Anthracycline Anticancer Drugs as Effective Ligands for Terbium(III) Ions

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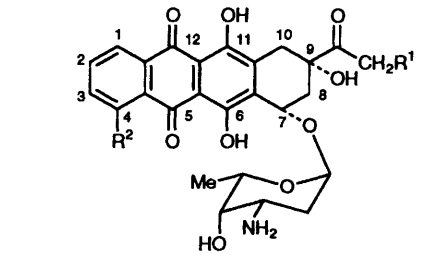
Spectroscopic measurements were carried out for terbium(III) complexes with the anthracycline anticancer drugs doxorubicin, aclarubicin, carminomycin and their simple model quinizarin-2-sulfonic acid (9,10-dihydro-1,4-dihydroxy-9,10-dioxoanthracene-2-sulfonic acid). Potentiometric titrations were carried out for the doxorubicin-, aclarubicin- and quinizarin-2-sulfonic acid-containing systems. Both stability constants and CD spectra indicate that interligand interactions stabilise the 1:2 metal-to-ligand complexes formed.

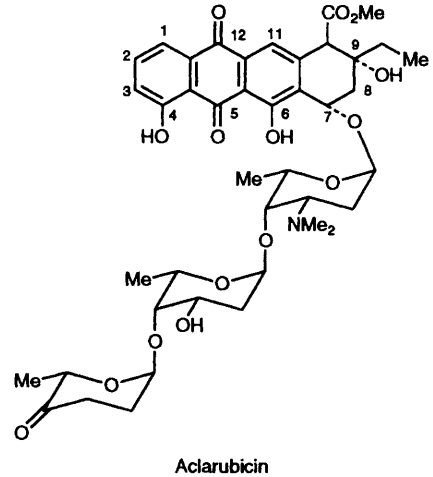
Anthracycline-based drugs are widely applied in the treatment of various tumour diseases. However, their high toxicity as well as the development of resistance in patients to these drugs has inspired the search for new analogues. The modification of the sugar and aglycon moieties by organic synthesis methods has brought many new anthracycline analogues with promising pharmacological behaviour.[1] The metallic derivatives of some anthracyclines were found to behave differently than the parent organic drugs and they may represent a new class of anthracycline antitumour agents.[2-6]

Biochemical and medical applications of lanthanides are already well established.[7] Although there is no essential role proposed for rare-earth elements, the use of paramagnetic lanthanides in NMR imaging or as the spectroscopic probes for biologically essential calcium is widely known. Terbium(III) with its luminescence properties was also used as a probe to study the interactions of anticancer drugs, including anthracyclines, with tumour cells[8,9] and calcium binding sites.[10]

Lanthanides were shown to co-ordinate rather effectively to doxorubicin, the most well known member of the anthracycline family.[8,11-14] The stoichiometry of the complexes formed, however, is controversial and although the recent work of Canada and Carpentier[8] clarified some doubts precise equilibrium data as well as the metal-ion binding sites have not been fully characterised. Thus, in this work spectroscopic methods (circular dichroism and absorption) and potentiometry, where possible, were employed to obtain a fuller description of the stability, stoichiometry and binding sites of Tb^{III} with anthracyclines, including the above-mentioned doxorubicin. Quinizarin-2-sulfonic acid (9,10-dihydro-1,4-dihydroxy-9,10-dioxoanthracene-2-sulfonic acid) was used as a simple model to evaluate the binding mode of the metal ion.



	R^1	R^2
Doxorubicin	OH	OMe
Carminomycin	H	OH
Daunorubicin	H	OMe



Aclarubicin

Experimental

The anthracycline antibiotics, doxorubicin (dox), aclarubicin (acl), and carminomycin (car), were kindly provided by Laboratoire Roger Bellon and Rhone Poulenc. Quinizarin-2-sulfonic acid (qnzs) was used as obtained from Sigma. Stock solutions of anthracyclines were prepared just before use to avoid degradation caused by oxygen and light. Their concentrations were determined by dilution to approximately 10^{-5} mol dm^{-3} and using $\varepsilon_{480} = 11\,500$ for dox, $\varepsilon_{430} = 11\,150$ for acl, $\varepsilon_{490} = 11\,500$ for car and $\varepsilon_{462} = 8\,000$ dm^3 mol^{-1} cm^{-1} for qnzs.

Stock terbium(III) solutions were standardised by use of ethylenediamine-*N*,*N*,*N*',*N*'-tetracetate (edta).

Spectroscopic Measurements.—Absorption spectra were recorded on a Cary 219 spectrophotometer and CD spectra on a Yvon Jobin Mark V dichrograph. Results are expressed in terms

of the molar absorption coefficient ε and molar CD coefficient $\Delta\varepsilon = \varepsilon_l - \varepsilon_r$. The values of both are related to the total anthracycline concentration which varied from 10^{-4} to 10^{-3} mol dm^{-3}. A broad range of metal-to-anthracycline molar ratios was used.

Potentiometric Studies.—Potentiometric titrations of doxorubicin, aclarubicin and quinizarin-2-sulfonic acid were performed with a Radiometer PHM 64 pH-meter and TTA 80 titration unit equipped with G20408 glass and K4040 calomel electrodes. The anthracycline concentration was 5×10^{-4} mol dm^{-3}, and the anthracycline-to-metal molar ratios were 0.5, 1, 2 and 3:1. The solutions containing Tb^{III} and dox were clear throughout the whole pH range studied, while in the case of Tb^{III} and acl slight foaming during argon bubbling was observed above pH 7 for free acl and above pH 5 for Tb-acl solutions. Thus the stability constant calculations were less precise than those for the dox-containing system. The real error in stability constants for Tb^{III}-acl is about 0.2–0.5 log unit so the data can be regarded as tentative.

The concentration stability constants, $\beta_{pqr} = [M_pA_qH_r]/[M]^p[A]^q[H]^r$, were calculated from the pH-metric curves by means of the PSEQUAD computer program.[15] Experiments were performed under argon at 25 °C and $I = 0.05$ mol dm^{-3} (KCl).

Results and Discussion

Terbium(III)–*Doxorubicin Complexes.*—Doxorubicin (H_2A^+) contains two dissociable protons in the pH range studied (2–10), one on the ammonium group of the sugar moiety (log K_1 = 8.49) and another on the phenolic hydroxyl of the 1,4-dihydroxyanthraquinone group (log K = 10.24). A detailed discussion of the pK values of dox is given in ref. 6.

The best fit between the experimental and calculated titration curves for Tb^{III}-dox solutions was obtained assuming the following species: $[Tb(HA)]^{3+}$, $[Tb(HA)_2]^{3+}$, $[TbA(HA)]^{2+}$ and $[TbA_2]^+$ (Fig. 1). The assumption of 1:1 species only, $[Tb(HA)]^{3+}$, $[TbA]^{2+}$ and $[TbAH_{-1}]^+$, gave very unsatisfactory results. The effect of a binary hydroxo-complex was also taken into account. Since no reliable stability constants have been published for terbium(III) ion the following (log β) data for the very similar erbium(III) ion were used in the calculations: −6.3 for $[TbH_{-1}]^{2+}$, −14.5 for $[TbH_{-2}]^+$ and −23.1 for $[TbH_{-3}]$.[16] The very similar complex-formation properties of Tb^{III} and Er^{III} allow us to assume that this would not introduce errors larger than the uncertainty of the stability constants reported in this work.

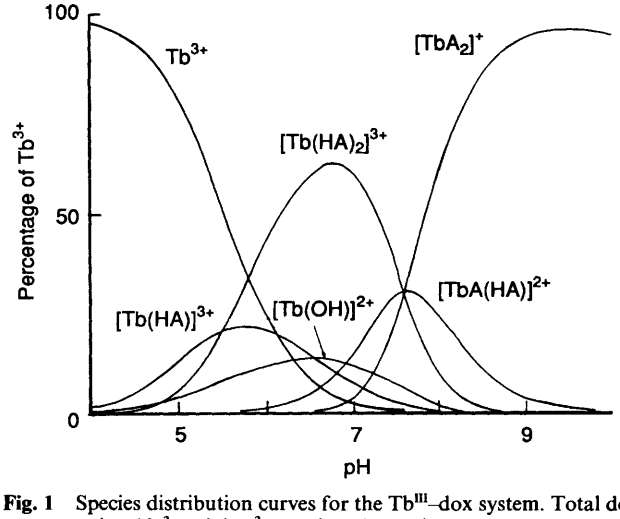


Fig. 1 Species distribution curves for the Tb^{III}–dox system. Total dox concentration 10^{-3} mol dm^{-3}, metal-to-dox ratio 1:2

The stability constants obtained for the best-fit model are collected in Table 1. As is seen from the speciation diagram $[TbHA]^{3+}$ is a minor species. This is also reflected in the negative value of log $[K_{TbHA}/K_{Tb(HA)_2}] = -0.4$. A similar observation has been made by Kiraly and Martin[11] for the complexes of Tb^{III} with daunorubicin.

Quinizarin-2-sulfonic acid is a simple model of dox having a donor system very similar to that of the anthracycline ring.[6] The species obtained as well as the stability constants correspond well to the complexes proposed above for the Tb^{III}–dox system (Table 1). Thus two important conclusions can be drawn: (*i*) 1:1 and 1:2 complexes are formed with the latter much more stable than the former and (*ii*) the binding mode in dox is similar to that of qnzs, *i.e.* a chelate is formed *via* O,O^- donors of the anthracycline ring with the sugar amine group being protonated in $[Tb(HA)]^{3+}$ and $[Tb(HA)_2]^{3+}$.

The spectroscopic results fully support this. Both CD and absorption spectra indicate the 1:2 species as the major complexes in the Tb^{III}–dox and Tb^{III}–qnzs systems.

Free dox exhibits a strong absorption band at 480 nm at pH < 9 assigned to the aromatic chromophore (Fig. 2). The metal co-ordination of dox starting around pH 4.5 causes drastic changes in the spectrum, shifting the main band towards 538 nm. This clearly indicates the involvement of the anthracycline ring in the binding. The metal ion upon substituting the phenolic proton forms a stable [C(12)=O,C(11)–O^-] chelate with the dox molecule. Similar behaviour was found for other metal ions.[2,6] Only one clear spectrum is observed (Fig. 2) at all dox to metal molar ratios. An isosbestic-like point observed around 510 nm is slightly disturbed as a result of a minor contribution from the 1:1 complex. There is only one type of CD spectrum for dox complexed by Tb^{III}. In the visible region the spectrum exhibits signals of the couplet type which disappear when the solvent is changed from water to water–ethanol (1:1). This strongly suggests that the excitonic coupling was caused by stacking of free and complexed dox molecules. The addition of ethanol removes the interactions between the dox molecules (see below).

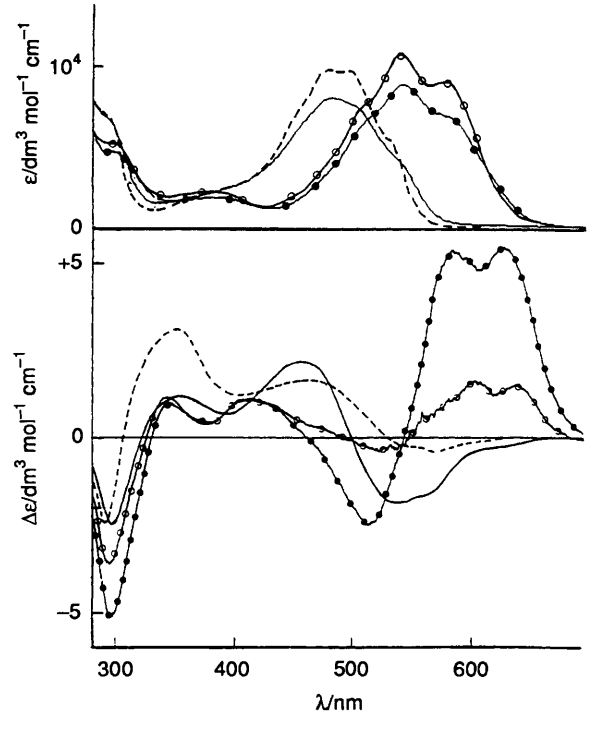


Fig. 2 Visible absorption (higher curves) and CD spectra (lower curves) of dox and its 1:2 (Tb:dox) complex. [dox] = 10^{-3} mol dm^{-3}, [Tb^{III}] = 5×10^{-4} mol dm^{-3}, pH 6.8. Solvent is either water (–dox; ●, complex) or water–ethanol (1:1) (- - -, dox; ○, complex)

Table 1 Values of log β for proton and terbium(III) complexes with dox, acl and qnzs at 25 °C and I = 0.05 mol dm^{-3} (KCl)

Species	dox	qnzs	acl
H_2A^+	18.73		17.66
	(8.49)[a]		(7.51)[a]
HA	10.24	9.30	10.15
$[Tb(HA)]^{3+}$	16.0(2)		16.3(2)
$[TbA]^{2+}$	(7.51)[b]	7.7(3)	(6.12)[b]
$[Tb(HA)_2]^{3+}$	32.4(2)		
$[TbA(HA)]^{2+}$	24.8(4)		25.5(4)
$[TbA_2]^+$	17.2(2)	16.26(2)	17.4(4)
	(9.66)[c]	(8.56)[c]	(11.28)[c]
$[TbA_2H_{-1}]$		7.5(2)	

[a] log K_1. [b] Calculated by subtracting the pK of the sugar amine group from log β for $[Tb(HA)]^{3+}$; refers to the reaction $Tb^{III} + HA \rightleftharpoons [Tb(HA)]^{3+}$. [c] $\log K_2 = \log \beta_{TbA_2} - \log \beta_{TbA}$.

The proton dissociation from amino-sugar of dox does not significantly influence the absorption spectra. The amino group is not involved in the metal-ion binding under any conditions used in this work. The stepwise deprotonation of $[Tb(HA)_2]^{3+}$ with pK value of around 7.6 could be characteristic of the parallel proton dissociation processes of non-co-ordinated sugar ammonium group(s) as well as co-ordinated water molecule(s). Neither of these deprotonations, however, effects the spectral properties of an anthracycline chromophore.

Similar behaviour was found for Tb^{III}–qnzs solutions. The absorption spectra show the presence of one dominant species of M:A molar ratio 1:2. Also in this case the isosbestic-like point is disturbed by the presence of minor 1:1 species.

Terbium(III)–*Carminomycin Complexes*.—Carminomycin is a close relative of daunorubicin (dnr). The amount of drug available was not sufficient to perform potentiometric titrations. However, the spectroscopic data allow the terbium(III) system to be described with some precision. The absorption and CD spectra clearly indicate the formation of one major complex having a well shaped isosbestic point. The spectra are best resolved for 1:2 molar ratio of the Tb^{III}:car and the dependence of Δε and ε on molar ratio indicates that the major complex formed is the 1:2 (Fig. 3). The negligible concentration of the 1:1 complex suggests the strong interactions between the car molecules favour the formation of the 1:2 complex, as was observed for the Tb^{III}–dox system (see above). The binding mode of car and dnr is expected to be the same as that of dox, *i.e. via* C(11)–O^- and C(12)=O donors.[2-6,17] Concerning the co-ordination ability, the major difference between dox and car lies at the C(4) site which in the case of dox contains a methoxy group, while car has a phenolic hydroxyl with a dissociable proton (pK = 7.8). The CD spectra in aqueous solutions at pH 6.5 at which C(4)–OH exists exhibit two Cotton effects, with vibronic structure, of opposite sign in the region of the $^1A \longrightarrow {}^1L_b$ transition. The character of these signals indicates exciton coupling of two transition dipole moments of two interacting ligand molecules.[18] Summation of the amplitudes of the two Cotton effects gives an A value (=$\Delta\varepsilon_1 - \Delta\varepsilon_2$, where $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$ are the values at the longer and shorter wavelength, respectively), equal to +9.5 dm^3 mol^{-1} cm^{-1}. The positive value indicates that a right-handed screw conformation exists between two transition dipole moments (parallel to the anthracycline ring long axis) corresponding to the $^1A \longrightarrow {}^1L_b$ transitions of the two molecules, *i.e.*, the chirality of the two interacting drug molecules is positive. This exciton interaction between the two chromophores does not disappear when the solvent is changed from water to water–ethanol (1:1) (Fig. 3). Since ethanol prevents drug dimerisation in metal-free solutions,[17] this result indicates that co-ordination promotes

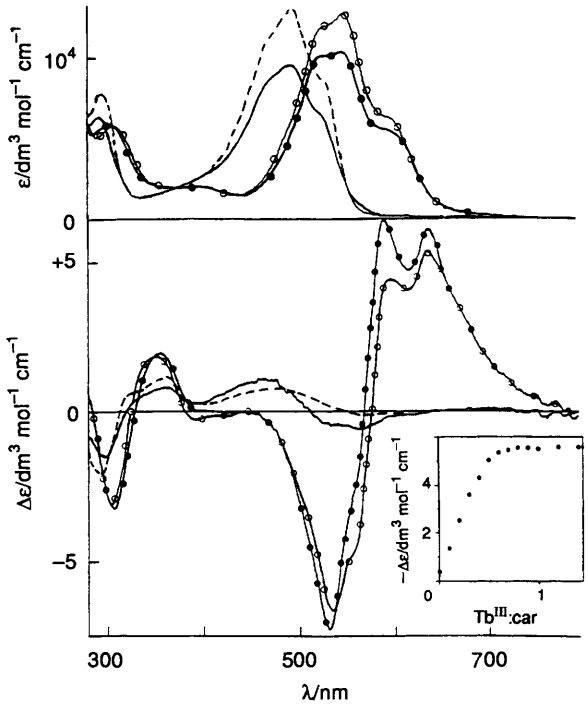


Fig. 3 Visible absorption (higher curves) and CD spectra (lower curves) of car and its 1:2 (Tb:car) complex. [car] = 10^{-3} mol dm^{-3}, [Tb^{III}] = 5 × 10^{-4} mol dm^{-3}, pH 6.5. Solvents as in Fig. 2. Inset: Δε at 532 nm plotted as a function of the Tb^{III}:car molar ratio

the interligand interactions. This is corroborated by the observation that free car exhibits a CD signal of the couplet type in pure aqueous solutions but not in water–ethanol (1:1) solutions.

The presence of the ligand-ligand coupled moiety explains the preference for 1:2 complexes in the studied systems. Co-ordination seems to promote such interaction at pH 6.5.

Increase in the pH above 8.0 causes some changes in the CD spectra. The vibronic structure of the positive band vanishes but the couplet-type signal is preserved and still centred at 540 nm. These variations are caused by deprotonation of the C(4) phenolic group of car.

Terbium(III)–*Aclarubicin Complexes*.—Aclarubicin lacks a C(11) phenolic group, the main binding site in the anthracyclines discussed above. Similarly to dox, acl dissociates two protons (H_2A^+), with pK_1 = 7.51 (phenolic group) and pK_2 = 10.15 (sugar tertiary ammonium group). The pK_1 value cannot be unambiguously ascribed to the phenolic OH at position 4 or 6, although the former seems to be favoured for deprotonation.[19] Loss of the first proton from the anthracycline ring makes the second phenolic group considerably more basic (pK > 12) due to electrostatic interactions and intramolecular hydrogen-bond formation. Owing to the slight foaming of the solutions, the evaluation of the titration curves is less precise than for the Tb^{III}–dox system. The real error in the stability constant is estimated to be around 0.2–0.5 log unit. Even the approximate results obtained for Tb^{III}–acl, however, indicate that contrary to dox the 1:2 complex with acl is not so highly favoured. The considerably larger space requirements necessary for acl co-ordination due to the presence of the bulky sugar moiety and metal binding close to the sugar–anthracycline link through C(4)–O^-, C(5)=O (see below) disfavour 1:2 complex formation and both 1:1 and 1:2 species are present as two major complexes in equilibrium (Fig. 4).

The absorption and CD spectra clearly show three distinct species formed in the Tb^{III}–acl solutions. The first complex, $[Tb(HA)]^{3+}$, favoured at pH 4.5–6 for low acl-to-metal molar

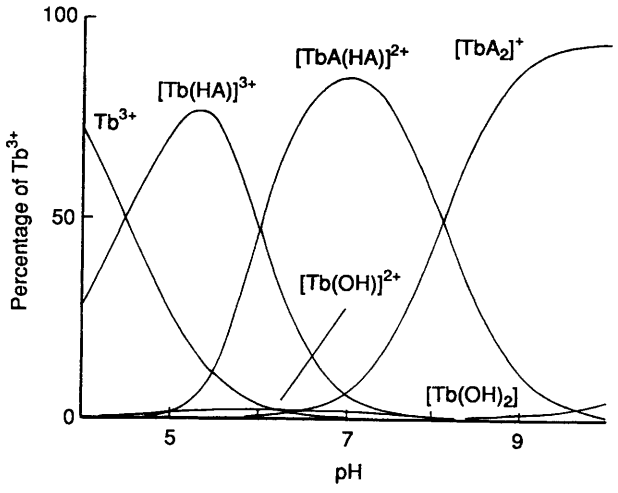


Fig. 4 Species distribution curves for the Tb^{III}–acl system. Total ligand concentration 1 × 10^{-3} mol dm^{-3}, metal-to-ligand ratio 1:2

ratios, does not effect the CD spectra significantly (Fig. 5). There is only a slight shift of the positive bands from 340 to 360 and from 430 to 470 nm when compared to the fully protonated free acl. The strong negative band at 290 nm is not affected at all. It should be mentioned that deprotonation of the acl C(4)–OH site has similar effects on the absorption and CD spectra. The stability constant log K = 6.15 for $[Tb(HA)]^{3+}$ (see Table 1) as well as spectroscopic data suggest chelate formation with involvement of the anthracycline C(4)–O^-, C(5)=O donors.

The formation of the $[TbA(HA)]^{2+}$ complex involves two simultaneous proton releases. Deprotonation of $[Tb(HA)]^{3+}$ and co-ordination to H_2A^+ in the manner discussed above for $[Tb(HA)]^{3+}$. This results in very considerable variations of the CD and also of the absorption spectra of co-ordinated acl (Fig. 5). The positive band centred at 470 nm {$[Tb(HA)]^{3+}$} splits and shifts to 550–570 nm, while the negative band moves from 290 {$[Tb(HA)]^{3+}$} to 300 nm ($[TbA(HA)]^{2+}$) decreasing considerably in intensity. The only possibility of very drastic changes of the acl spectrum at pH <7 may derive from deprotonation of the C(6)–OH phenolic group. Since this is very basic (pK > 12), the spectral changes observed at pH 6.5 may derive most likely from the involvement of this site in metal-ion binding. Since the sugar group remains protonated till pH about 9.5 (pK = 10.15) the further deprotonation step occurring above pH 7.5, $[TbA(HA)]^{2+} \longrightarrow [TbA_2]^+$, having p$K$ = 8.1 may correspond to deprotonation of bound water or to further variation in the acl protonation state. Since deprotonation of co-ordinated water is not expected to affect the anthracycline spectra, the strong shifts of CD bands from 300 ($[TbA(HA)]^{2+}$) to 320 nm ($[TbA_2]^+$) and from 550–570 ($[TbA(HA)]^{2+}$) to 570–595 nm ($[TbA_2]^+$) with distinct changes in Δε (Fig. 5) suggest involvement of the C(6)–OH group of the second bound ligand in co-ordination. It is noteworthy that the absorption spectrum of free acl at pH 13 with both C(4)–OH and C(6)–OH phenolic groups deprotonated is centred at 520 nm, *i.e.* the same wavelength as that of the $[TbA_2]^+$ species recorded at pH 7.5. This strongly supports involvement of C(6)–O^- in co-ordination in both 1:2 complexes formed in the Tb^{III}–acl system. The CD spectra of acl bound in $[TbA_2]^+$ and free acl at pH 13 are, however, considerably different. A different mode of co-ordination of acl when compared to dox is suggested also by the unusually high value of log K_2 = log β_{TbA_2} − log β_{TbA} (see Table 1), which for acl equals to 11.3, *i.e.* almost twice as high as log K_{TbA}. A comparison with the corresponding value for the Tb^{III}–dox complex cannot be made as in the latter case parallel hydroxo complex formation reactions also take place. In the Tb^{III}–acl system these processes might be strongly suppressed by the much higher charge density due to the four negatively charged phenolate O^- groups, and also by the more 'closed' co-ordination around the metal ion.

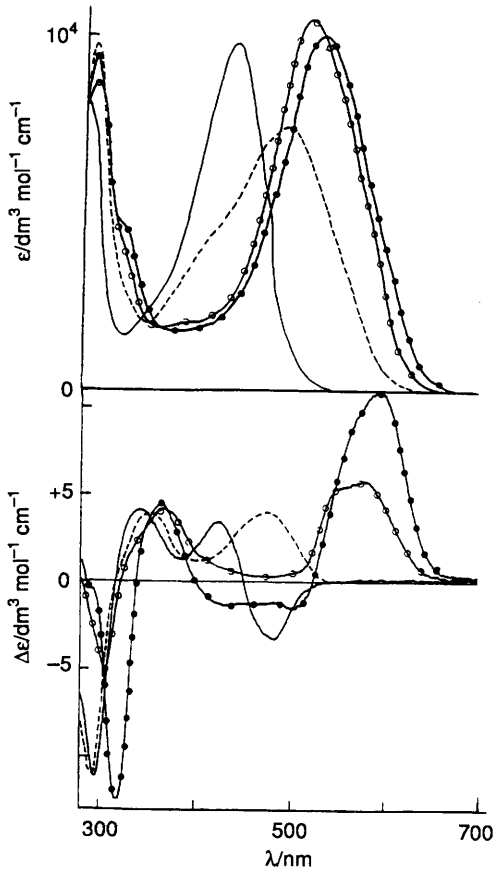


Fig. 5 Visible absorption (higher curves) and CD spectra (lower curves) of acl and Tb^{III}–acl complexes. [acl] = 10^{-3} mol dm^{-3} and [Tb^{III}] = 0, pH 5.5 (—), [Tb^{III}] = 10^{-3} mol dm^{-3}, pH 5.5 (– – –), [Tb^{III}] = 5 × 10^{-4} mol dm^{-3}, pH 7 (○), and [Tb^{III}] = 5 × 10^{-4} mol dm^{-3}, pH 9 (●)

Thus, the binding mode in Tb^{III}–acl solutions changes as follows: C(4)–O^-, C(5)=O in $[Tb(HA)]^{3+}$; mixed C(4)–O^-, C(5)=O and C(6)–O^-, C(5)=O in $[TbA(HA)]^{2+}$; and 2[C(6)–O^-, C(5)=O] in $[TbA_2]^+$. The amino sugar moiety remains protonated in all these complexes below pH 9.5.

Conclusion

The anthracyclines are very efficient ligands towards Tb^{III}, co-ordinating *via* their phenolic and quinone oxygen-donor atoms. The specific feature of all the anthracycline drugs studied is their ability to stabilise considerably the 1:2 complex due to intraligand interactions, most effectively for car. The interligand interactions lead to exciton coupling which is characterised by a couplet type of CD signal observed very clearly in the Tb^{III}–car system. In this case the interactions between the bound ligand molecules is not affected by ethanol, which prevents such interactions in metal-free solutions.

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