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Solution Chemistry and DNA Binding Properties of MEN 10755, A Novel Disaccharide Analogue of Doxorubicin

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Abstract—The behavior under physiological conditions of MEN 10755, a novel disaccharide analogue of doxorubicin, was investigated in detail by a variety of spectroscopic techniques including spectrophotometry, fluorescence, and ^{1}H NMR. The pH dependent properties of MEN 10755 were also analysed by spectrophotometry and potentiometry within the pH range 5–11. It is found that MEN 10755 behaves very similarly to doxorubicin and reproduces closely its pH dependent pattern. Like doxorubicin, MEN 10755 undergoes dimerization with a significantly smaller association constant. The interaction of MEN 10755 with calf thymus DNA was studied in detail. Spectrophotometric and fluorescence titrations of MEN 10755 with calf thymus DNA show spectral patterns almost identical to those obtained with doxorubicin implying that the binding mechanism and the stability of the resulting adducts are very similar. An apparent affinity constant of 1.2×10^6 was determined for the interaction of MEN 10755 with calf thymus DNA to be compared with the value of 3.3×10^6 measured for doxorubicin, under the same conditions. The effects of both anthracyclines on the thermal denaturation profiles of calf thymus DNA were also analyzed; both compounds turned out to stabilize to a similar extent the DNA double helix and to give rise to a characteristic two-step melting profile. The implications of the present results for the pharmacological activity and the mechanism of action of this novel and promising antitumor compound are discussed.

Introduction

Given the central role of anthracyclines in antitumor chemotherapy, there are continuous efforts to design and develop new anthracyclines with improved clinical properties and lower systemic toxicity.[1–3] In fact, it is well established that even modest chemical modifications of the classical structure of doxorubicin produce important consequences on the biological and pharmacological properties of these compounds.[1,2] Recently, a disaccharide analogue of doxorubicin, MEN 10755 (Scheme 1), has been developed and characterized by Menarini Ricerche Spa, that exhibits promising antitumor activity toward human tumor xenografts including doxorubicin-resistant xenografts.[4]

In particular, MEN 10755 exhibited a striking in vivo antitumor activity in all preclinical models examined, including tumor systems refractory to doxorubicin treatment[5] in spite of an apparently reduced cellular accumulation. Cellular accumulation of MEN 10755 was evaluated in a panel of human tumor cell lines by fluorescence measurements in comparison with doxorubicin.[4] The results indicated that at 30, 60, and 120 min of incubation the intracellular quantity of MEN 10755 is about 2 times lower than doxorubicin and that the latter one is retained longer than MEN 10755. Furthermore, the optimal antitumor cumulative dose that can be administrated to mice with tumor is greater with MEN 10755 (30 mg/kg) than with doxorubicin (21 mg/kg); such results were ascribed to a shorter half-life and a lower tissue distribution of MEN 10755 compared to doxorubicin.[6] Despite these pharmacokinetic parameters, the ability of MEN 10755 to elicit a marked antitumoral activity in a wide number of xenografts suggests that it is endowed with intrinsic greater potency and/or better pharmacodynamic properties than doxorubicin.

Some features of the solution properties of MEN 10755 were previously elucidated through ^{1}H NMR spectroscopy and molecular mechanics, and shown to be substantially similar to those of doxorubicin up to the second sugar ring.[7] With the help of a variety of physico-chemical techniques (spectrophotometry, fluorescence, potentiometry, etc.), we have further extended

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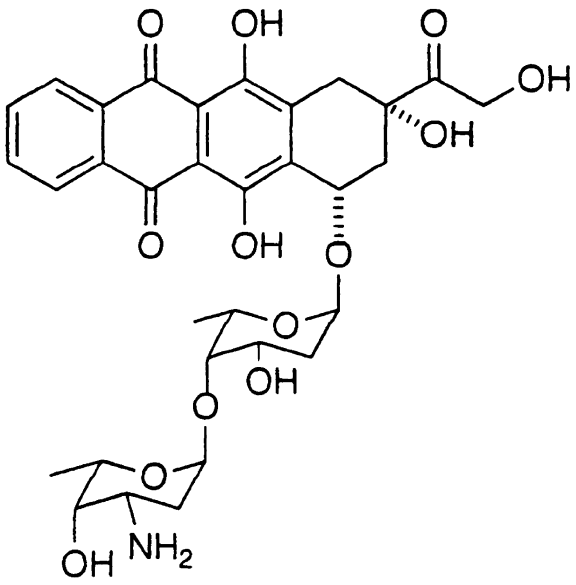
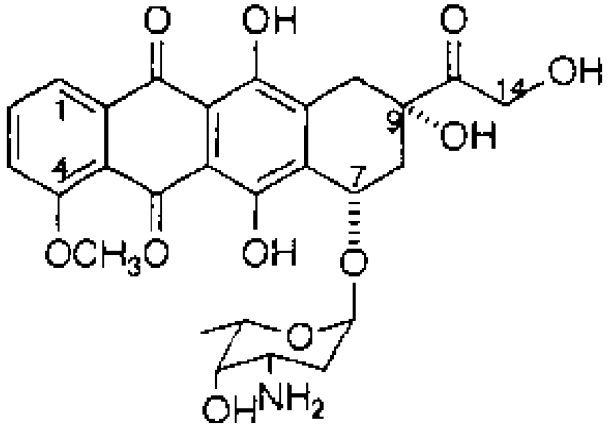
the investigation of the solution properties of MEN 10755. This study has been carried out in parallel to doxorubicin to reveal possible differences in the solution chemistry of the two anthracyclines, particular emphasis being given to the description of the pH dependent properties.

Moreover, we have extensively investigated the reaction of MEN 10755 with DNA, the established biomolecular target of anthracyclines, using calf thymus DNA as reference compound. The reaction of MEN 10755 with calf thymus DNA was mainly analyzed through spectrophotometric and fluorescence methods in comparison to doxorubicin; direct information on the apparent DNA binding constants and on the mechanism of drug–DNA interaction is gained. Also, the effects that both anthracyclines produce on the thermally induced helix-to-coil transition of calf thymus DNA were considered in detail.

MEN 10755



DOXORUBICIN



Scheme 1.

Results

Solution chemistry of MEN 10755

The behavior of MEN 10755 under physiological conditions. The electronic spectra of MEN 10755 and doxorubicin recorded within the BPES buffer pH 7.4, are very similar to one another being dominated by the intense visible bands of the anthracycline aglycone[8] (Fig. 1a). The minor differences that are detected between the visible spectra of the two compounds are very likely a consequence of the fact that doxorubicin, at variance with MEN 10755, bears a methoxy group in position 4; larger differences are observed in the fluorescence spectra (Fig. 1b). Notably, the visible spectra of MEN 10755 do not undergo significant changes when monitored over a period of several hours at room temperature, implying that both compounds are pretty stable under physiological conditions.

Analogously to daunomycin and doxorubicin, MEN 10755 undergoes aggregation in aqueous and buffer solutions[9,10] due to stacking of the anthracycline rings. As in the case of the two mentioned anthracyclines,[10] aggregation leads to important concentration-dependent hypochromic effects. Although aggregation mainly consists of dimerisation, it cannot be ruled out that more complex aggregation processes may take place. For instance, Chaires et al. proposed that an indefinite association model rather than dimerisation better fits the experimental data.[9] In addition, it was shown that an increased ionic strength favors doxorubicin association.[11] Analysis of the concentration dependent spectral modifications of MEN 10755 according to a simple dimerisation process allowed us to estimate a value $\approx 2850\,M^{-1}$ for the association constant to be compared with the values of 13,000 and $23{,}000\,M^{-1}$ previously determined for daunomycin and doxorubicin[10] (the actual values of the extinction coefficients for MEN 10755 are the following $\epsilon_{monomer} = 10{,}500$; $\epsilon_{dimer} = 7500$).

1-D and 2-D NMR spectra of MEN 10755 were carried out in buffer solution that led to the complete assignment of the ^1H NMR resonances (data not shown); overall the ^1H NMR spectrum in the buffer does not differ significantly from the spectrum, previously reported, of MEN 10755 dissolved in DMSO[7] (Fig. 2).

pH Dependent properties. The pH dependent properties of MEN 10755 were first analyzed spectrophotometrically. Visible spectra of MEN 10755, in the pH range 5–11, are shown in Figure 3a; for comparison purposes the visible spectra of doxorubicin within the same pH range are shown in Figure 3b. Both anthracyclines exhibit a very similar pH-dependent spectral pattern; indeed, the visible spectra of both compounds are quite stable in the low pH range but show large changes upon moving to alkaline pH. Upon increasing

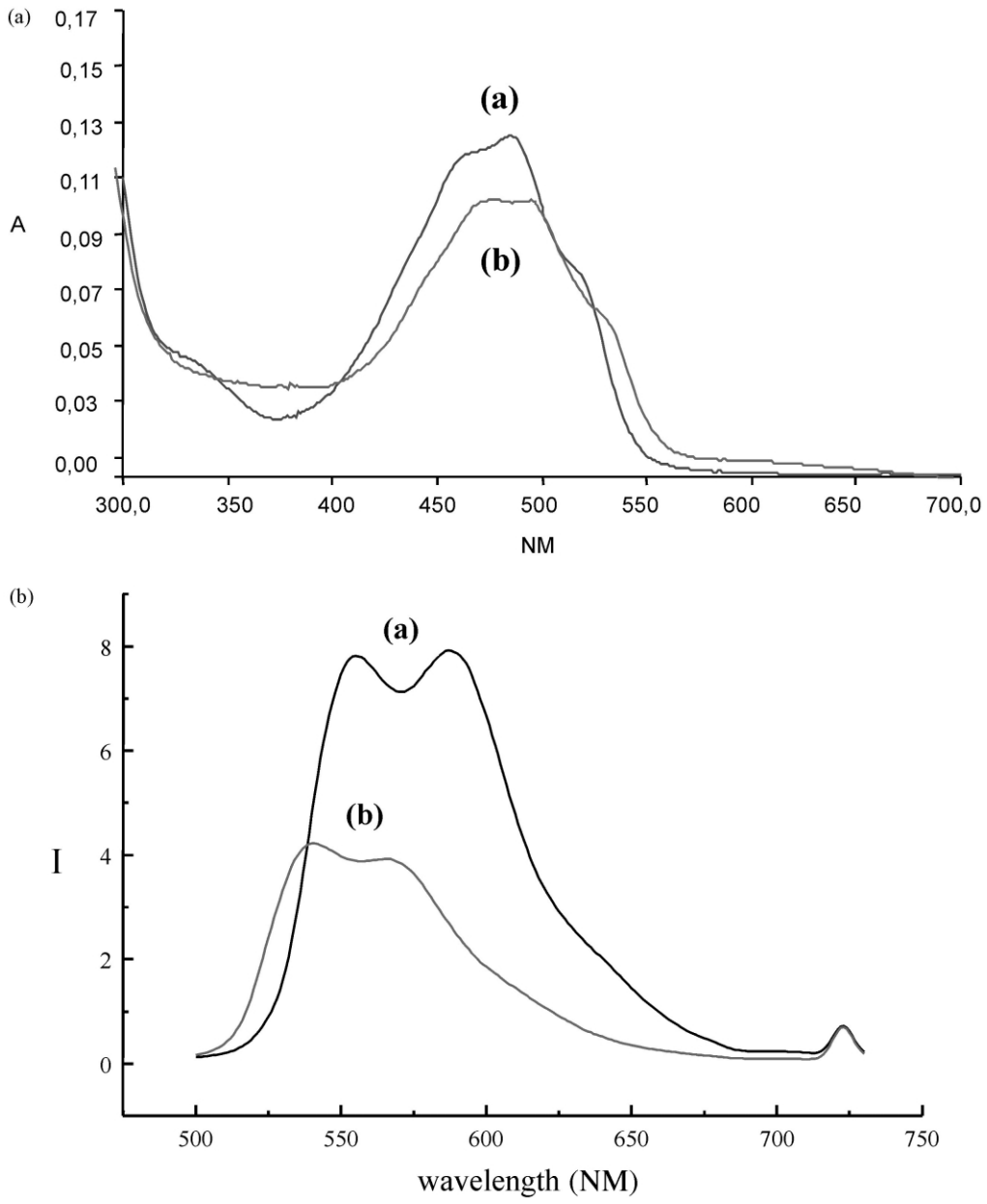


Figure 1. (a). Electronic absorption spectra of MEN 10755 (a) and doxorubicin (b), (10 μM) in buffer BPES Na_2HPO_4 (6 mM), NaH_2PO_4, (2 mM), EDTA (1 mM), NaCl (185 mM), pH 7.4; (b) fluorescence spectra of MEN 10755 (a) and doxorubicin (b), (1 μM) in buffer BPES, pH 7.4.

pH from 7 to 10, a rapid decrease of the band at 490 nm is observed in concomitance with the appearance of a new band at 550 nm. These spectral changes are attributed to deprotonation of the phenolic OH group on the aglycone, as previously proposed.[12]

The pH-dependent properties of both compounds were further analyzed with the help of potentiometry. Potentiometric curves of MEN 10755 and doxorubicin are shown in Fig. 4. Notably, potentiometry permits identification of an additional pK_a value falling at 2.6; moreover, potentiometric results point out that the spectral changes observed in the high pH region ($8 < pH < 11$) depend on the presence of two ionizable groups that very likely correspond to the protonated amino group and to the phenolic OH.[12] The individual pK_a values for these groups were carefully determined for both MEN 10755 (8.6 and 10.44) and doxorubicin (8.15 and 10.16) (Fig. 4).

The interaction with DNA

Electronic and fluorescence spectra. In the second part of this study, we investigated whether the subtle chemical differences detected in solution between MEN 10755 and doxorubicin might result into a different mode of interaction with DNA, the established biomolecular target of anthracyclines.[1,2]

The reaction of MEN 10755 with calf thymus DNA was first analyzed spectrophotometrically. Addition of increasing amounts of purified calf thymus DNA to a solution of MEN 10755, under physiological conditions, produces progressive hypochromic effects of the intense

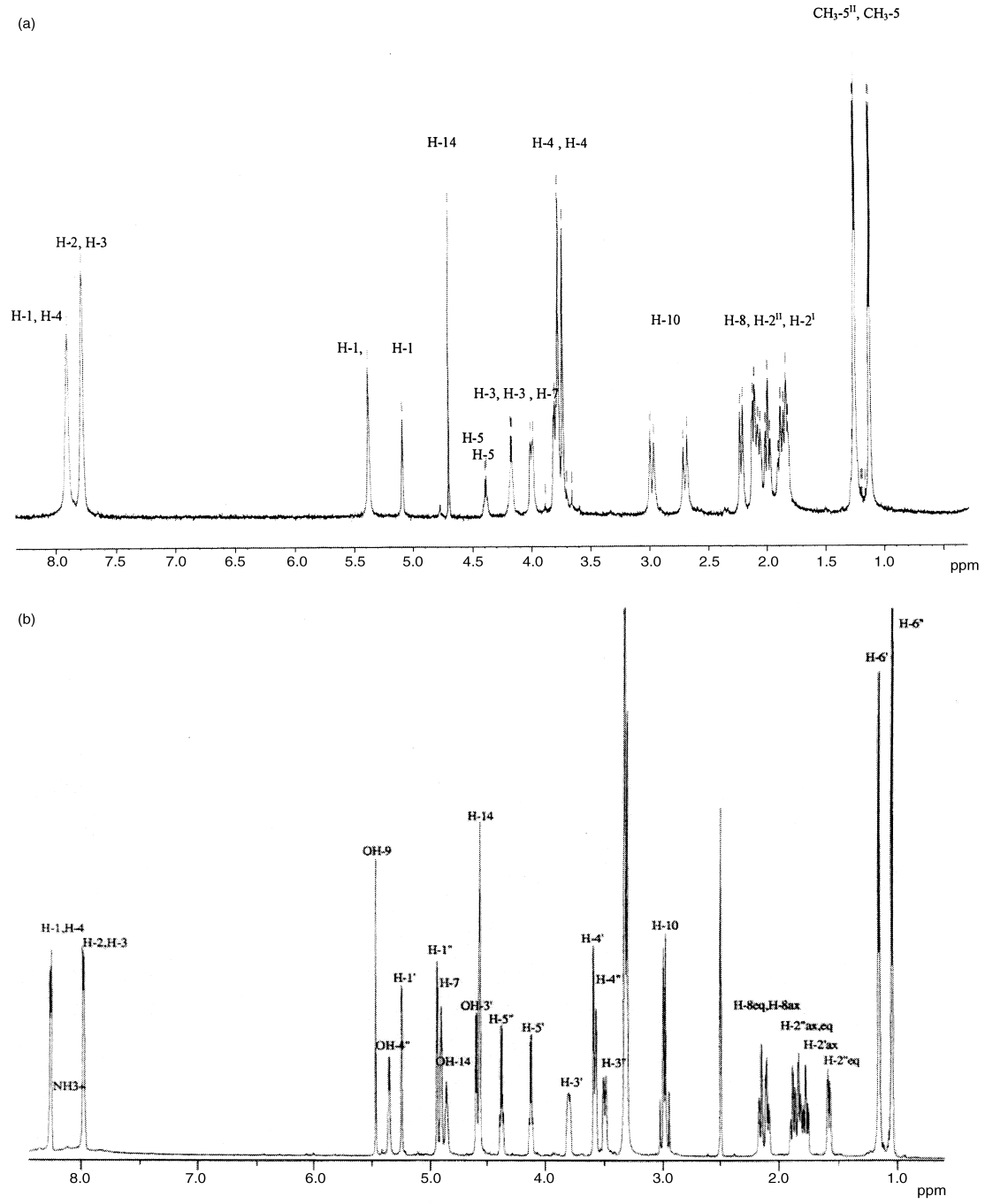


Figure 2. (a) 600 MHz ^{1}H NMR spectrum of MEN 10755 in D_2O. (b) For comparison purposes, the ^{1}H NMR spectrum previously reported for MEN 10755 in Me_2SO-d_6 is shown.[5]

visible transitions of MEN 10755, that manifest soon after mixing. The pattern of spectral modification in the visible region is very similar for both MEN 10755 and doxorubicin (Fig. 5) reflecting an essentially similar interaction with the DNA double helix.[8,13,14]

Further evidence in support of this hypothesis was obtained from fluorescence studies. Anthracycline intercalation to DNA is known to produce a dramatic quenching of the intrinsic fluorescence of the anthracycline chromophore.[8,15] A drastic quenching of MEN fluorescence was indeed detected upon adding increasing amounts of calf thymus DNA to a solution of MEN 10755 in agreement with intercalative binding (Fig. 6a). Again, the resulting spectral pattern is very similar to that obtained in the case of doxorubicin (Fig. 6b).

Careful analysis of the spectrophotometric and fluorescence titration profiles of both anthracyclines with DNA permits to determine with accuracy their respective apparent binding constants to calf thymus DNA. Data analysis was carried out according to the McGhee–van Hippel formalism;[16,17] results are shown in Table 1. Notably, both methods—spectrophotometry and fluorescence—point out that MEN 10755 binds DNA with an affinity constant very similar to that

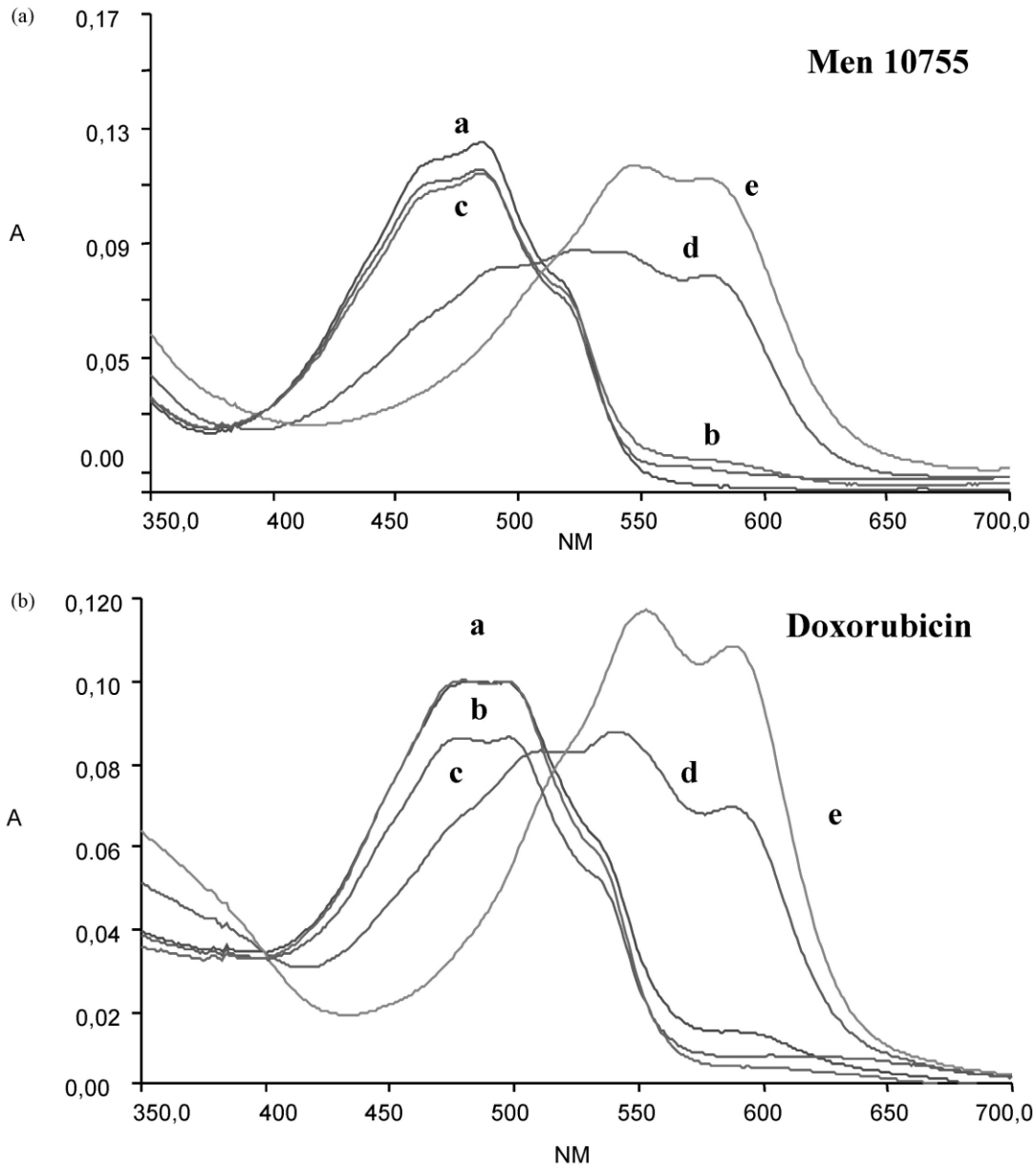


Figure 3. pH dependence of the electronic spectra of MEN 10755 (a) [(a) pH 6; (b) pH 7.4; (c) pH 8.5; (d) pH 10; (e) pH 11.5] and doxorubicin (b) [pH 6; (b) pH 7.4; (c) pH 8.5; (d) pH 10; (e) pH 11.5].

measured for doxorubicin implying that the different spatial distribution of the charges, the presence of a second sugar moiety and the absence of the methoxy group in 4 do not modify substantially the energetics of this interaction. Specifically these results mean that the presence of a second sugar moiety plays a minor role in the interaction of the anthracycline with DNA, if any.

Melting profiles. The consequence of intercalation on the stability of the DNA double helix are best demonstrated by DNA melting experiments.[18] DNA melting studies, carried out in our laboratory according to the reported procedure,[19] show that addition of MEN 10755 produces, as expected, a large stabilization of the DNA double helix. Melting profiles remind those previously obtained by Chaires with daunomicin. Notably, at low ratio, the profile is distincly biphasic (see Fig. 7); at higher ratios the profile is essentially monophasic with a much larger stabilisation effect.[18]

Discussion and Conclusions

The spectroscopic results reported in this study shed light on the solution behavior and the DNA binding properties of MEN 10755 in comparison to those of the popular parent compound doxorubicin. An extensive analysis of the solution properties of MEN 10755 is largely justified by the promising biological properties that MEN 10755 has recently shown in various in vivo models.[4,5] Thus, detection of even slight differences in the solution behavior or in the interaction with DNA might be particularly meaningful to understand the molecular bases of the different pharmacological properties of MEN 10755 and doxorubicin.

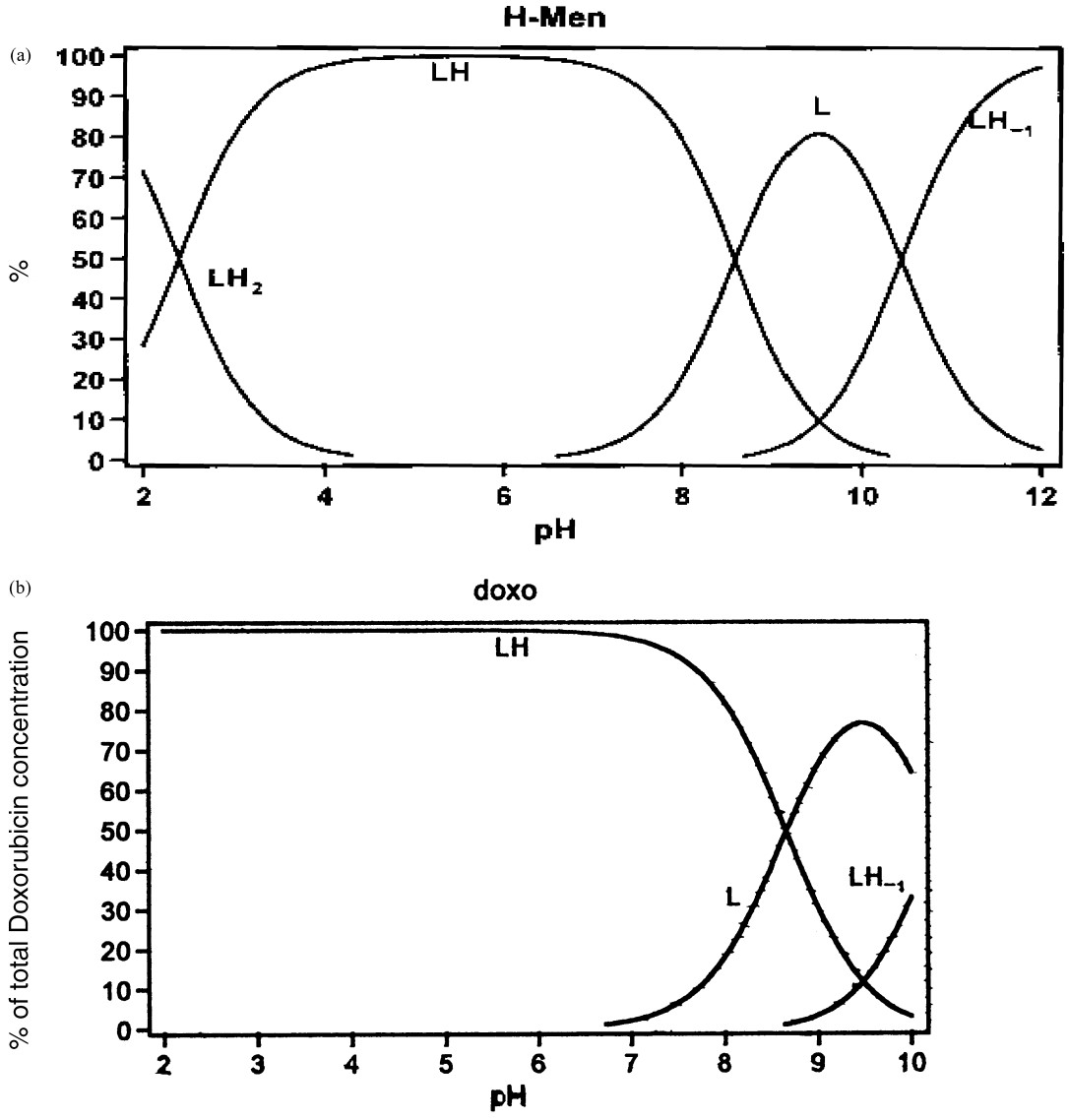


Figure 4. Potentiometric profiles of (a) MEN 10755 and (b) doxorubicin 1 mM, in the pH range 2.5–10.5, upon titration with TMA hydroxide (TMA chloride 0.1 M).

Solution behavior of MEN 10755

The spectral studies of MEN 10755 in solution, here reported, show a behavior that is essentially similar to that of doxorubicin. The electronic spectra of both compounds are almost identical being dominated by the intense visible bands of the anthracycline chromophore. The minor spectral differences that are detected are ascribed to the presence of a methoxy group on the aglycone of doxorubicin (in position 4) at variance with MEN 10755. MEN 10755, like daunomycin and doxorubicin, undergoes aggregation in aqueous solutions; notably the dimerisation constant determined for MEN 10755 is much lower than in the case of daunomycin or doxorubicin, probably as a consequence of a bulkier sugar moiety.

Since doxorubicin and MEN 10755 possess the same ionizable groups, it may be anticipated that they will exhibit similar spectral profiles for the pH dependence; indeed the pattern of the pH dependence of the electronic spectra of the two anthracyclines is nearly the same. In the light of these results, the acid-base equilibria taking place in solution in the pH range 8–11 may be interpreted according to the scheme previously proposed by Sturgeon for doxorubicin.[12] Sturgeon proposes first ionization of the amino group on the aminosugar, then deprotonation of the phenolic group of the hydroquinone (Scheme 2). These ideas are confirmed by our potentiometric data.

The interaction with DNA

The spectral results obtained when monitoring the interaction of MEN 10755 with DNA closely resemble those of the doxorubicin/DNA interaction. In both cases, the electronic spectra of the anthracycline chromophore undergo a significant red shift and a large hypochromic effect upon addition of increasing amounts of DNA. The dramatic intensity decreases observed in the fluorescence spectra of MEN 10755

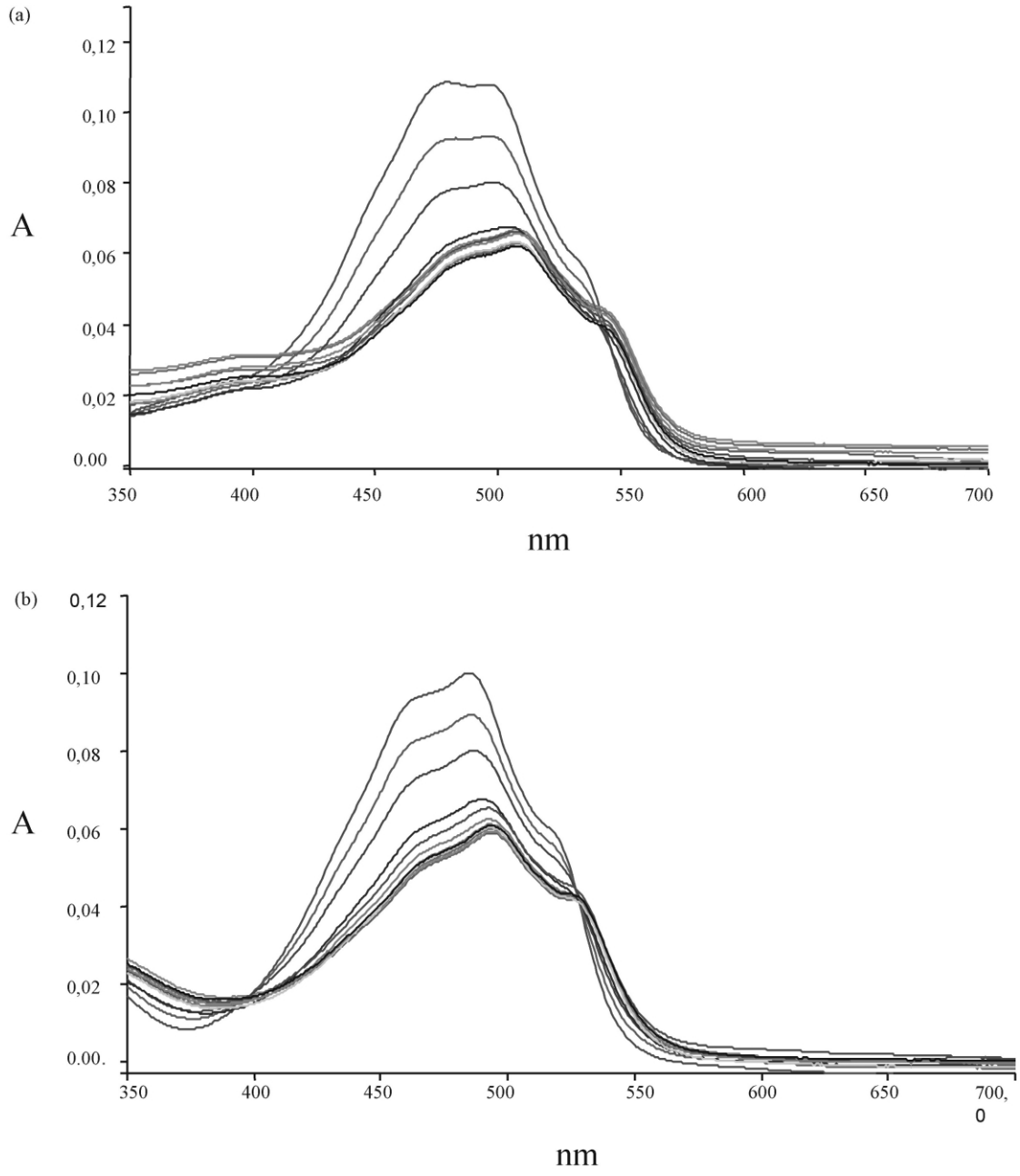


Figure 5. Spectrophotometric titrations of (a) doxorubicin and (b) MEN 10755, with increasing amounts of calf thymus DNA in BPES buffer, pH 7.4 (during the titration, r varies from 1.5 to 0.05).

following DNA addition are surely diagnostic of intercalative binding.[8]

The spectrophotometric and fluorescence titration profiles, analyzed according to the McGhee–van Hippel formalism, allowed us to determine the affinity constant and the exclusion parameters for the interaction of MEN 10755 with DNA. Notably, the obtained values of the affinity constants and of the exclusion parameters match those found for doxorubicin even if the affinity constant of the MEN is smaller by a factor ≈2 (Table 1). This implies that the presence of a second sugar moiety and the different spatial location of the charged amino group do not modify appreciably the stability of the DNA adducts. Also, thermal denaturation profiles of MEN 10755/DNA and doxorubicin/DNA adducts carried out at $r = 0.05$ do not reveal major differences in the effects produced by the two anthracyclines; indeed, a relevant thermal stabilization and a characteristic biphasic melting profile are found in both cases.

Possible implications for the pharmacological properties

Two main results emerge from this study that are of potential relevance to interpret the pharmacological properties of MEN 10755 compared to those of doxorubicin:

i. the solution chemistry of MEN 10755 and its pH dependent properties are virtually superimposable to those of doxorubicin.

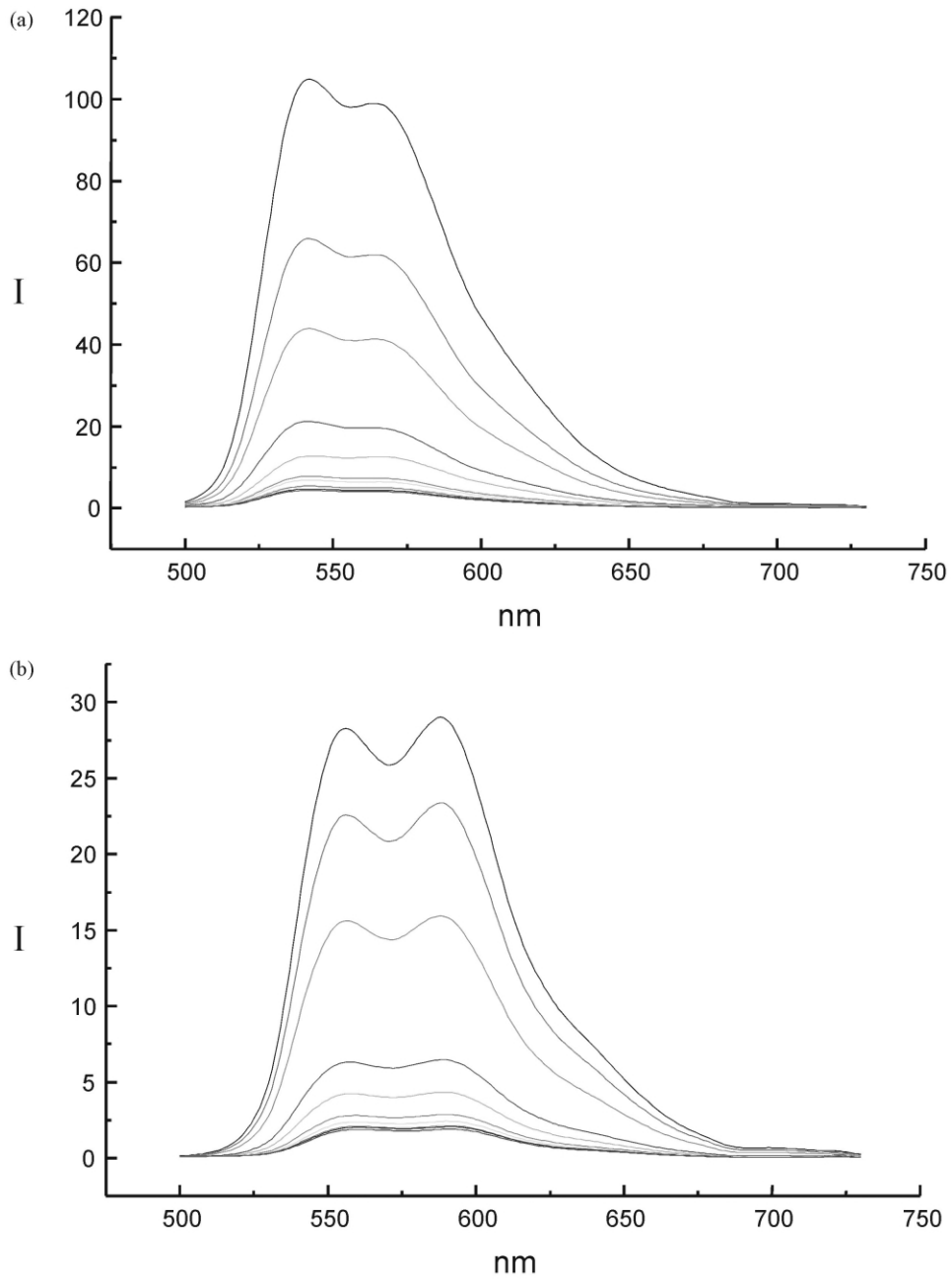


Figure 6. Fluorescence quenching of (a) MEN 10755 and (b) doxorubicin upon addition of increasing amounts of calf thymus DNA in BPES buffer, pH 7.4 during the titrations, *r* varies from 1.5 to 0.05.

ii. the binding mechanism of MEN 10755 to calf thymus DNA and the stability of the resulting adducts are very similar to those of doxorubicin, although the affinity constant for calf thymus DNA is slightly smaller.

In the light of these results, we can expect that the behavior in physiological solution of the two anthracyclines must be very similar; thus the effects on the presumed biomolecular target—DNA—must be, again, very similar. Indeed, both compounds act as inhibitors of topoisomerase II, although MEN 10755 seems to be somehow more effective.[20] This means that the differences observed in the pharmacological behavior[5] between MEN 10755 and doxorubicin might arise from a different biodistribution of the two anthracyclines,[6] from a different ability to enter the cell nucleus,[4] from different pharmacokinetics, different interactions with complex target molecules—like proteins, membrane pumps, enzymes, and so on—that are capable to discriminate the two anthracyclines.

Some indications in this sense come from preliminary studies of D'Aranno et al. that reveal significant differences in the pharmacokinetic behavior of MEN 10755 compared to doxorubicin.[21]

Table 1. Conditional affinity constants and exclusion parameters for the binding of MEN 10755 and doxorubicin to calf thymus DNA

	K_i (M^{-1})	n	NaCl (M)	Method	Source of DNA
MEN	1.2×10^6	3.1	0.185	F	CT
10755	7.9×10^5	2.6	0.185	A	CT
DOXO	3.3×10^6	3.8	0.185	F	CT

Abbreviations: A, absorbance; F, fluorescence; CT, calf thymus.

Concluding remarks

Overall, the present results permit to state that the behavior of MEN 10755 in solution essentially resembles that of doxorubicin. Dimerization processes take place for both compounds with somehow different association constants; the pH dependent properties of the two derivatives are nearly the same as a consequence of the fact that both compounds possess the same ionizable groups. In spite of the significant structural differences existing between these two anthracyclines—the spatial arrangement of the ionizable groups in the two compounds is different; MEN 10755 possesses two sugar moieties; there is a methoxy group in doxorubicin—no major differences could be detected in the mechanism of DNA binding and in the stability of the resulting adducts. Just small differences in the affinity constants for calf thymus DNA were detected.

Materials and Methods

Materials

MEN 10755 was prepared by Laboratori Guidotti, Pisa, Italy according to the reported method.[4] Doxorubicin and calf thymus DNA were purchased from SIGMA Chemical Company. Where not differently stated, experiments were performed in BPES buffer containing Na_2HPO_4 (6 mM), NaH_2PO_4 (2 mM), EDTA (1 mM), NaCl (185 mM), (pH 7).

Spectroscopic measurements

Visible absorption spectra were carried out with a Perkin–Elmer Lambda Bio 20 instrument. The measurements were done at room temperature (25 °C). Fluorescence spectra were performed with a Jasco FP-750 spectrofluorimeter in room temperature with $\lambda_{ex} = 480$ nm. ^{1}H NMR spectra were recorded on a Bruker Avance 600 instrument.

Potentiometric studies

Potentiometric studies were carried out with a Crison micropH 2002 instrument, using tetramethylammonium hydroxide as titrant in constant ionic strenght. Data analysis was carried out using the HYPERQUAD program.[22]

DNA binding studies

Spectrophotometric titrations were performed by adding aliquots of a concentrated DNA calf thymus solution directly into a cuvette containing a fixed drug amount. Each addition was followed by a 5-min equilibration period after which absorbancies at 480 and 540 nm were recorded. Fluorescence titration experiments were performed using a Jasco FP-750 spectrofluorimeter. The fluorescence intensity exhibited by MEN 10755 and doxorubicin in the absence and in the presence of DNA, were determined by exciting at 480 nm. Binding data were cast into the form of a Schatchard plot of r/C_f versus r, where r is the number of the moles of drug bound per mole of DNA bp and C_f is the concentration of free drug, and computer fitted according to the neighbour exclusion model:[8]

$$r/C_f = K_i\,(1\text{-}nr)\,[(1\text{-}nr)/(1\text{-}(n\text{-}1)r]^{(n-1)},$$

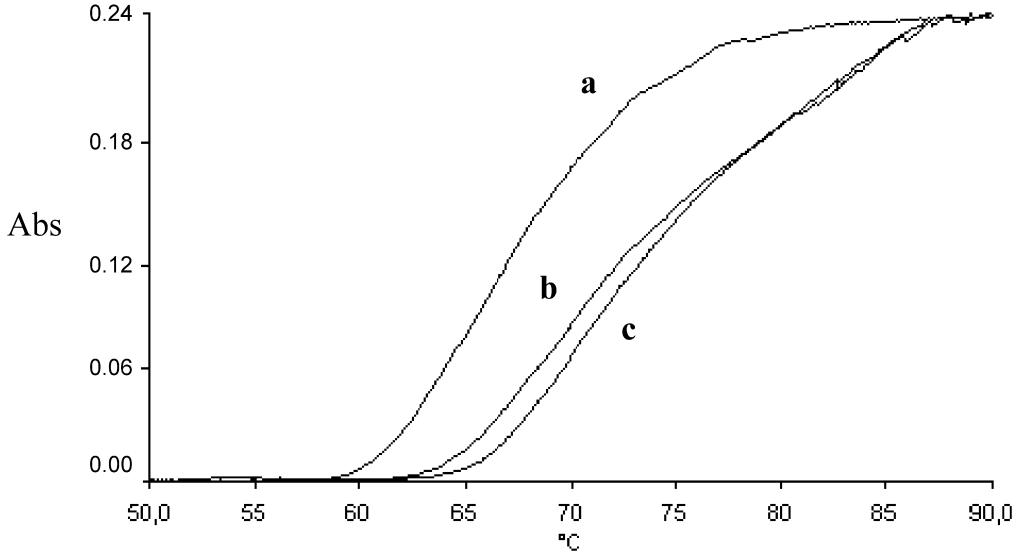
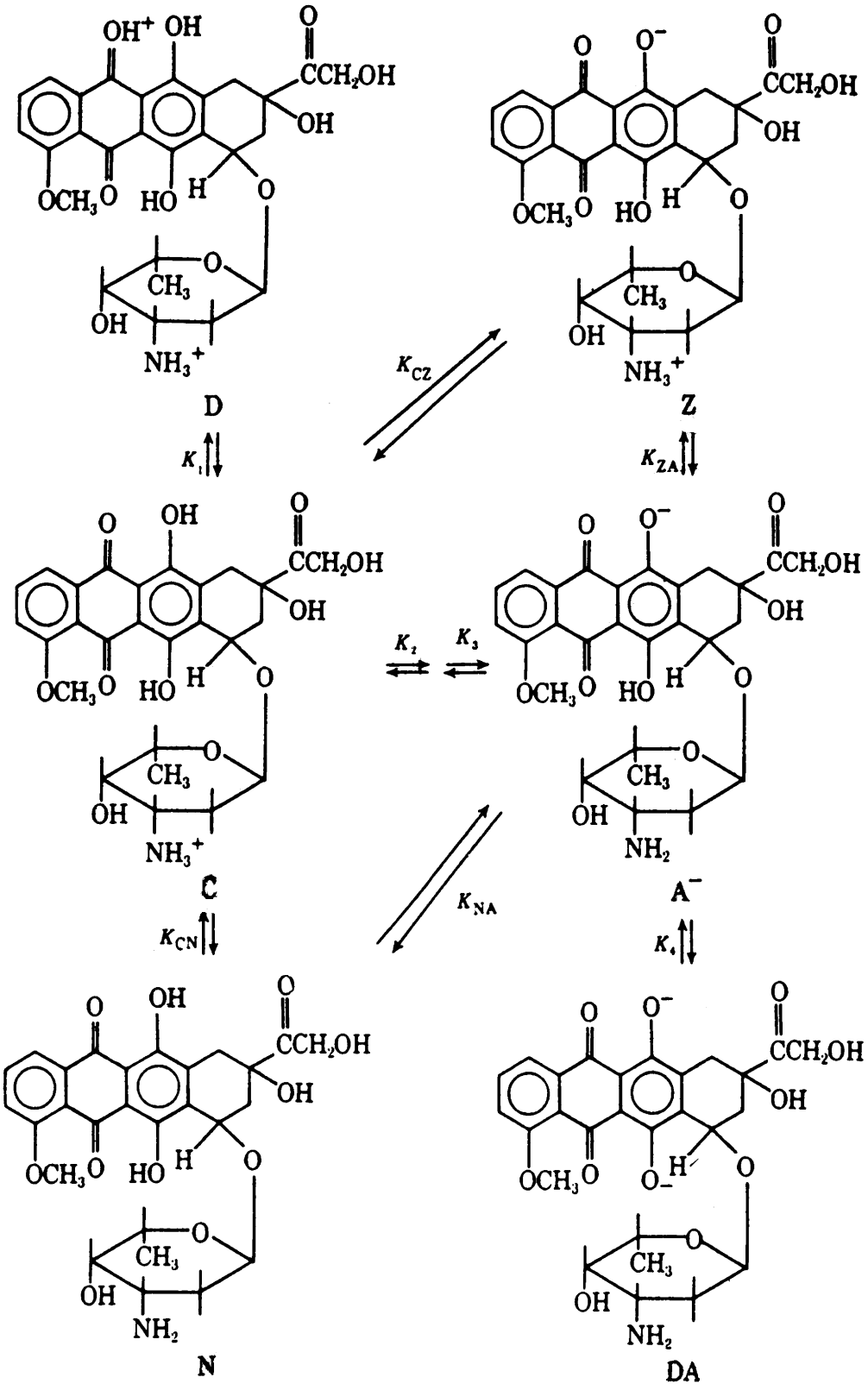


Figure 7. Thermal denaturation profiles of (a) calf thymus DNA, and its adducts with (b) MEN 10755 and (c) doxorubicin, in buffer $NaClO_4$ (10^{-2} M) and NaCl (10^{-3} M), pH 7.0; at $r = 0.05$.



Scheme 2.

where K_i is the intrinsic binding constant and n is the exclusion parameter.

Melting curves

Thermal denaturation experiments were carried out with a Perkin-Elmer Lambda Bio 20 spectrophotometer equipped with a thermostated cuvette.[8] Samples were continuously heated at 0.5 °C/min. Absorbance changes at 260 nm were monitored. The buffer used for this experiment is $NaClO_4$ (10^{-2} M) and NaCl (10^{-3} M), pH 7.0. The initial DNA concentration was (5×10^{-5} M).

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