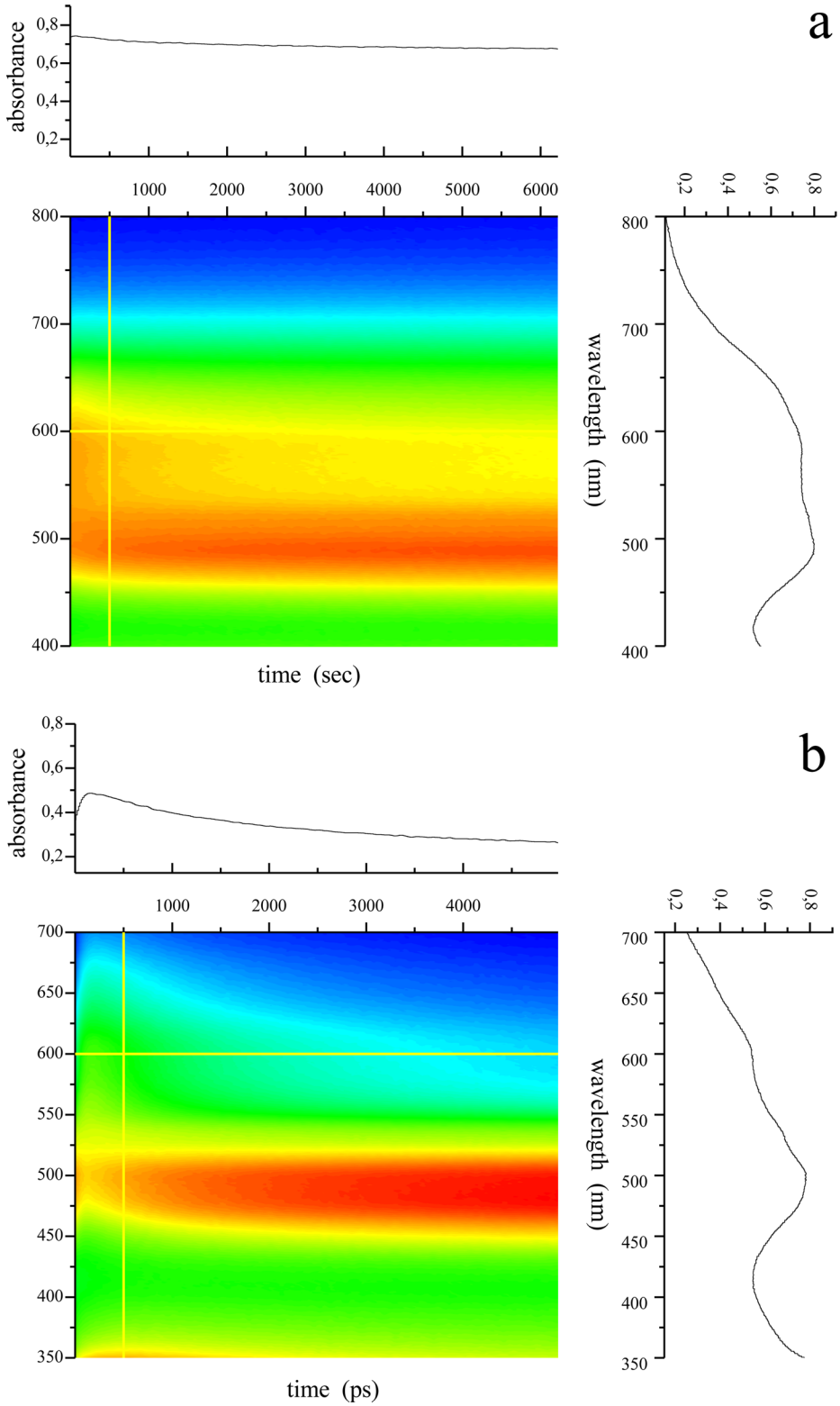


**Figure 4.** Contour plots showing time-dependent changes in the absorbance spectra of the anthracyclines: (a) data for the $Fe^{3+}$:idarubicin system; (b) data for the $Fe^{3+}$:daunorubicin system. The color gradient reflects absorbance values from high (red) to low (blue). Above each contour plot is the kinetics traces for absorbance at 600 nm. The full spectrum of the complex at ~500 s is shown in the panels to the right of the contour plots.

we have added methyl β-L-daunosamine to a buffered solution of daunorubicin. Our motivation for performing this experiment is that the pendent daunosamine group on the anthracyclines can be cleaved hydrolytically, leaving the free species in solution to potentially influence the formation and dynamics of the $Fe^{3+}$ complex. The methyl group in the daunosamine derivative we used is attached to the position where the daunosamine binds to the anthracyclines. For this experiment we set the concentration of free methyl β-L-daunosamine in solution to be 10 times larger than the


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anthracycline concentration. Kinetics data were recorded following the addition of ferrous ions (vide infra). We show in Figure 5 a comparison plot between the daunorubicin–iron(III) complex in solution, with (red) and without (black) methyl β-L-daunosamine. The kinetics profiles of the two solutions are functionally identical. Thus, methyl β-L-daunosamine does not compete to any significant extent with daunorubicin for iron(III) in solution.

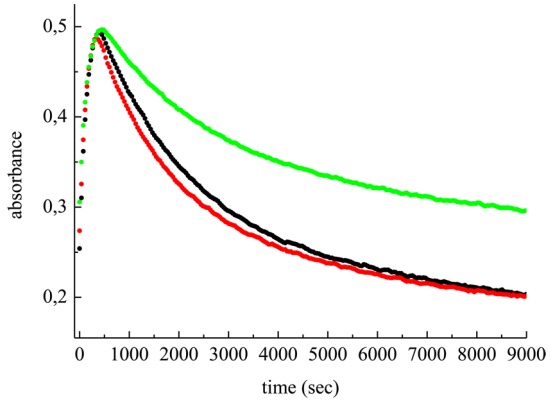


**Figure 5.** Kinetics plots of absorbance change at 600 nm for the $Fe^{3+}$:daunorubicin complex for fresh daunorubicin solution (red), fresh daunorubicin solution with free methyl β-L-daunosamine (black), and aged daunorubicin solution with the daunosamine functionality cleaved (green).

The fact that free daunosamine does not exhibit chemical interactions with $Fe^{3+}$ that might affect anthracycline binding kinetics does not necessarily mean that it does not influence $Fe^{3+}$ binding in any manner. Steric interactions between the pendent daunosamine moiety and the quinone–hydroquinone region of the anthracycline may give rise to interference with $Fe^{3+}$ binding, analogous to that seen for the methoxy group. To address this potential issue, we compared the kinetics of iron binding for daunorubicin containing the daunosamine moiety and the aglucone form of daunorubicin. It is known that the daunosamine group can be hydrolytically cleaved from anthracyclines.[21,22] In our study we compared the behavior of fresh daunorubicin solution with a daunorubicin solution that had been aged for 7 days. The daunorubicin loss of the daunosamine group was confirmed by rotational diffusion measurements (Figure 6), showing the orientational relaxation time of fresh daunorubicin solution to be 339 ± 27 ps and the analogous data for the aged solution to be 251 ± 14 ps. This difference in reorientation time is a factor of 1.35 ± 0.18 change, and the difference between the hydrodynamic volumes of the daunosamine-containing and daunosamine-free anthracycline chromophore predicts a factor of 1.33 difference in reorientation times.[23] The kinetics plot of aged daunorubicin solution is shown in green in Figure 5. The aged daunorubicin solution behaves differently from fresh solution. The lack of daunosamine makes the hydrolysis kinetics of the anthracycline:$Fe^{3+}$ complex slower than it is for daunorubicin possessing the pendent daunosamine group. The steric effect of the daunosamine is not as pronounced as that of the methoxy group, but it is still important in mediating the binding of iron to the anthracycline. The complex of daunosamine's aglucone is significantly more stable over time, in support of this assertion.

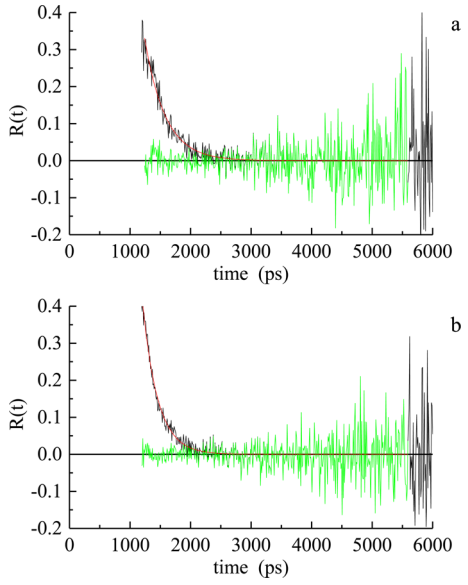


**Figure 6.** Fluorescence anisotropy decay plots for (a) fresh daunorubicin solution ($\tau_{OR}$ = 339 ± 27 ps) and (b) aged daunorubicin solution ($\tau_{OR}$ = 251 ± 14 ps).

## CONCLUSIONS

We have examined the effect of pendent methoxy and daunosamine groups on anthracycline–iron(III) complex formation and stability. The interactions of anthracyclines with $Fe^{3+}$ depend sensitively on the structure of the anthracycline. The methoxy substituent at the 4-position interacts with the adjacent quinone oxygen, affecting both $Fe^{3+}$ binding efficiency and the fluorescence lifetime of the anthracycline chromophore. Free rotation of the methoxy group is hindered by the presence of the quinone oxygen. The height of the barrier to rotational motion is calculated to be ~120 kcal/mol, and this barrier likely limits the extent to which methoxy group rotation is able to reduce the fluorescence lifetime of the anthracycline chromophore. The free daunosamine moiety does not exhibit measurable interactions with $Fe^{3+}$, but when this group is attached to the anthracycline ring system, it does mediate the strength of interaction with $Fe^{3+}$, as can be seen through changes in complexation upon hydrolytic cleavage of the daunosamine. The data contained in this and the preceding paper[11] demonstrate collectively that there are a range of structural interactions possible between $Fe^{3+}$ and anthracyclines[24] and that subtle structural variations between anthracyclines can have a significant effect on their chemical reactivity and likely also on their biological activity.

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### Notes

The authors declare no competing financial interest.

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